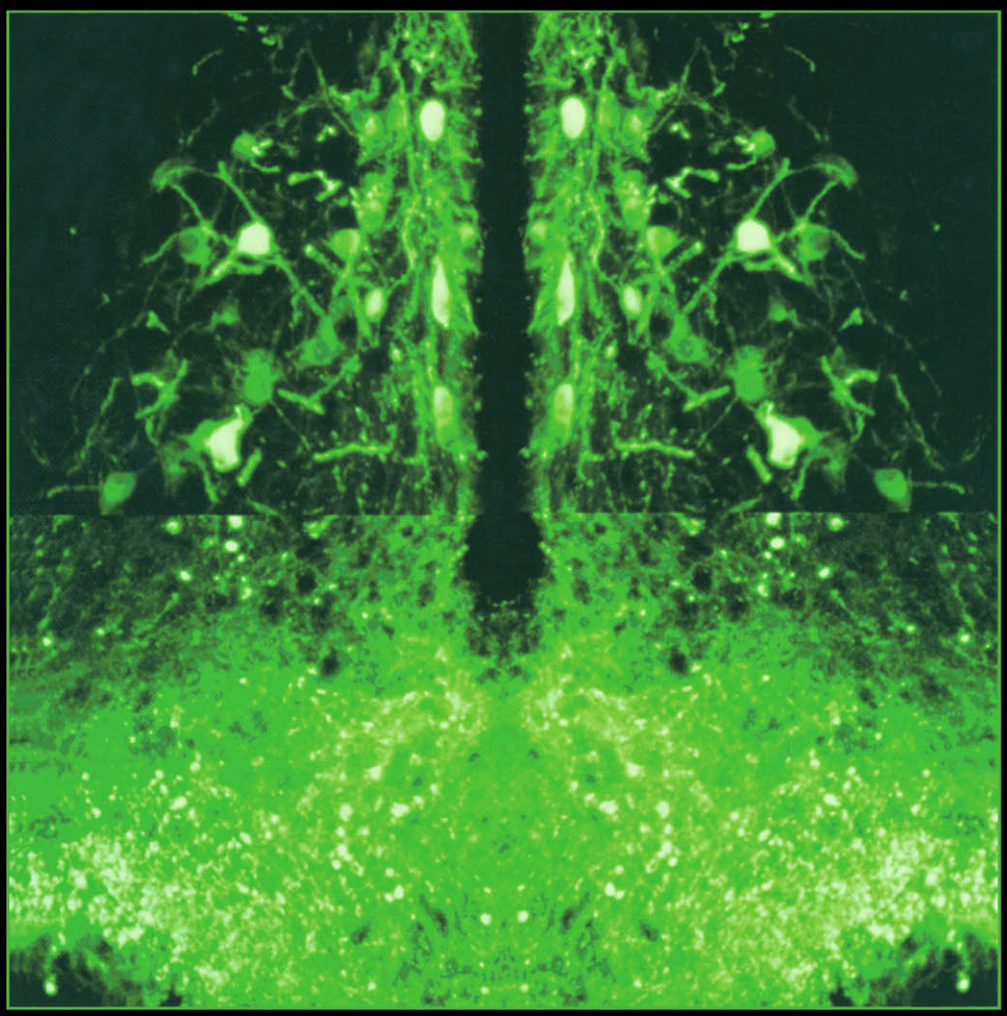
Neuroendocrinology in Physiology and Medicine

EDITED BY

P. Michael Conn
Marc E. Freeman



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Edited by

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PREFACE

We as scientists and physicians were trained with "classic" comprehensive texts that we continue to cherish as reference books in our libraries. These are the texts that first introduced us to the fundamentals of our discipline and continue to serve us as sources of professional refreshment.

The present text, *Neuroendocrinology in Physiology and Medicine*, is designed to fill a major void in the discipline. There is no comprehensive clinical or basic science text covering the topic. Moreover, owing to constraints of length, none of the general neuroscience or endocrinology texts have been able to provide comprehensive coverage of the topic. Though the discipline is relatively young, the growth in the continually burgeoning science of neuroendocrinology now justifies such a comprehensive text.

Neuroendocrinology in Physiology and Medicine has been designed to provide systematic coverage of analytical, anatomical, functional, clinical, and pathologic topics in neuroendocrinology by experts in the field who are also recognized as skilled teachers. The text is intended for the most advanced undergraduate students as well as for beginning graduate and medical students, fellows, residents, and those in clinical practice. Textual coverage is supported by clear, comprehensive tables and figures. Taken together, *Neuroendocrinology in Physiology and Medicine* should serve as a classroom text as well as provide reference support for the interested reader.

P. Michael Conn
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PART I
THE INTERACTIONS BETWEEN THE NERVOUS AND ENDOCRINE SYSTEMS

1 The Hypothalamus as an Endocrine Organ

The Science of Neuroendocrinology

Béla Halász, MD

CONTENTS

1. INTRODUCTION

Although some faint indications about the influence of the central nervous system (CNS) on endocrine functions can be traced back to the end of the previous century, neuroendocrinology started to become a fairly independent scientific field around the 1940s when neurosecretion was discovered and it had been postulated that the CNS control of the anterior pituitary is exerted via a neurohumoral mechanism.

In the last 50 years, researchers working in this field have made outstanding contributions resulting in a very rapid and spectacular development of the discipline; many of the basic questions of neuroendocrinology have been solved over the years. We gained a vast amount of important new information, which in essence, confirmed and proved the original basic concepts of neuroendocrinology.

However, it also led to a significant revision of our views about the discipline in general and about some of its special areas in particular. Originally, neuroendocrinology meant primarily neurosecretion as a peculiar phenomenon, the supraoptico- and paraventriculo-hypophysial system producing vasopressin and oxytocin, and the neural control of the anterior pituitary gland. Now, the discipline covers much more. Neuroendocrinology deals with all interactions between hormones and nerve structures. There are an infinite number of such interactions that exist at very

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different levels. Therefore, it is almost impossible to draw definite boundaries of the discipline and to give a more detailed definition of neuroendocrinology.

After a brief historical background, this chapter provides an overview of the hypothalamus as an endocrine organ secreting neurohormones. Furthermore, it discusses briefly the feedback action of hormones on the CNS, and the basic organization of the hypothalamus as a key structure of the neuroendocrine system. Finally, the close link between neuroendocrinology and immunology is considered and that neuroendocrinology fertilized other fields of neuroscience.

2. HISTORICAL BACKGROUND: BASIC OBSERVATIONS WHICH CONTRIBUTED TO THE DEVELOPMENT OF NEUROENDOCRINOLOGY AS A SCIENTIFIC DISCIPLINE

Nearly all that we know about this system has been gathered during the twentieth century, building on fragmentary notions from earlier times. Galen regarded the pituitary as a sump for waste products (phlegm = pituita) derived in the brain from distillation of "animal spirit." He assumed that the waste products funneled down the infundibular stalk to the pituitary gland, which then dispersed them through ducts in the sphenoid and ethmoid bones to the nasopharynx, where they appeared as nasal mucus or pituita. This view of pituitary function persisted through the Middle Ages and Renaissance and even through Vesalius until the middle of the seventeenth century, when it was shown that the foramina in the cribriform plate of the ethmoid bone transmitted olfactory nerves rather than ducts. It had been recognized as early as at the end of the eighteenth century that the rabbit ovary developed corpora lutea only after mating, but the involvement of the pituitary gland in this reflex was not demonstrated until the end of the 1920s. The term hypothalamus was coined at the end of the nineteenth century, but nothing was known of the function of this part of the brain.

The first experimental demonstration of a hormonal effect was made by Berthold (1849), who showed that male sexual and aggressive behavior and sex accessories were maintained or restored by testicular transplants in caponized cocks, whose behavior and accessories were otherwise eunuchoid. At the beginning of the twentieth century, it turned out that the adiposogenital syndrome, which was considered to be a case of pituitary tumor, was not related to the pituitary, but was rather because of the damage to the hypothalamus. This view was proven by the experimental induction of polyuria and the adiposogenital syndrome in dogs without hypophysectomy, by merely puncturing the base of the hypothalamus transbuccally with a needle.

Recognition of pituitary trophic hormones dates back to the second decade of the twentieth century, when the existence of a growth-promoting factor in the anterior pituitary had been demonstrated. Subsequently, it had been reported that the pituitary gland produced hormones that stimulate the thyroid, adrenal cortex, gonads, and growth. Complete hypophysectomy by the parapharyngeal approach depressed all these functions.

By this time, the anterior pituitary was considered as an independent organ and as the leader of the endocrine orchestra. However, an increasing number of data have indicated that activity of this gland depended on the presence or absence of target-organ hormones and the concept of reciprocal action, later called negative feedback, was proposed.

At the beginning, it was assumed that the action of gonadal hormones was exerted directly on the hypophysis. However, it was found that following ovariectomy, so-called castration cells failed to develop in the pituitary when it was transplanted under the kidney capsule, i.e., separated from the hypothalamus. It had been proposed that a so-called hypothalamic sex center was involved in the feedback circuit. It also became evident that in the case of estrogen and progesterone, there was also a positive feedback mechanism. It had been discovered that ovulation and the development of corpora lutea can be induced in juvenile rats with a single injection of estradiol. This effect was found to be specific for females. The induction of ovulation in persistently estrous rats by progesterone was also demonstrated. Furthermore, it had been shown that it is possible to manipulate the length of the estrous cycle of normally cycling rats by the appropriate differentially timed injection of either estrogen or progesterone.

During the 1930s, the anatomy and physiology of the hypothalamus were intensively studied. New discoveries of hypothalamic function were made possible with the use of a stereotaxic instrument. It had been reported that localized lesions interrupting the supraopticohypophysial tract induced polyuria. This alteration was interpreted as an effect of denervation of the pituicytes of the posterior pituitary, which were supposed to secrete the antidiuretic hormone.

At this time, Ernst and Berta Scharrer had been staining secretory granules in the preoptic region of

the fish brain. The supraoptic nuclei in mammals also contained granules. The Scharrers suggested that hormones might be produced in such neurons. They called these nerve cells neurosecretory neurons and the mechanism neurosecretion. The concept was greatly supported by other researchers who applied Gomori's chrome alum hematoxylin-phloxine stain, and observed that the whole supraoptico- and paraventriculo-neurohypophysial systems were stained. Cutting the stalk caused a piling up of the Gomori-positive granules on the proximal side of the cut. The system could be manipulated by changing the water intake of the animals. These and other observations indicated that the antidiuretic hormone was synthesized in the large supraoptic neurons, and was carried down along their axons to the posterior pituitary. Shortly afterwards, the chemical structures of vasopressin and oxytocin were determined.

At the end of the 1930s, it had been reported that reflex induction of ovulation in the rabbit (mentioned earlier) required that the pituitary remains *in situ* for nearly an hour after coitus. Furthermore, it has been observed that electrical stimulation of the basal hypothalamus would induce ovulation in the rabbit, but similar stimulation of the pituitary gland was ineffective in this regard. The sparse innervation of the anterior lobe, unlike the richly innervated neurohypophysis, led to speculation as to how the coital stimulus, as well as hypothalamic electrical stimulations, might be transmitted to anterior pituitary cells. Concerning the mechanism of the hypothalamic action on the anterior pituitary, a significant progress had been made in this line by the description of the hypophysial portal vascular system. On the basis of these findings, it had been assumed that the anterior pituitary was controlled not by nerve fibers, but humorally.

John Green, and particularly Geoffrey Harris, became the great champions of the neurovascular concept of the hypothalamic control of the anterior pituitary, postulating that hypophysiotropic substances are produced by hypothalamic neurons projecting to the median eminence. The substances released at the axon terminals enter the portal capillaries and are carried by the portal circulation to the cells of the anterior lobe. They recognized the importance of the neurovascular concept and set out to demonstrate it by every means at their disposal. They showed that in several species, the upper or proximal capillary plexus on the median eminence filled before the portal veins themselves. They demonstrated nerve fibers ending in relation to the proximal capillary plexus and resolved the conflicting claims of results after section of the pituitary stalk by suggesting that restoration of function was effected by regeneration of the portal vessels. It has been proposed that complete hypophysectomy and pituitary transplantation would be necessary to support this assumption. These experiments were done and they demonstrated conclusively that the regenerating portal vessels have a restorative effect in hypophysectomized animals bearing a pituitary transplant under the median eminence. Pituitary grafts placed under the temporal lobe remained inactive, whereas full-pituitary function was restored in transplants under the median eminence as new portal vessels grew out to them. Furthermore, it was demonstrated that cyclic reproductive function was lost in the rat by transplanting the pituitary beneath the kidney capsule, and later restored by reimplanting it under the median eminence in the same animal. Additional support to the concept about the importance of the portal system in the control of anterior pituitary function was provided by other neuroendocrinologists studying ducks in which the vascular stalk was uniquely separated from the neural stalk. Transection of the vascular stalk interrupted the effect of light on testicular growth, whereas cutting the neural stalk did not interfere with this gonadotrophic process.

The concept of the neurovascular link (Fig. 1) was based on two postulates; the first being that the hypothalamus produces substances that act on the cells of the anterior pituitary and the second being that these substances are carried by the portal circulation to the hypophysis. In order to get information in this line, a campaign started in the 1950s to isolate the hypophysiotropic substances from the brain, to identify the molecular structure of the hypothalamic factors transported in the portal system to control anterior pituitary secretion. These investigations were very successful, and led to the isolation of the various pituitary tropic hormone releasing and release inhibiting factors (now called hormones, as their chemical structure is known). The concentration of these hypothalamic neurohormones has been demonstrated to be significantly higher in the hypophysial portal blood than in the general circulation. It turned out that besides these "classical" releasing and inhibiting hormones, there are several other compounds, mainly peptides, that are present in the median eminence and may act on the anterior pituitary. It became also evident that the neurons producing the "classical" hypothalamic neurohormones are not restricted to the hypothalamus, but are widely distributed, are present not only in the CNS, but also in the peripheral nervous system and even also in nonneuronal elements.

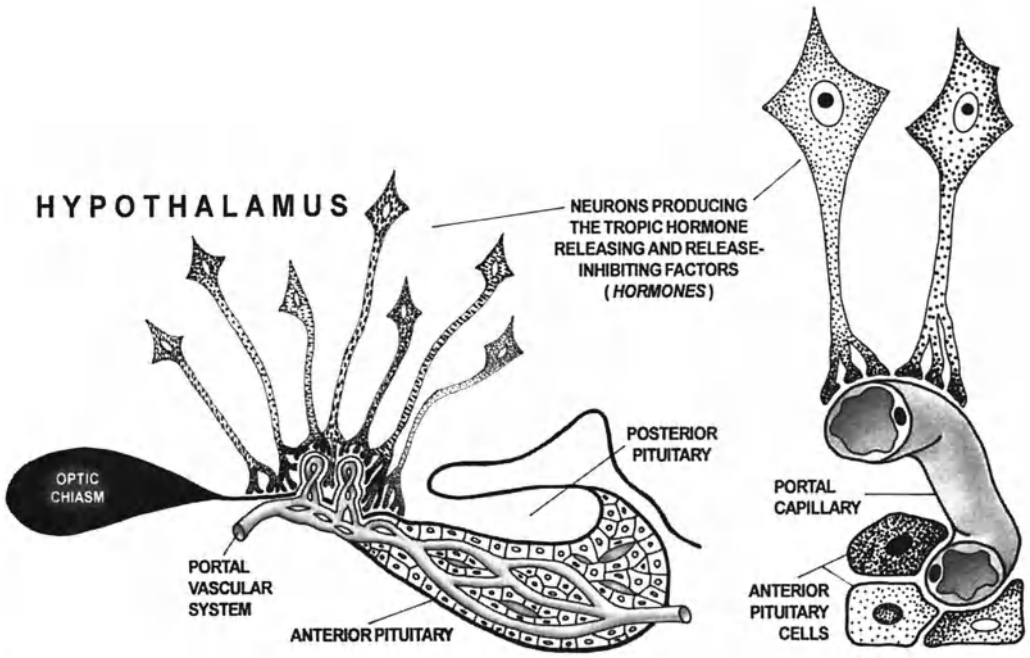

Fig. 1. Schematic illustrations of the neurohumoral mechanism controlling anterior pituitary function.

Because of their isolation, a vast amount of information accumulated about the hypophysiotropic neurohormones. Also, our knowledge about posterior pituitary hormones, and in general about neuroendocrinology, increased very significantly. At present the synthesis of a number of neurohormones is already known in details, starting with the genetic code, transcription and translation of the genetic information up to the posttranslational processes of a neurohormone. Further, the mechanism of action of many neurohormones has been considerably clarified. Remarkably, we learned about the receptors binding the neurohormones and about the second messenger systems involved in the intracellular mediation of the action of a neurohormone. It also became evident that neuroendocrinology is closely linked to other disciplines, first of all, to immunology. Further, we learned that autocrine and paracrine mechanisms are also operating within endocrine glands and these can be modulated by the nerve fibers innervating the gland. In addition, diffusible gases, nitric oxide, and carbon monoxide as well as endothelins appear to be involved in the control of neuroendocrine functions.

3. THE HYPOTHALAMUS SYNTHESIZING THE POSTERIOR PITUITARY HORMONES

In mammals, the posterior pituitary hormones are vasopressin and oxytocin. They are synthesized in the cell bodies of the magnocellular neurons of the supraoptic and paraventricular nuclei and in parvocellular neurons of the paraventricular nucleus, and transported down the axons of these neurons to their endings in the posterior pituitary (Fig. 2). Here they are secreted in response to electrical activity in the endings. Thus, oxytocin and vasopressin are typical neural hormones, being produced by nerve cells and secreted into the circulation. The neurons in the supraoptic and paraventricular nuclei projecting to the posterior pituitary are forming the so-called supraopticohypophysial and paraventriculo-hypophysial tract. Some of the neurons make oxytocin and others synthesize vasopressin. Oxytocin-containing and vasopressin-containing cells are evident in both cell groups.

The term neurosecretion was originally coined to describe the secretion of hormones by neurons, but the term is now somewhat misleading, because it became evident that almost all neurons secrete chemical messengers.

Both vasopressin and oxytocin are synthesized as part of larger precursor molecules. Vasopressin and oxytocin each have a characteristic neurophysin associated with them in the granules in the nerve cells (neurophysin I is associated with oxytocin, whereas neurophysin II is associated with vasopressin). The neurophysins appear to be simply parts of the precursor molecules, which are synthesized in the ribosomes

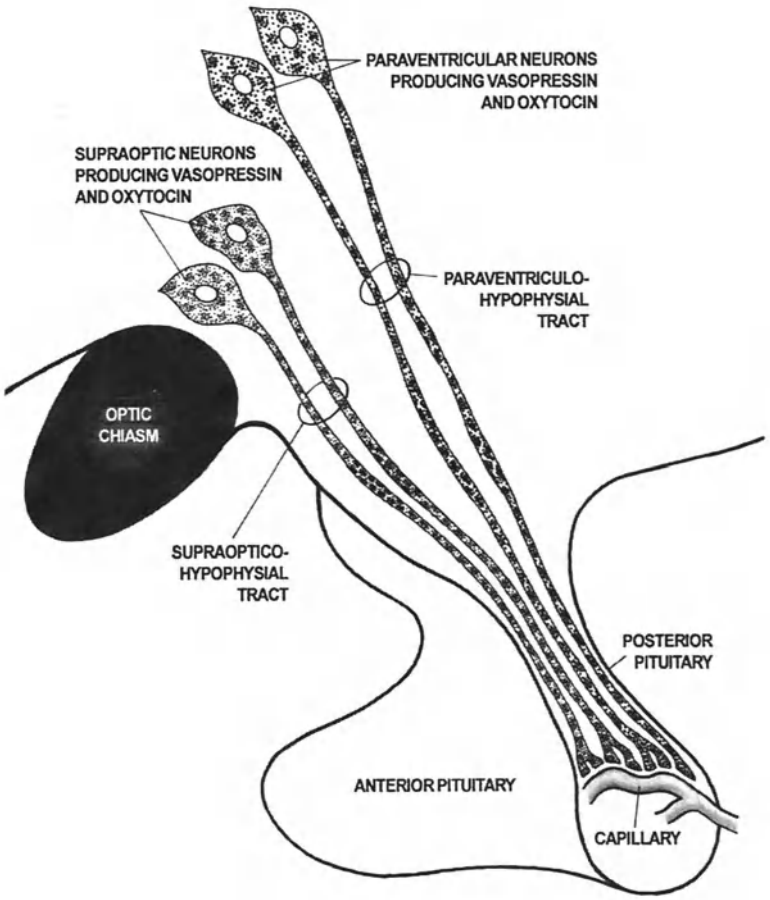

Fig. 2. The supraoptico- and paraventriculo-hypophysial system synthesizing vasopressin and oxytocin.

of the cell bodies of the neurons. The precursor molecules have their leader sequences removed in the endoplasmic reticulum, are packaged into secretory granules in the Golgi apparatus, and are transported down the axons by axoplasmic flow to the endings in the posterior pituitary. Cleavage of the precursor molecules occurs as they are being transported, and the storage granules in the endings contain free vasopressin and oxytocin and the corresponding neurophysin.

Vasopressin and oxytocin producing neurons of the paraventricular nucleus project not only to the posterior pituitary, but also to the brain stem and spinal cord, and may be involved in cardiovascular control. Vasopressin and oxytocin are also synthesized in the gonads and the adrenal, and there is oxytocin also in the thymus.

The main effects of vasopressin are: antidiuretic activity exerted on specific receptors (vasopressin receptor type 2) in the kidney, vasopressor activity (mediated by vasopressin receptor type 1), which together with other peptide hormones controls, systemic arterial pressure, modulation of the stress response by direct stimulation of ACTH release and by enhancement of ACTH release by corticotropin releasing hormone (CRH).

Oxytocin acts primarily on the breasts and uterus. In mammals, oxytocin causes contraction of the myoepithel cells that line the ducts of the breast. This squeezes the milk out of the alveoli of the lactating breast into the large ducts and thus the nipple (milk ejection). In most species, milk ejection requires oxytocin. Milk ejection is normally initiated by a neuroendocrine reflex. Impulses generated in the touch receptors in the breast, especially around the nipple, are conveyed to the supraoptic and paraventricular nuclei of the hypothalamus. Discharge of the oxytocin containing neurons releases oxytocin from the posterior pituitary.

Oxytocin causes contraction of the smooth muscle of the uterus. The sensitivity of the uterine musculature to oxytocin is enhanced by estrogen and inhibited by progesterone. Oxytocin secretion is increased during labor. Descent of the fetus down the birth canal

initiates impulses in the afferent nerves that are relayed to the supraoptic and paraventricular nuclei, causing secretion of sufficient oxytocin to enhance labor.

The vasopressin-secreting and oxytocin-secreting neurons generate and conduct action potentials, and action potentials reaching their endings trigger release of hormone from them by Ca^{2+}-dependent exocytosis.

Stimulation of the vasopressin-secreting neurons by a stimulus such as hemorrhage causes an initial steady increase in firing rate followed by a prolonged pattern of phasic discharge in which periods of a high-frequency discharge alternate with periods of electrical quiescence. These phasic bursts are generally not synchronous in different vasopressin-producing neurons. They appear to be well suited to maintain a prolonged increase in the output of vasopressin. Stimulation of the nipples causes a synchronous, high-frequency discharge of the oxytocin neurons after a latency of a few minutes. This discharge causes release of a pulse of oxytocin and a consequent milk ejection.

4. THE HYPOTHALAMUS SECRETING TROPHIC HORMONE-RELEASING AND RELEASE-INHIBITING HORMONES

The known hypophysiotropic neurohormones are corticotropin-releasing hormone (CRH), gonadotropin-releasing hormone (GnRH or luteinizing hormone-releasing hormone, LHRH), growth hormone-releasing hormone (GHRH), somatotropin release-inhibiting hormone (SRIH, or growth hormone release-inhibiting hormone), thyrotropin-releasing hormone (TRH), prolactin release-inhibiting factor (PIF).

Because the first experimental observations on the existence of hypothalamic substances acting on pituitary trophic hormone secretion were published, several attempts were made to localize the structures producing these substances:

(1) Lesioning various hypothalamic regions and determining the trophic hormone-releasing or hormone release-inhibiting activity of the median eminence-pituitary stalk region.
(2) Cutting the hypothalamus into small pieces and measuring trophic hormone-releasing activity of the pieces.
(3) Implanting anterior pituitary tissue into the hypothalamus and investigating its structure and hormone secretion.
(4) Completely or partially cutting around certain hypothalamic regions and testing pituitary trophic function in various ways.

All of these investigations provided some information about the site of production of the hypophysiotrophic substances, but did not clarify the question of location.

Immunocytochemistry furnished more direct evidence about the location of neurons synthesizing these neurohormones. However, neurons containing the trophic hormone-releasing and release-inhibiting neurohormones are widely distributed in the CNS (some of them are even present in nonneural tissues), and not all of these neurons terminate in the hypothalamic median eminence and pituitary stalk. Furthermore, in most cases, it is impossible to trace the axon from its origin to its terminal arborization. Therefore, in recent years, immunocytochemistry has been combined with retrograde tracing, injecting the tracer into the median eminence. At present, this is the most direct approach to find out which of the neurons producing one or the other releasing hormone project to the median eminence and which send their axons elsewhere.

Double-label immunocytochemistry has made possible the demonstration of two different substances in the same section. By means of this technique, we learned that the hypophysiotrophic neurons may also contain other chemical messengers. The double-label approach has also provided significant information about the immunocytochemically identified afferent connections of the hypophysiotrophic neurons. In addition, there are many other neurons in the hypothalamus containing chemical messengers different from the abovementioned neurohormones. Most of them are also peptides, and some of them terminate in the median eminence. The functional significance of these other chemical messengers is not known. The possibility of interactions of the various substances at the median eminence level exists, but there is only one report on synaptic contacts between nerve terminals of the median eminence.

4.1. Corticotropin-Releasing Hormone

The most prominent corticotropin-releasing hormone (CRH)-containing cell group of the hypothalamus is the paraventricular nucleus, mainly its parvocellular part (Fig. 3). Besides the paraventricular nucleus, aggregates of CRH-immunoreactive perikarya are in the supraoptic, medial, and periventricular preoptic and premammillary nuclei of the hypothalamus and in several extrahypothalamic regions.

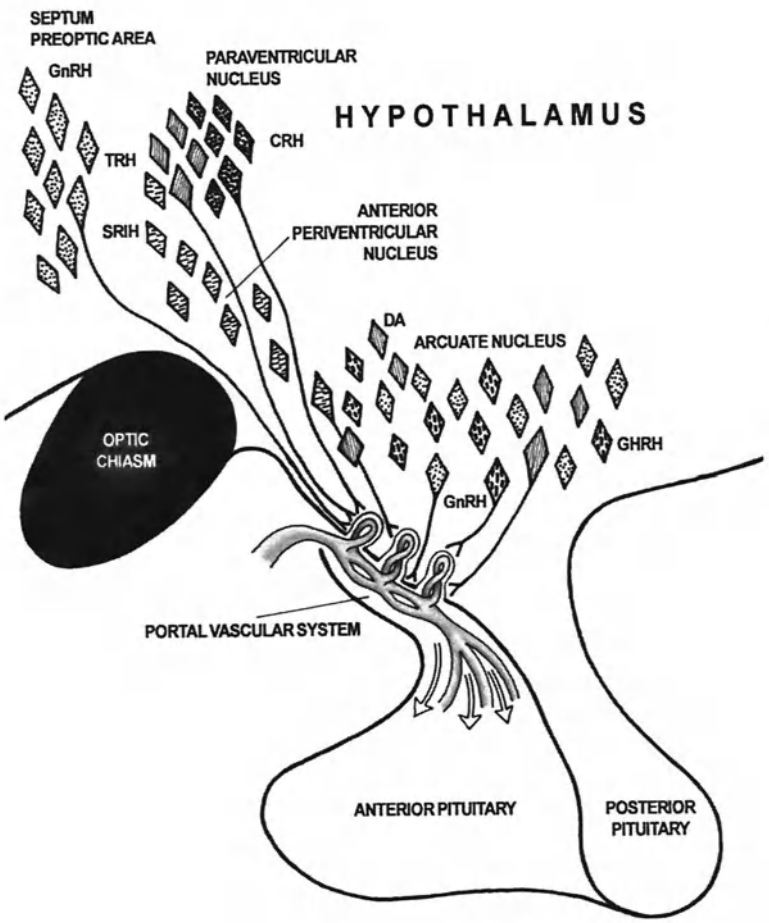

Fig. 3. Localization of neurons producing hypophysiotrophic neurohormones and projecting to the surface zone of the median eminence. Schematic drawing of midsagittal section of the hypothalamo-pituitary unit.

Although CRH neurons are widely distributed in the brain, the vast majority of the CRH terminals in the median eminence and pituitary stalk arise from paraventricular neurons. Several data indicate that other chemical messengers—largely peptides, such as vasopressin, neurotensin, enkephalin, and γ-aminobutyric acid (GABA)—are also present in at least some of the CRH neurons in the hypothalamic paraventricular nucleus.

It appears that the activity of the CRH neurons in the parvocellular paraventricular nucleus may be modulated by many chemical messengers evident in the afferent fibers terminating on CRH perikarya or dendrites. There are CRH-CRH connections and reciprocal communication between the parvocellular CRH and the magnocellular oxytocin- and vasopressin-containing neurons of the nucleus. Also neuropeptide Y, proopiomelanocortin, GABAergic and monoaminergic (serotoninergic, adrenergic, and presumably also other catecholaminergic) fibers terminate on CRH neurons of the cell group. There are glucocorticoid receptor immunoreactive sites in the nuclei of parvocellular paraventricular neurons, that express CRH immunoreactivity in their cytoplasm.

4.2. Gonadotropin-Releasing Hormone

In general, there are two areas that contain a significant amount of immunoreactive gonadotropin-releasing hormone (GnRH) nerve cell bodies: (1) the septal-preoptic-suprachiasmatic region and (2) the mediobasal area of the tuber cinereum, especially the infundibular and premammillary nuclei (Fig. 3). But there are great variations in the number of such cells in these two regions of various species.

The septal-preoptic-suprachiasmatic and the tuberal regions are not the only areas containing GnRH perikarya. Also, such elements were observed in several other structures of the brain, such as the olfactory bulb, indusium griseum, and hippocampus.

GnRH neurons originate in the medial olfactory

placodal epithelium of the developing nose, migrate across the nasal septum, and enter the forebrain with the nervus terminalis, a cranial nerve that is a part of the accessory olfactory system and projects directly from the nose to the septal-preoptic area and hypothalamus. This migratory route for GnRH-expressing neurons could explain the deficiency of gonadotropins seen in hypogonadotropic hypogonadism with anosmia.

By using combined retrograde tracing and immunocytochemical identification, several investigators found that in the rat and mouse, approximately 70% of the GnRH neurons in the septum, preoptic, and anterior hypothalamus project to the median eminence, and the rest may project to other GnRH targets within the brain or be involved in local circuits. The two populations are intermixed.

A large amount of information accumulated on the afferent connections of the GnRH neurons in the septal-preoptic-hypothalamic region. In both rat and monkey, GnRH-containing terminals are in synaptic contact with GnRH neurons. Also, catecholaminergic, serotoninergic, and GABAergic fibers as well as axons containing neuropeptide Y, substance P, neurotensin, CRH, vasopressin, galanin, terminate on GnRH neurons in regions projecting to the median eminence. Sex-steroid receptors have not been observed on GnRH neurons.

4.3. Growth Hormone-Releasing Hormone

The majority of the growth hormone-releasing hormone (GHRH)-immunoreactive cell bodies are in the arcuate nucleus and the medial perifornical region of the lateral hypothalamus (Fig. 3). Scattered cells are in the laterobasal hypothalamus, the medial and lateral portions of the ventromedial nucleus, and the dorsomedial and paraventricular nuclei.

Retrogradely labeled GHRH neurons were identified in the arcuate nucleus and the laterobasal hypothalamus, but not in the perifornical area, suggesting that the location of GHRH neurons projecting to the median eminence is confined within the arcuate nucleus and the laterobasal hypothalamus.

GHRH and dopamine, and GHRH and galanin were colocalized in cell bodies of the arcuate nucleus and in nerve fiber varicosities in the external layer of the monkey median eminence.

GHRH and somatotropin release-inhibiting hormone containing nerve terminals were found in synaptic contact with GHRH dendrites and perikarya in the arcuate nucleus. GHRH neurons are also innervated by axons containing TRH, substance P, enkephalin, and catecholamines.

4.4. Somatotropin Release-Inhibiting Hormone

Somatotropin release-inhibiting hormone (SRIH)-immunoreactive cells are widely distributed. They occur not only in the CNS, but also outside it—in the pancreas, the wall of the intestines, and elsewhere. Within the brain, such perikarya were found both within and outside the hypothalamus. In the hypothalamus, the majority of such neurons is concentrated in the periventricular region, forming one to three rows in the ventricular wall and extending from the middle of the optic chiasm to the rostral margin of the median eminence (Fig. 3). Such cells are also evident in the parvicellular part of the paraventricular nucleus, anterior hypothalamic nucleus, perifornical region, and lateral hypothalamus. In the preoptic area, positive cells were detected in the preoptic magnocellular nucleus as well as in the lateral region of the lateral preoptic nucleus. Outside the hypothalamus, such elements are present in the neocortex, caudate nucleus, putamen, several limbic structures (piriform cortex, amygdala, hippocampus, septum, etc.), and various lower brain stem areas.

By employing a combination of immunocytochemistry and retrograde tracing from the median eminence, double-labeled neurons were found in the medial preoptic area, anterior periventricular area, and paraventricular nucleus. About 70% of the SRIH neurons in the medial preoptic area, anterior periventricular area, and paraventricular nucleus project to the median eminence.

SRIH-immunoreactive nerve cells in the anterior periventricular nucleus receive afferents with different chemical messengers. SRIH-containing axons terminate on perikarya and dendrites containing the same peptide. There are reciprocal connections between SRIH and GHRH neurons. Furthermore, CRH, neuropeptide Y-, and GABA-containing axons form synaptic specializations on SRIH neurons in the anterior periventricular nucleus.

4.5. Thyrotopin-Releasing Hormone

Thyrotropin-releasing hormone (TRH) was the first hypothalamic-releasing factor structurally characterized. It is widely distributed in the central and peripheral nervous system. Within the hypothalamus, TRH-immunoreactive perikarya are mostly gathered in the parvocellular division of the paraventricular nucleus

(Fig. 3). Such neurons are also in the suprachiasmatic portion of the preoptic nucleus, the dorsomedial nucleus, and the laterobasal hypothalamus. TRH nerve cells are also present outside the hypothalamus; among others in the raphe nuclei, the olfactory bulb, diagonal band of Broca, septal nuclei, and central gray matter of the mesencephalon. The distribution suggests that TRH functions both as a hypophysiotrophic neurohormone and as a neurotransmitter or neuromodulator.

TRH neurons of the parvocellular paraventricular nucleus receive a rich innervation. Neuropeptide Y, proopiomelanocortin, norepinephrine, serotonin-containing fibers were found to be in intimate anatomic proximity, and often forming synaptic contacts with TRH cell bodies or dendrites of the paraventricular nucleus. In addition, there are axodendritic connections between TRH-containing structures, that is, both the pre- and the postsynaptic element is TRH immunoreactive.

4.6. Prolactin-Inhibiting Factor: Tuberoinfundibular Dopaminergic System

It is currently generally accepted that the prolactin release-inhibiting factor, at least one of it, is dopamine and that this dopamine is produced by the tuberoinfundibular dopaminergic neurons, which are situated in the arcuate nucleus and in the ventral part of the anterior periventricular nucleus (Fig. 3). Most of these dopamine cell bodies are in the anterior part of this cell group; although these dopaminergic neurons represent only a small percentage of the total amount of cell bodies present in this nucleus.

CRH and serotoninergic axon terminals forming synaptic specializations on tuberoinfundibular dopaminergic neurons were demonstrated.

The following conclusions may be drawn from the observations summarized in this part of the chapter.

Neurons producing different hypophysiotrophic neurohormones can be divided into two subpopulations: one having access to the portal vessels and acting rather selectively on anterior pituitary function, and another without access to the portal system, but projecting to other nervous structures. These two are intermingled.

It appears that the suprachiasmatic region and the rostral part of the retrochiasmatic area are very rich in elements producing hypophysiotrophic neurohormones and projecting to the median eminence. The majority of CRH, TRH, SRIH neurons, and a significant number of GnRH and dopamine (DA) synthesizing cells are situated in this region. The tuberal region (periventricular nucleus, arcuate nucleus) contains GHRH, DA, and in certain species, GnRH producing elements.

Axons containing one or the other neurohormone form synaptic connections with neurons synthesizing the same peptide. This may be the structural basis for an ultrashort-feedback mechanism (*see* Section 5) or may indicate an intrinsic circuit. There are reciprocal connections between certain hypophysiotrophic nerve cells.

The hypophysiotrophic neurons receive a very significant neural input mediated by several chemical messengers. The presence of synaptic connections between various peptidergic, monoaminergic, and GABAergic axons on one side and trophic hormone-releasing or release-inhibiting hormone-synthesizing perikarya and dendrites on the other side may provide the structural basis for the assumption that the actions of the different neurotransmitters or neuromodulators observed so far are, at least partly, exerted directly on the structures producing the hypophysiotrophic neurohormones.

For details about hypophysiotrophic neurohormones, see Chapter 3.

5. THE HYPOPHYSIAL PORTAL VASCULAR SYSTEM CONVEYING THE TROP HORMONE-RELEASING AND RELEASE-INHIBITING HORMONES TO THE ANTERIOR PITUITARY

The portal vascular system represents the key structure required for the operation of the neurohumoral mechanism controlling pituitary tropic functions. It transports the substances released from the nerve terminals in the median eminence to the pituitary. For details, see Chapter 2.

The main features of the portal vascular system can be summarized as follows (Fig. 4). The so-called superior hypophysial arteries form a dense plexus, largely precapillary in character, within the pars tuberalis of the adenohypophysis (the adenohypophysis is subdivided into three parts: pars tuberalis surrounding the median eminence and pituitary stalk, pars distalis or anterior lobe, and pars intermedia or intermediate lobe). This plexus is especially dense on the contact surface between the median eminence and the pars tuberalis (mantleplexus). From this plexus arises the capillary loops that penetrate into the tissue of the median eminence and infundibular stem. The mantleplexus and the capillary loops drain toward the portal vessels (some of the capillary loops drain toward the

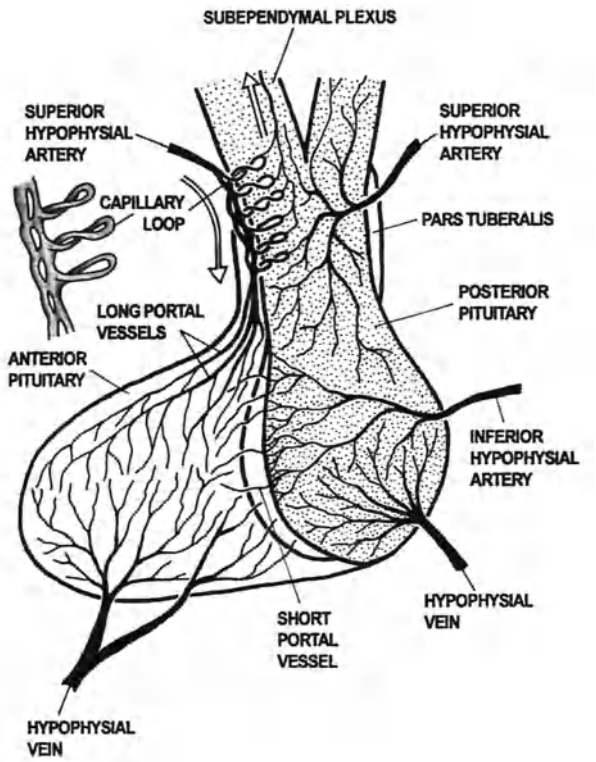

Fig. 4. Schematic illustration of the hypophysial portal vascular system.

subependymal plexus of the third ventricle) that lie on the ventral surface of the stalk. These vessels are called the long portal vessels. Part of the blood from the posterior pituitary reaches the anterior pituitary by way of vessels known as short portal vessels.

The presence of trophic hormone-releasing and release-inhibiting substances in the portal blood is well documented, as well as the concentration of these substances is much higher in the portal blood than in the peripheral plasma, and that changes occur under certain experimental conditions.

The majority of the portal blood is directed from the median eminence toward the pituitary, where some blood may flow in the reverse direction toward the hypothalamus. Blood collected from a single portal vessel through a microcannula, the tip that points toward the hypothalamus, contains several times higher concentrations of LH, TSH, ACTH, and prolactin in portal plasma than in arterial plasma. There is experimental evidence for the view that anterior pituitary hormones can influence their own secretion via the CNS. However, more studies are needed to verify whether a retrograde transport from the pituitary to the hypothalamus exists at all under physiologic circumstances and what is the functional significance of the retrograde transport.

6. THE HYPOTHALAMUS AS A SITE OF HORMONAL FEEDBACK: HORMONE RECEPTORS IN THE HYPOTHALAMUS

Hormones of the target endocrine glands (thyroid, adrenal, and gonads) exert a feedback action on the secretion of the hormone produced by the anterior pituitary and stimulating the target endocrine gland. In general, there are two sites of the hormonal feedback: the pituitary gland itself and structures of the CNS (Fig. 5).

The feedback action of hormones is mediated by hormone receptors, which are evident both in the pituitary as well as in various regions of the CNS, in the hypothalamus and in extrahypothalamic structures. There are corticosteroid receptors on CRF neurons in the paraventricular nucleus indicating that corticosteroids may have a direct feedback action on CRF release. This is not the case for GnRH; estrogen receptors were not found on such neurons, but only on other nerve cells in the region. The picture is not clear for thyroid hormone receptors on TRH neurons.

The fact that hormone receptors are widely distributed in the CNS indicates clearly that the action of hormones on the brain is not limited to the feedback action through which the various hormones control their own secretion, but is much broader. Let us take as an example corticosteroid hormones. Because of their lipophilic nature, corticosteroid hormones easily pass the blood-brain barrier and thus are uniformly distributed in the brain, and as true endocrine messengers, only retain at those sites where steroid receptors are present. This holds not only for steroid hormones that are produced in the adrenal gland, but also for other steroids including a recently described class of brain-born steroids: the neurosteroids (pregnenolone and dehydroepiandrosterone) which are synthesized from cholesterol in certain brain cells. Corticosteroids can bind to two receptor types in the brain. The first type is the mineralocorticoid receptor (MR) which displays, in vitro, high affinity for the natural mineralocorticoid aldosterone and also for corticosterone. The second type is the glucocorticoid receptor (GR) which has a 10-fold lower affinity for corticosterone and very low affinity for aldosterone. The GR, however, effectively binds synthetic glucocorticoids such as dexamethasone. The structure of these two receptors in the brain is identical to the MR and GR in peripheral organs such as the kidney and liver. The MRs in the brain are enriched in limbic structures such as the hippocampus and septal area and motor nuclei of the brainstem. The GRs are more widely

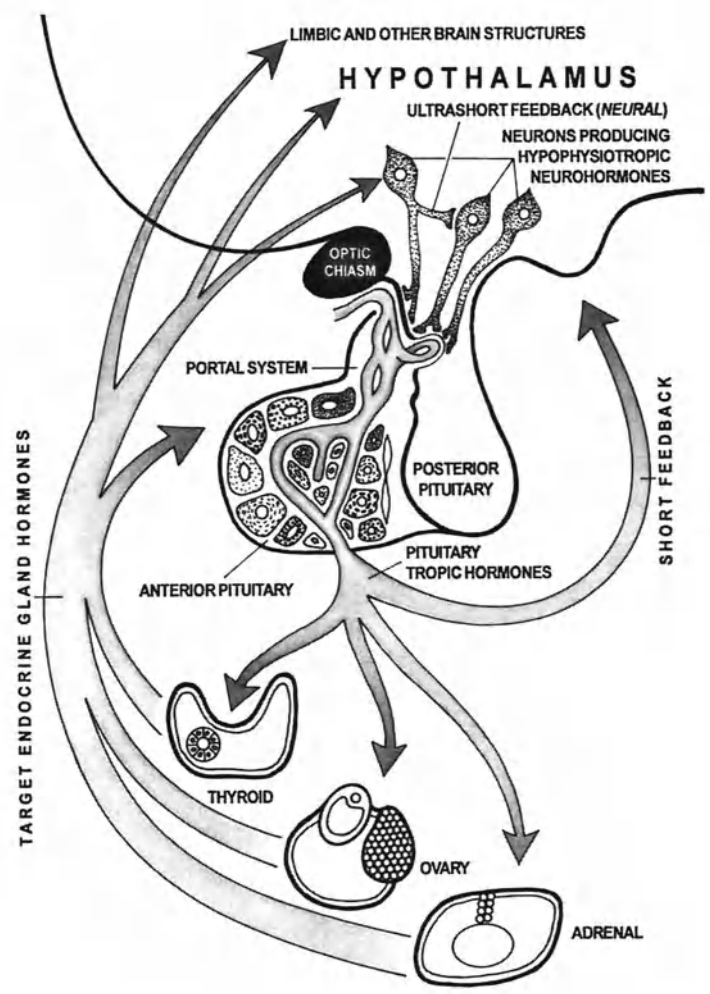

Fig. 5. This figure shows the sites of feedback action of target endocrine gland hormones, anterior pituitary hormones (short feedback), and of hypophysiotrophic neurohormones (ultrashort feedback). Humoral pathway of ultrashort feedback is not indicated.

distributed: the hypothalamic paraventricular nucleus and the hippocampus have a particularly high density of GRs.

Steroid receptors are localized in the cytoplasm or nucleus. Nuclear hormone receptors share two characteristic features; namely, a DNA binding domain containing a zinc finger motif and a hydrophobic C-terminal responsible for ligand binding. The steroid receptor is activated as a transcription factor by hormone binding. On binding of the steroid to its receptor, the steroid-receptor complex is activated and exhibits an enhanced affinity to certain sequences of the genomic material: the hormone-responsive elements. Binding of hormone-receptor dimers to the responsive elements results in changes of gene transcription, leading to altered protein synthesis within the cell. These proteins in turn may be involved in membrane characteristics that are essential for the neuronal excitability. Because of this genomic mechanism of action, steroid effects mediated by intracellular receptors are slow in onset and of prolonged duration. The steroids thus supply the possibility of a delayed neuronal reaction to environmental changes or to primary changes in the circulating steroid levels (in both sex- and corticosteroid level in the blood). The steroid receptors mediated effects on gene transcription are expected to alter the mRNA expression and protein synthesis of brain cells. Some of these proteins may contribute to the electrical properties of the cell membrane. These proteins may affect voltage-dependent ion channels in the membrane, transmitter systems, comprising G protein-coupled receptors or ligand-gated ion channels, and ion transporters. Estrogens, in addition to their primary role to stimulate or repress rates of specific gene transcription, may also regulate posttranscriptional events and other non-genomic events.

Gonadal steroids are known to play an important role during both development and adulthood, in modulating the size, morphology, and synaptic density of sex-steroid responsive structures in the CNS. As levels of gonadal steroids differ between male and female

animals, both neonatally and postpubertally, sexually dimorphic synaptic patterns develop naturally in several areas of the CNS.

Gonadal steroids exert organizational effects on steroid-responsive tissues in the CNS. The structural sexual dimorphism that results from the organizational effects of sex steroids exist at many morphological levels including neuron numbers, dendritic length, neuronal membrane organization, synaptic formation, and neuronal connectivity. Gonadal steroids, affecting virtually all structural parts of the synaptic formation, influence synaptic remodelling in specific areas of both the peripheral and central nervous systems of the adult mammal under both physiological and experimental conditions. Gonadal hormones influence the pattern of synaptic connectivity in diencephalic and telencephalic structures that control reproductive behavior, such as the ventromedial hypothalamic nucleus, lateral septum, and the amygdala. In addition, extensive evidence suggests that testosterone and/or estradiol modulate synaptic connectivity in the neuroendocrine diencephalic regions that control the release of pituitary hormones, such as the hypothalamic arcuate nucleus. There is a dendritic growth of arcuate neurons projecting to the median eminence following orchidectomy, and the length and geometry of the dendritic trees of oxytocin- and vasopressin-synthesizing neurons are altered during lactation.

Besides target endocrine hormones, pituitary tropic hormones as well as trophic hormone-releasing hormones appear to exert a direct feedback action (called short and ultrashort feedback, respectively) on the CNS structures involved in the control of pituitary tropic hormone secretion (Fig. 5). In the case of ultrashort feedback, this action could be mediated either via direct neuronal or humoral pathway. The close apposition of neuronal processes and somata containing the same hypophysiotrophic hormone may be the anatomical indication of a neuronal ultrashort feedback mechanism. The humoral ultrashort feedback could be exerted via the vascular connections between the hypophysial portal vascular system and the vessels of the medial basal hypothalamus (for details, *see* Chapter 2).

Pituitary hormone receptors as well as receptors for trophic hormone-releasing hormones are evident in the brain, and show a fairly wide distribution, indicating that pituitary hormones and hypophysiotrophic hormones can influence not only their own secretion, but also various brain functions. For example, besides the anterior and intermediate lobes of the pituitary, CRH receptors are present in the cerebral cortex (involved in cognitive function), amygdala, hippocampus (involved in emotion and stress responses), in brain stem regions such as locus coeruleus and nucleus of the solitary tract (regulating autonomic function). There are CRH receptors also in the cortex of the cerebellum and in the olfactory bulb. In addition to a physiological role for CRH in integrating the responses of the brain and endocrine systems to physiological stimuli, recent clinical data indicate CRH in the etiology and pathophysiology of various endocrine, psychiatric and neurologic illnesses.

7. BASIC ORGANIZATION OF THE HYPOTHALAMUS AS A KEY STRUCTURE OF THE NEUROENDOCRINE SYSTEM

Our knowledge concerning the morphology of the hypothalamus increased considerably. This progress may first of all be ascribed to the introduction of new experimental techniques such as immunocytochemistry, *in situ* hybridization, new tract tracing methods, etc. More than 20 neuropeptides (brain born) have been localized in the endocrine hypothalamus including hypophysiotrophic hormones, oxytocin, vasopressin, opioid peptides (enkephalins, endorphins, dynorphins), vasoactive peptides (neurotensin, bradykinin, atrial and brain natriuretic peptides, angiotensin II) and gastrointestinal peptides (vasoactive intestinal polypeptide, VIP, pituitary adenylate cyclase activating polypeptide, PACAP, cholecystokinin, tachykinins, galanin, and neuropeptide Y).

These peptides, besides neurohormonal activity, may have neurotransmitter (neuromodulator) activity influencing hypothalamo-hypophysial regulatory mechanisms and/or may serve as a neuronal link between the endocrine hypothalamus and extrahypothalamic brain regions, especially the limbic system and autonomic regulatory regions in the lower brain stem.

7.1 Characteristics of the Hypothalamic Cell Groups

A crude quantitative analysis, based on the cell density, shape, and size of the neurons, reveals that the known hypothalamic cell groups are not at all homogeneous and several subgroups can be distinguished. This became even more evident from the data obtained by immunocytochemistry. For example, the paraventricular nucleus contains vasopressin, oxytocin, SRIH, GHRH, CRH, TRH, and immunoreactive neurons. The suprachiasmatic nucleus, repre-

senting a key structure of the biological clock, is composed of vasopressin, VIP, SRIH, substance P, GABA, and gastrin-releasing peptide containing nerve cells. In the arcuate nucleus, there are dopamin, GHRH, enkephalin, galanin, substance P, GABA, atrial natriuretic peptide (ANP), neurotensin, gastrin-releasing peptide, and glutamat-containing neurons. The situation is further complicated by the colocalization of various neuropeptides already referred to. In the paraventricular nucleus there are magnocellular vasopressinergic neurons which may contain dynorphin, angiotensin II, enkephalin, galanin, VIP; oxytocinergic cells containing also CRH, dynorphin, enkephalin and parvocellular CRH neurons in which there is also vasopressin, enkephalin, neurotensin, galanin, and GABA. Similar colocalizations of neuropeptides are known for neurons of the suprachiasmatic, arcuate, and other hypothalamic cell groups. The functional significance of colocalization of substances in the nerve cells needs to be clarified.

The paraventricular nucleus is unique among hypothalamic cell groups in housing substantial populations of cells that participate in the control of anterior and posterior pituitary secretions. This nucleus is the predominant source of CRH in hypophysial portal plasma. In addition, the paraventricular nucleus is acknowledged, along with the supraoptic nucleus as a principal seat of magnocellular neurosecretory neurons, which synthesize the nonapeptide hormones oxytocin and arginine vasopressin for release into the general circulation from terminals in the posterior lobe. A third major cell type comprises neurons that give rise to long descending projections to the brain stem and spinal cord that include sensory and motor structures associated with the autonomic nervous system. These three visceromotor populations are essentially separate and exhibit a high degree of topographic organization. Upon this high degree of anatomical organization is imposed a somewhat imprecise manner of chemical coding.

Concerning connections between neuronal elements in the hypothalamus quantitative analysis of the synaptic organization of the rat supraoptic nucleus revealed that there are over five million boutons on each side of this nucleus, an average of 600 per neuron. There is a sexual dimorphism in the neuropil of the rat preoptic area and this depends on neonatal androgen. In the normal female, the number of non-amygdaloid synapses on dendritic spines in the area is higher than in the male. Interestingly, castration of the male within 12 h after birth causes an increase in the number of spine synapses compared with females of similar age. Conversely, females treated on d four (but not on day 16) with testosterone propionate have a low number of spine synapses within the male range. In accordance with these findings, neonatal estradiol treatment results in an increased number of axodendritic synapses in the arcuate nucleus.

The neurons of a nucleus display rich intrinsic connections. A local network of fibers seems to be a common feature of neuroendocrine hypothalamic nuclei. For example, a significant part of axon terminals in the supraoptic nucleus appear to be of intranuclear or otherwise of local origin. This could be because of either numerous intranuclear axon collaterals or interneurons with richly arborising axons, or possibly both. There are intranuclear synaptic connections between hypophysiotrophic neurons producing the same neurohormone or synthesizing different peptides.

Local circuit neurons may synchronize the activities of peptidergic neurons in a hypothalamic nucleus to integrate or coordinate them as a functional unit. Recurrent axon collaterals of hormone-producing peptidergic neurons may provide the morphological basis for an ultrashort feedback mechanism.

7.2. Intrahypothalamic Connections

Information on the intrahypothalamic connections is rather scanty. Studies with lesions or using autoradiography indicate that there may be a large number of such connections. It is well corroborated by both neurohistological and electrophysiological data that arcuate neurons, for example, besides projecting to the external layer of the median eminence, also project to several other regions including the ventromedial nucleus, lateral hypothalamic area, anterior hypothalamic, and preoptic area, and receive afferents from, among others, the medial preoptic area, the ventromedial nucleus and presumably from several other hypothalamic regions. Peptidergic projections from the arcuate nucleus to other hypothalamic neurons are also known. There is evidence that the ventromedial nucleus has abundant intrahypothalamic connections. Ventromedial neurons project to the arcuate nucleus, anterior hypothalamic nucleus, preoptic area, suprachiasmatic nucleus, lateral hypothalamus, supramammillary area.

The abundant intranuclear connections and in addition the rich connections between the various hypothalamic cell groups support the general impression that the hypothalamus should be considered a neuronal network of quasirandom internal connections.

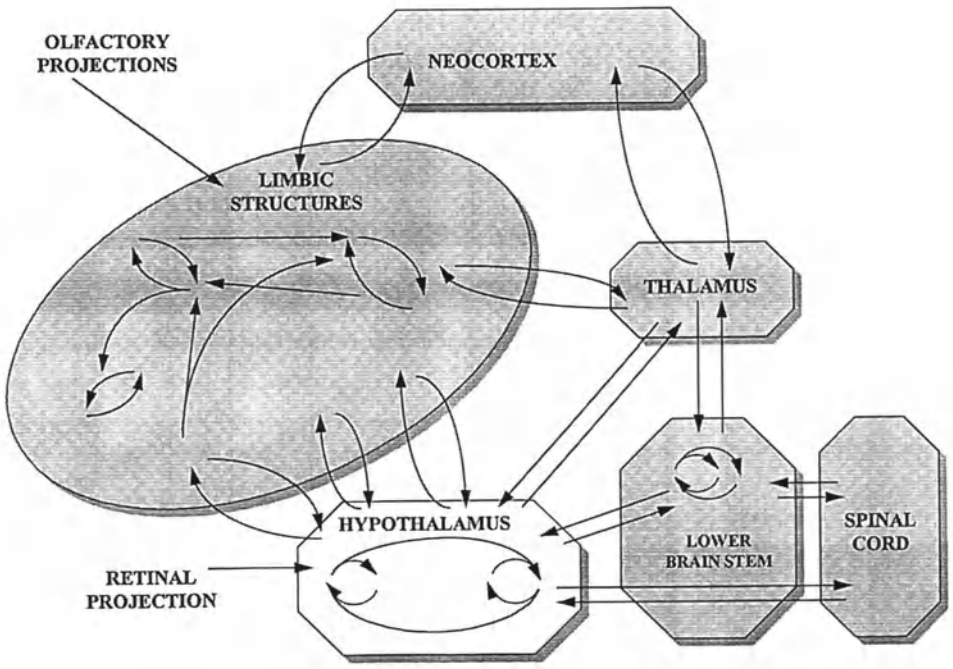

Fig. 6. A very simplified scheme of the reciprocal neural connections within the hypothalamus (intranuclear and internuclear) and between hypothalamic and extrahypothalamic structures.

In this network, in which the impulses leave the hypothalamus through the main axons, excitation can spread from a given focus in any direction and can establish an infinite number of closed, self-reexciting chains.

7.3. Hypothalamic Output and Input Channels

Both output and input channels of the hypothalamus are of two kinds, i.e., humoral and neural.

7.3.1. Output Channels

7.3.1.1. Humoral Output Channels. These channels, represented by the classical magnocellular neurosecretory supraoptico- and paraventriculo-hypophysial system producing oxytocin and vasopressin and the neurosecretory system secreting trophic hormone-releasing and release-inhibiting hormones, have been described briefly in former sections.

7.3.1.2. Neural Output Channels (Fig. 6). Anatomically, the connections between the hypothalamus and the rest of the brain are dominated by certain limbic structures and the lower brain stem. The efferent pathways of the hypothalamus appear to reciprocate several of the major afferent hypothalamic connections. Many such reciprocating connections are contained in the medial forebrain bundle, the dorsal longitudinal fasciculus, the stria medullaris, and the stria terminalis. These pathways appear to close neuronal circuits between the hypothalamus, on the one hand, and the mesencephalon, pons, thalamus and several of the limbic forebrain structures on the other. The major brain structures receiving hypothalamic efferents are the amygdala, hippocampus, septum, thalamus, lower brain stem, and spinal cord.

7.3.2. Input Channels

As the hypothalamus is deeply involved in homeostatic adjustments, it needs abundant information from both the external and internal environment, and a rich afferent input is indeed evident. The information that flows continuously into the hypothalamus is carried in two ways along humoral and neural pathways.

7.3.2.1. Humoral Input Channels. Several parameters of the blood influence hypothalamic functions. One of these is the blood hormone level. As discussed briefly in Section 6, various hormone receptors exist within the hypothalamus. The estrogen and androgen binding cells are concentrated in the medial preoptic area, medial anterior hypothalamus, ventromedial nucleus, arcuate nucleus, and ventral premammillary nucleus.

The sex-steroid feedback is partly involved in pituitary gonadotropic hormone secretion, exerting both a negative and a positive feedback action, and partly in sexual behavior. With regard to the latter function,

the preoptic area and the anterior hypothalamus appear to be primarily involved. Direct implantation of estrogen into these regions induces lordosis in ovariectomized rats. Corticosteroids do not appear to be concentrated in the cells of the hypothalamus in contrast to certain limbic structures.

As mentioned in Section 6, pituitary tropic hormones appear to exert a direct feedback action (short feedback) on the basal region of the hypothalamus. FSH, LH, PRL, GH, and ACTH implanted into the medial basal hypothalamus influence the hormone secretion of the adenohypophysis. Also hypothalamic releasing hormones may have a direct feedback action (ultrashort feedback) on the hypothalamus itself.

In addition to hormone receptors, there are also other receptors such as cytokine receptors, thermoreceptors, glucoreceptors, leptin receptors (leptin is a protein secreted by adipocytes and implicated in the maintenance of energy balance) in the hypothalamus.

7.3.2.2. Neural Input Channels (Fig. 6). A large number of regions are projecting to the hypothalamus including the amygdaloid complex, hippocampus, septum, thalamus, basal ganglia, cortex, lower brain stem, and spinal cord. Although the hypothalamus receives a large amount of sensory input through the regions mentioned, there are also some more or less direct pathways. There is a direct projection from the retina to the hypothalamus, primarily to the suprachiasmatic nucleus. The effect of light on the hypothalamus, particularly on its control of the adenohypophysis, may be mediated by this pathway. Inputs from the olfactory bulb have a relatively free access to the hypothalamus, although direct connections from the olfactory bulb to the hypothalamus are not known. The piriform cortex, which receives fibers from the olfactory bulbs, sends projections directly to the hypothalamus. Other olfactory pathways reach the hypothalamus mainly via the amygdala.

An increasing body of evidence suggests that there are afferent and efferent neural connections between the gonads and the hypothalamus as well as the adrenal gland and the hypothalamus.

Before summarizing the structural features of the hypothalamus, mention should be made of the overlap in the regions involved in the various hypothalamic functions—such as the regulation of body temperature, food and water intake, control of the anterior pituitary, and the role of the hypothalamus in sexual behavior. Presumably, there is a link between several functions such as temperature regulation and the control of pituitary TSH secretion, pituitary gonadotropic function and sexual behavior, water intake and the magnocellular neurosecretory system, and so on.

The following conclusions may be drawn on the basis of the organization of the hypothalamus.

The hypothalamus functions partly as an endocrine organ and partly as nervous tissue (Fig. 7). As an endocrine organ, it synthesizes hypophysiotrophic neurohormones and posterior pituitary hormones. It receives an abundance of information streaming in continuously along neural and humoral channels. Most of the hypothalamic connections are reciprocal. Neuronal networks, presumably forming circuits, may exist at all structural levels within hypothalamic cell groups, between various hypothalamic nuclei and areas, as well as between hypothalamic and different extrahypothalamic regions. The circuits are presumably closely interconnected and are partly excitatory and partly inhibitory. This arrangement suggests that the hypothalamus is an open-loop system.

The reciprocal connections of the hypothalamus with limbic forebrain structures and the lower brain stem are of such magnitude that it appears possible to interpret the hypothalamus, at least partly, as a way-station in both the ascending and descending limbs of a polysynaptic neural circuit that extends between the limbic forebrain, on the one hand, and the primarily paramedian mesencephalic region on the other. It may be assumed that the functional state of the hypothalamus is determined, to a significant extent, by the neural events that take place in the limbic structures and the lower brain stem; both having a very integrated structural organization with several reciprocal interconnections and neural circuits. In addition, they receive a vast amount of information from both the external and internal environment flowing in along neural and humoral pathways (there are hormone receptors in the hippocampus, amygdala).

The extremely complex neuronal network of the hypothalamus and its reciprocal connections suggest that, apart from a few exceptions such as the supraoptico- and paraventriculohypophysial system, there are not well-defined regions and pathways that are specifically and exclusively concerned with a discrete hypothalamic function. Of course, this does not exclude the predominance of one or the other hypothalamic area in the involvement of a particular hypothalamic function. Instead of a mosaic type pattern, the hypothalamus can rather be envisaged as some kind of computer. This computer has a number of built-in programs, and its elements are involved in several processes. It elaborates the solution for each actual situation on the basis of a wealth

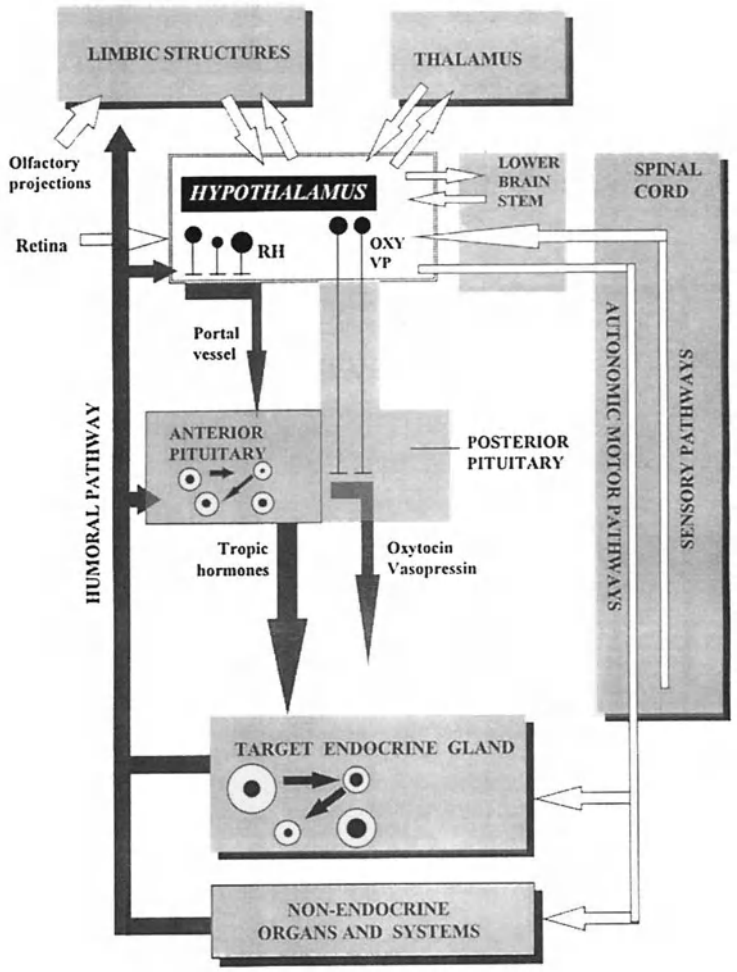

Fig. 7. Summary of the main output and input channels of the hypothalamus. Both are humoral (marked with gray arrows) and neural pathways (empty arrows). Black arrows within ANTERIOR PITUITARY and TARGET ENDOCRINE GLAND box indicate autocrine and paracrine mechanisms. RH: releasing hormone producing neurons; OXY, VP:nerve cells producing oxytocin and vasopressin and releasing it into the capillaries of the posterior pituitary.

of information that is partly stored and partly streaming in continuously by way of neural and humoral channels. The results are then distributed over a number of neural and humoral output channels.

Details on the structural organization of the hypothalamus are dealt with in Chapter 2.

8. NEUROENDOCRINOLOGY BEING CLOSELY LINKED TO IMMUNOLOGY

There is a close link between the neuroendocrine system and the immune system. Originally, it was a fairly generally accepted view that the immune system is a totally autonomous and self-regulating unit. Later on, it was assumed, on the basis of Selye's observation of thymic involution during stress, that steroid hormones are the sole factors in neuroendocrine modulation of the immune system. Recent studies indicated clearly that the two systems are linked to each other reciprocally and profoundly. According to our present knowledge:

1. Peptide hormones are produced by the immune system.
2. Hormones and neurohormones are involved in the regulation of the immune system.
3. Cytokines produced by the immune system are influencing the neuroendocrine system.

8.1. Peptide Hormones Synthesized by Cells of the Immune System

Lymphocytes produce various peptide hormones including anterior pituitary hormones, trophic hormone-releasing and release-inhibiting hormones, posterior pituitary hormones, and many other neuropeptides. There is evidence that all anterior pituitary hormones (i.e., proopiomelanocortin-derived ACTH and β-endorphin, TSH, LH, FSH, GH, and prolactin) are synthesized by lymphocytes. Likewise, lymphocytes produce CRH, GHRH, and GnRH as well as arginine vasopressin and oxytocin. A number of other neuropeptides including VIP, SRIH, neuropeptide Y, calcitonin gene-related peptide, and insulin-like

growth factor I (IGF-I), are also synthesized by cells of the immune system. Preliminary results indicate that there may be some cell specificity within the immune system to certain hormonal responses. However, very little information is available on the production of peptide hormones by particular cell types within the immune system. No doubt, the cellular origin of the peptide appears to depend on the particular hormone and the stimulus for production.

Peptide hormones derived from cells of the immune system, may function as endogenous regulators of the immune system exerting autocrine or paracrine immunoregulatory functions. There are peptide hormone receptors on lymphocytes. Cells of the immune system possess arginine vasopressin receptor (V1 receptor), corticotropin receptor, β-endorphin receptor, GH receptor, prolactin receptor, TSH receptor, SRIH receptor, CRH receptor, GHRH receptor, and GnRH receptor. Many of these receptors have characteristics that are identical to those receptors found on cells of the neuroendocrine system. Data on the human immune system have convincingly demonstrated that prolactin acts as a cytokine in that it is released within the immune system and regulates the lymphocyte response by paracrine and autocrine mechanisms. Peptide hormones synthesized by cells of the immune system may also act on cells of the neuroendocrine system.

8.2. Action of Peptide Hormones on the Immune System

It is clear that peptide hormones can directly modulate immune functions. Arginine vasopressin, TSH, ACTH, GH, prolactin, SRIH, VIP, and endorphins can control many important immune functions such as affecting cytokine production (cytokines are locally released factors regulating the immune response), stimulating or inhibiting antibody production, as well as of lymphocyte cytotoxicity and proliferation, modulation of macrophage and neutrophil leukocyte functions, initiation of hypersensitivity events and possibly some immune complex diseases, and enhancement of thymic regeneration. It remains to be clarified to what extent are these actions exerted by peptide hormones derived from cells of the immune system or from cells of the neuroendocrine system.

8.3. Action of Cytokines on the Neuroendocrine System

Cytokines can affect directly the function of the neuroendocrine system. Interleukin-1 (IL-1) is a very potent activator of the hypothalamic pituitary adrenal axis acting both at the hypothalamic (on CRH release) as well as at the pituitary level. IL-1 may also act on TSH, GH, PRL, and LH release. There are cytokine receptors in the CNS and in endocrine glands. Furthermore, astrocytes and microglia cells as well as the adrenal gland, synthesize cytokines. Besides cytokines, peptide hormones produced by cells of the immune system may also act on the neuroendocrine system. In this latter case, these actions may be in concert with the action of cytokines produced by lymphocytes and influencing neuroendocrine functions.

In summary, there is a profound bidirectional communication between the immune system and neuroendocrine system (Fig. 8). The neuroendocrine peptides are endogeneous to the immune system and may be used for both intraimmune system regulation as well as for bidirectional communication between immune and neuroendocrine system. Likewise, cytokines of the immune system are evident in neural and endocrine tissues and can exert profound effects on the neuroendocrine system.

9. NEUROENDOCRINOLOGY FERTILIZING OTHER FIELDS OF NEUROSCIENCE

The demonstration of hypophysiotrophic neurohormones in nerve cells of various brain regions outside the hypothalamus significantly contributed to the broad interest of peptide research in neuroscience. It stimulated, among others, neuroanatomical investigations, which led to the notion that a very large number of peptides and peptide receptors are present in neurons; not only in regions known to be involved in the neural control of pituitary hormone secretion, but also in structures participating in various other brain functions. Thus, they may be considered as neurotransmitters or neuromodulators. The wide distribution of peptides within the CNS had a very stimulatory effect, not only on neuroanatomy, but also on neurophysiology, neurochemistry, neuropharmacology, neuropsychology, developmental neurobiology, and neuropathology, mentioning just a few fields of neuroscience. Studies on the effect of various peptides on the CNS indicated, among others, effects on learning and memory processes, on social behavior, maintenance, maternal and sexual behavior, on temperature, nociception, rewarded behavior, and drug tolerance. Some of the peptides exhibited neurotrophic effects,

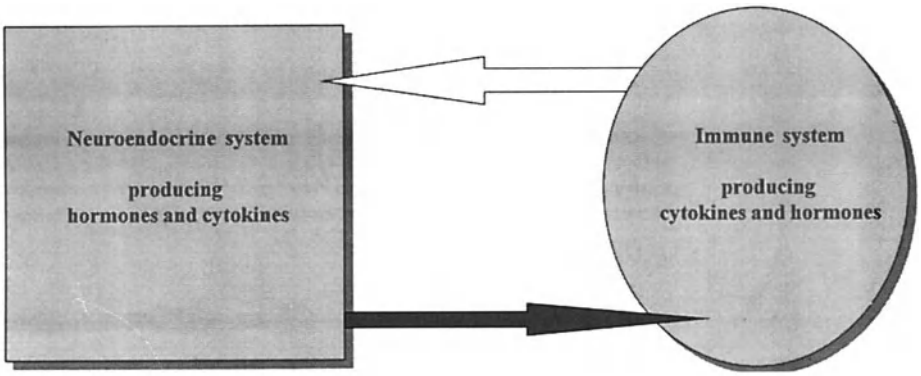

Fig. 8. The neuroendocrine system and the immune system affect each other, both are synthesizing hormones and cytokines. The action of the neuroendocrine system on the immune system is mediated by hormones, and in the opposite direction, presumably mainly by cytokines.

whereas others exhibited neuroleptic-like properties as well.

10. CONCLUSION

The hypothalamus functions partly as an endocrine organ and partly as nervous tissue. The endocrine organ is represented by the magnocellular neurons of the supraoptic and paraventricular nuclei synthesizing oxytocin and vasopressin and terminating in the posterior pituitary, and by the nerve cells producing various hypophysiotrophic neurohormones and terminating in the median eminence. Both the supraoptic and paraventricular nuclei are heterogeneous, particularly the latter cell group, synthesizes many different compounds, and their neurons end not only in the posterior pituitary, but also in various regions of the CNS. Nerve cells synthesizing trophic hormone-releasing or release-inhibiting neurohormones are widely distributed. They are present not only in the CNS, but also in nonneural tissues, and usually do not form a solid cell group, but are intermingled with other neurons. The hypothalamus as nervous tissue is characterized by neuronal networks existing at all structural levels within and between cell groups. Furthermore, there are reciprocal connections between the hypothalamus and extrahypothalamic regions, primarily limbic forebrain structures and lower brain stem. The functional state of the hypothalamus is determined partly by factors from the external or internal environment acting directly on the hypothalamus via neural or humoral pathways and partly by the neural events that take place in the limbic structures and the brain stem both having a very integrated structural organization and receiving a vast amount of information from both the external and internal environment flowing in along neural and humoral pathways.

The spectacular development of neuroendocrinology in the last 50 years fertilized different fields of neuroscience. This progress led to a gradual integration of neuroendocrinology into neuroscience. Furthermore, it became evident that neuroendocrinology is also closely linked to other disciplines such as immunology.

SELECTED READINGS

Blalock JE, ed. Neuroimmunoendocrinology. In Ishikaza K, Lachmann PJ, Waksman BH, eds. Chemical Immunology, Vol. 52. New York: Karger, Basel, 1992.

Brann DW, Bhat GK, Lamar CA, Mahesh VB. Gaseous transmitters and neuroendocrine regulation. Neuroendocrinol. 1997; 65:385.

Chadwick DJ, Marsh J, eds. Functional Anatomy of the Neuroendocrine Hypothalamus. Chichester, West Sussex, England: Wiley, 1992.

De Souza EB. Corticotropin-releasing factor receptors: physiology, pharmacology, biochemistry and role in central nervous system and immune disorders. Psychoneuroendocrinol 1995; 20:789.

Ehrhart-Bornstein E, Hinson JP, Bornstein SR, Scherbaum WA, Vinson GP. Intraadrenal interactions in the regulation of adrenocortical steroidogenesis. Endocrine Rev 1998; 19:101.

Flerkó B, Merchenthaler I, Sétáló Gy. Short and ultrashort feedback control of gonadotropin secretion. In: Leung PCK, Armstrong DT, Ruf KB, Moger WH, Friesen HG, eds. Endocrinology and Physiology of Reproduction. New York: Plenum, 1987; 37.

Garcia-Segura LM, Chowen JA, Párducz A, Naftolin F. Gonadal hormones as promoters of structural synaptic plasticity: cellular mechanisms. Prog Neurobiol 1994; 44:279.

Gerendai I, Halász B. Neuroendocrine asymmetry. Front Neuroendocrinol 1997; 18:354.

Halász B. Neuroendocrinology in 1992. Neuroendocrinol 1993; 57:1196.

Imura H, ed. The Pituitary Gland. In: Martini L, ser.ed. Comprehensive Endocrinology. New York: Raven, 1994.

Meites J, Donovan BT, McCann SM, eds. Pioneers in Neuroendocrinology, Vol. I. New York and London, England: Plenum, 1975.

Meites J, Donovan BT, McCann SM, eds. Pioneers in Neuroendocrinology, Vol. II. New York and London, England: Plenum, 1978.

Sawyer CH. Anterior pituitary. Neural control concepts. Endocrinol: People and Ideas. Amer Physiol Soc, 1988: 23.

Stojilkovic SS, Catt KJ. Expression and signal transduction pathways of endothelin receptors in neuroendocrine cells. Front Neuroendocrinol 1996; 17:327.

2 Micro- and Macroscopic Structure, Innervation, and Vasculature of the Hypothalamus

Miklós Palkovits, MD

CONTENTS

1. TOPOGRAPHICAL ORGANIZATION OF THE HYPOTHALAMUS

The topographical organization of the hypothalamus has been the subject of many fine books and book chapters. In the rat, the nuclear pattern of the hypothalamus was first described more than 60 years ago by Gurdjian (1927) and Krieg (1932), and not much can be added to those earlier accounts. A detailed description of the cytoarchitecture of the human hypothalamus has been given by Diepen (1962), Crosby and Showers (1969).

1.1. Major Hypothalamic Units

The hypothalamus may be divided into four major rostro-caudally organized regions: preoptic area, anterior, middle and posterior hypothalamus (Fig. 1). Each area can be further divided into medial and lateral hypothalamic areas. Although, developmentally, the preoptic area is not a part of the hypothalamus (it is of forebrain origin), functionally, and topographically, it is a part of the neuroendocrine hypothalamus. In contrast to the preoptic area, the mamillary body, which is topographically a portion of the hypothalamus, functionally it belongs to the limbic system. Because well-defined nuclei can be localized in the cell-dense medial hypothalamus, the lateral hypothalamus is more loosely arranged and dominated by the fibers of the medial forebrain bundle, therefore, referred to as the lateral hypothalamic area.

1.1.1. PREOPTIC AREA

The *preoptic area* is bordered by the basal forebrain and the lamina terminalis anteriorly, and extends caudally as far as the rostro-caudal half of the optic chiasm (0.6 mm caudal to the level of the bregma—in adult rats), and passes into the anterior hypothalamus (Fig. 1). Dorsally it is bordered by the anterior commissure and the bed nucleus of the stria terminalis. The preoptic area occupies about one fourth of the total hypothalamus in rats (Table 1).

The cell-dense medial preoptic area contains the median (periventricular), medial, and suprachiasmatic preoptic nuclei, whereas the lateral area is occupied by the fibers and intrinsic cells of the medial forebrain bundle. In the midline, the organum vasculosum lami-

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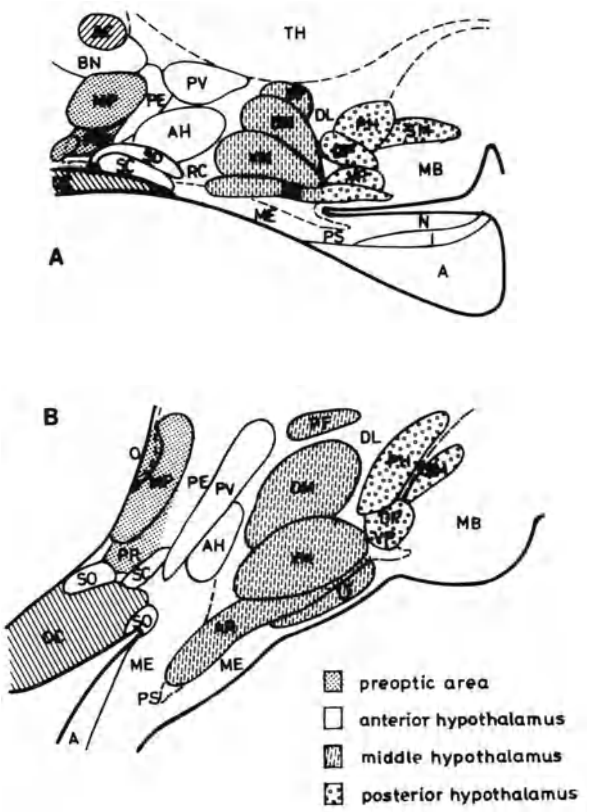

Fig. 1. Topographical distribution of hypothalamic nuclei and major areas in a sagittal view. **(A)** rat hypothalamus; **(B)** human hypothalamus. The third ventricle is outlined by dotted lines. Abbr.: A—anterior pituitary, AC—anterior commissure, AH—anterior hypothalamic nucleus, AR—arcuate nucleus, BN—bed nucleus of the stria terminalis, DL—dorsolateral hypothalamic area, DM—dorsomedial nucleus, DP—dorsal premamillary nucleus, I—intermediate pituitary lobe, LT—lateral tuberal nuclei, MB—mamillary body, ME—median eminence, MP—medial preoptic nucleus, N—posterior pituitary, O—organum vasculosum laminae terminalis, OC—optic chiasm, PE—hypothalamic periventricular nucleus, PF—perifornical nucleus, PH—posterior hypothalamic nucleus, PP—preoptic (median) periventricular nucleus, PS—pituitary stalk, PV—paraventricular nucleus, RC—retrochiasmatic area. SC—suprachiasmatic nucleus, SM—supramamillary nucleus, SO—supraoptic nucleus, TH—thalamus, VM—ventromedial nucleus, VP—ventral premamillary nucleus.

nae terminalis (it is also referred as "supraoptic crest") is located around the rostral tip of the third ventricle. This circumventricular organ, which is outside of the blood-brain barrier, may serve as an open gate for humoral inputs to the preoptic area.

1.1.2. Anterior Hypothalamus

The *anterior hypothalamus* is a caudal continuation of the preoptic area. It extends caudally as far as the rostral beginning of the median eminence (Fig. 1). The medial part of the anterior hypothalamus consists of the periventricular, suprachiasmatic, anterior hypothalamic, and paraventricular nuclei, whereas its lateral part contains the supraoptic nucleus and the cells and fibers of the medial forebrain bundle. Several magnocellular neurons form small islands in both the medial and lateral part of the anterior hypothalamus. Caudally, the medial and lateral retrochiasmatic area occupy the most ventral part of the anterior hypothalamus.

Table 1
Weights of the Hypothalamic Regions of Adult (200 ± 10 g) Rats, *n* = 14

Regions	*Weight (mg) Mean ± S.E.M.*	*Percentage of Total Hypothalamus*
Preoptic area	6.63 ± 0.35	24.18
medial 2.81 ± 0.22		
lateral 3.82 ± 0.47		
Anterior hypothalamus	8.30 ± 0.31	30.27
medial 4.03 ± 0.38		
lateral 4.27 ± 0.40		
Middle hypothalamus	8.41 ± 0.22	30.67
medial 4.41 ± 0.29		
lateral 4.07 ± 0.27		
Posterior hypothalamus	4.08 ± 0.10	14.88
medial 2.11 ± 0.18		
lateral 1.97 ± 0.17		
Total hypothalamus	27.42 ± 0.54	

1.1.3. Middle Hypothalamus

Three parts are recognized in the *middle hypothalamus:* the medial-basal, dorsal, and lateral hypothalamus. Almost 40% of the total hypothalamus belongs to this part (Table 1). The middle hypothalamus starts from the retrochiasmatic area, rostrally, and ends at the level of the separation of the pituitary stalk, caudally (Fig. 1). A vertical cut through the fornix and the mamillothalamic tract divides the medial and the lateral parts. The medial one can be further divided by a horizontal cut bisecting the third ventricle into a medial-basal and a dorsal part. The *medial-basal hypothalamus* includes the median eminence, the arcuate and the ventromedial nuclei, whereas the dorsal part is completely occupied by the dorsomedial nucleus. At the border of the medial and lateral parts, the perifornical nucleus is located just dorsal to the fornix. Laterally, the middle part of the hypothalamus is bordered by the optic tract and the internal capsule. At the caudal level of the middle hypothalamus, the median eminence is separating from the hypothala-

mus and it follows further down as the pituitary stalk (Fig. 1).

1.1.4. Posterior Hypothalamus

This is a relatively small part of the hypothalamus (called also premamillary region) which includes the ventral and dorsal premamillary, tuberomamillary, supramamillary and posterior hypothalamic nuclei and the postinfundibular portion of the median eminence (Table 1). The *posterior hypothalamus* is bordered by the mamillary body, ventrally, and the midbrain, caudally (Fig. 1). The posterior hypothalamic nucleus is continuous with the periaqueductal central gray. Two portions of the third ventricle are present in the posterior hypothalamus: the caudal portion of the ventricle that leads into the cerebral aqueduct, and the inframamillary recess with a dead end in the mamillary body.

1.1.5. Lateral Hypothalamus

The *lateral hypothalamus* consists of a fiber system (medial forebrain bundle) which connects the medial neuroendocrine hypothalamus with other major brain areas by cortical, limbic, and autonomic afferent and efferent fibers. Although the cell density in the lateral hypothalamus is lower than that is in the medial hypothalamus, several thousand intrinsic (relay) neurons are located in each portions (preoptic, anterior, middle, and posterior) of the lateral hypothalamus.

2. HYPOTHALAMIC NUCLEI AND AREAS

The topography of the hypothalamic nuclei in the rat has been mapped by several investigators during the past seven decades (Gurdjian, 1927; Krieg, 1932; Bleier and colleagues, 1979; Swanson, 1992). Here, only a brief summary is given about their major topographical and neurochemical parameters.

2.1. Topography of the Hypothalamic Nuclei

Brain nuclei refer to areas where the density of neurons is higher than in the adjacent regions. Based on this definition, 16 hypothalamic nuclei can be distinguished (Table 2). Their rostro-caudal extensions are summarized in Fig. 2. (In the rostro-caudal coordinates of the hypothalamus, the rostral zero plane lies in the bregma level. The bregma is the point of the surface of the skull at the junction of the coronal and sagittal sutures. The bregma level is a vertical plane that corresponds to the bregma outside on the skull, and to the crossover of the anterior commissure inside the brain. This plane is perpendicular to the cortical surface of the brain.)

Table 2
Volume of the Hypothalamic Nuclei of Adult (200 ± 10 g) Male Rats (mm^3), $n = 7$

Nuclei	*Volume*
Preoptic area	
medial preoptic nucleus	1.17 ± 0.06
preoptic periventricular nucleus	0.52 ± 0.06
Anterior hypothalamus	
periventricular nucleus	0.43 ± 0.04
suprachiasmatic nucleus	0.26 ± 0.01
anterior hypothalamic nucleus	1.65 ± 0.05
supraoptic nucleus	0.30 ± 0.02
paraventricular nucleus	0.45 ± 0.03
medial retrochiasmatic area	0.10 ± 0.01
Middle hypothalamus	
arcuate nucleus (I–III parts)	0.60 ± 0.02
ventromedial nucleus	1.67 ± 0.08
dorsomedial nucleus	0.88 ± 0.05
perifornical nucleus	0.32 ± 0.02
median eminence	0.24 ± 0.01
Posterior hypothalamus	
arcuate nucleus (IV–V parts)	0.34 ± 0.02
tuberomamillary nucleus	0.24 ± 0.04
ventral premamillary nucleus	0.16 ± 0.01
dorsal premamillary nucleus	0.19 ± 0.01
posterior hypothalamic nucleus	0.62 ± 0.03
supramamillary nucleus	0.21 ± 0.03

2.1.1. Preoptic nuclei

The *medial preoptic nucleus* is a relatively large group of cells with medial and lateral subdivisions. The medial one includes the so-called "preoptic sexually dimorphic nucleus." Caudally, the medial preoptic nucleus merges the anterior hypothalamic nucleus. In mammals, except primates and humans, luteinizing (gonadotropin) hormone-releasing hormone (LH-RH)-synthesizing neurons are located in this nucleus and their long axons run caudally to the median eminence.

The *preoptic periventricular nucleus* is also called *median preoptic nucleus.* More precisely, the median nucleus is a subdivision of the periventricular one, like the preoptic suprachiasmatic nucleus. All these portions contain cells in a few layers along the third ventricle, which are continuous with the hypothalamic periventricular nucleus, caudally.

The *lateral preoptic area* is a relatively large (Table 2) and diffusively arranged area. It can be divided into a medial, relatively cell-dense area and a lateral area that is occupied by the fibers of the

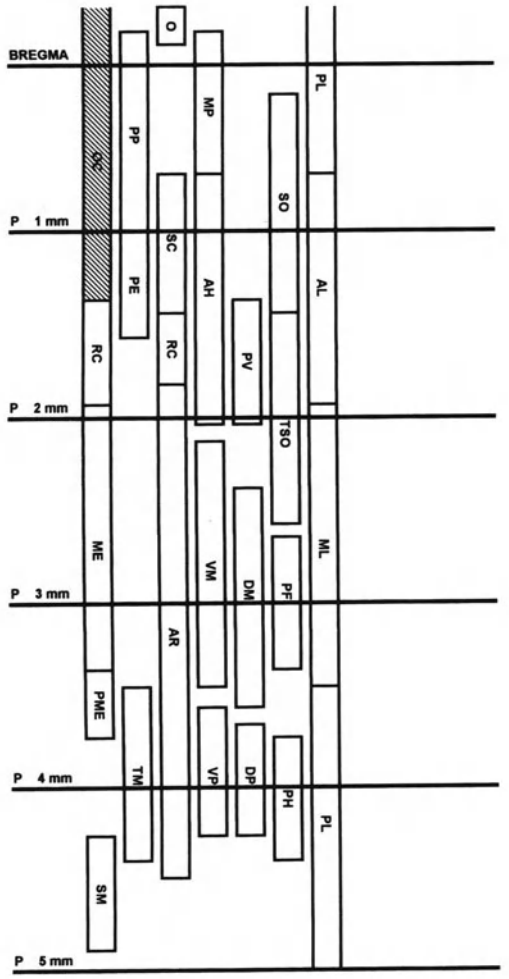

Fig. 2. Rostrocaudal extension of the hypothalamic nuclei of adult (200 g) rats. The zero plane = level of the bregma. P = caudal (posterior) to the bregma in mm. For abbreviations *see* Fig. 1., and lateral hypothalamic area in the preoptic area (PL), in the anterior (AL), middle (ML), and posterior (PL) hypothalamic regions. PME = postinfundibular median eminence, TSO—tuberal supraoptic cells.

medial forebrain bundle. Several fiber systems arise from here, or pass through it in both rostral and caudal directions.

2.1.2. Nuclei of the Anterior Hypothalamus

The *periventricular hypothalamic nucleus* is the caudal continuation of the preoptic periventricular nucleus. It is comprised of a narrow band of cells lying along the third ventricle. This nucleus includes somatostain synthesizing neurons that project to the median eminence and a part of the cells of the A14 catecholaminergic (dopaminergic) cell group. The periventricular cells extends the paraventricular and arcuate nuclei, caudally.

The *suprachiasmatic nucleus,* which serves as a biological clock for neuroendocrine regulations, is found besides the third ventricle, immediately above the optic chiasm. It extends caudally as far as the caudal edge of the chiasm. It comprises a variety of peptidergic neurons.

The *anterior hypothalamic nucleus* is one of the largest hypothalamic nuclei with four subdivisions (Table 2). Rostrally, it is bordered by the medial preoptic nucleus, and caudally by the ventromedial nucleus.

Most of the cells that comprise the *supraoptic nucleus* extend along the lateral edge of the optic chiasm. These cells are exclusively large neurosecretory cells with posterior pituitary projections. Oxytocin-containing cells occupy the dorsal, whereas the vasopressin cells are mainly located in the ventral portion of the nucleus.

The *paraventricular nucleus* lies at the caudal portion of the anterior hypothalamus immediately on the two sides of the third ventricle, just dorsal to the anterior hypothalamic nucleus. Magnocellular neurosecretory and parvocellular subdivisions can be distinguished in the nucleus. Among several neuropeptides, the paraventricular nucleus contains corticotropin-releasing hormone (CRF) and thyrotropin-releasing hormone (TRH) producing neurons with projections to the median eminence. A group of the paraventricular neurons comprise medium-sized neurons that give rise to long descending projections to the brainstem and spinal cord.

The *retrochiasmatic area* is caudal to the optic chiasm at the base of the hypothalamus on either side of the midline. It extends caudally as far as the beginning of the median eminence. This area consists of loosely packed cells and the fibers of the supraoptic decussations (*see* Section 4.1.2.). It can be divided into medial and lateral parts. The *lateral retrochiasmatic area* serves as a gate for fibers entering the median eminence (*see* Section 2.5.2 and Fig. 3).

The *lateral hypothalamic area* in the anterior hypothalamus contains relatively few neuronal cells but numerous nerve fibers. The rostro-caudally oriented *medial forebrain bundle* fibers are crossed by others arching from the paraventricular nucleus to the lateral retrochiasmatic area (paraventriculo-neurohypophyseal tract). Several accessory magnocellular cell groups are embedded in the lateral hypothalamic area.

2.1.3. Nuclei in the Middle Hypothalamus

The *arcuate nucleus* is an elongated cellgroup in the most ventromedial part of the middle and posterior

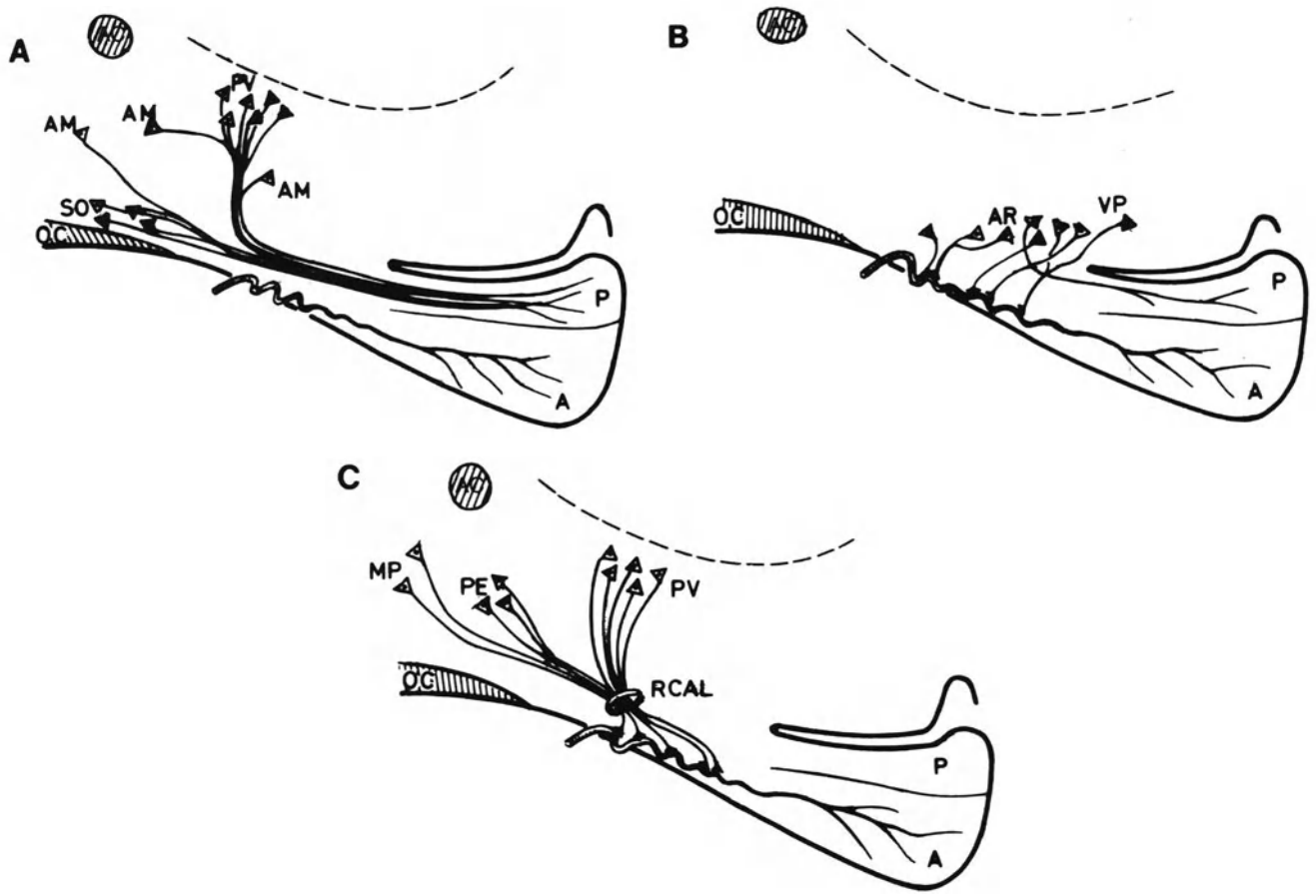

Fig. 3. Hypothalamic projections to the median eminence and the posterior pituitary. **(A)** *magnocellular neurosecretory projections* from the paraventricular, supraoptic, and accessory magnocellular nuclei pass through the internal layer of the median eminence on their way to the posterior pituitary, where oxytocin and vasopressin are released into the systemic circulation. **(B)** *tuberoinfundibular neurons* in the arcuate nucleus project to the external layer of the median eminence. Among others, these cells produce growth hormone releasing hormone, pro-opiomelanocortin, and substance P. **(C)** Neurons mainly in the parvicellular paraventricular, periventricular, medial preoptic nuclei enter the median eminence through the *lateral retrochiasmatic area.* Abbr.: A—anterior pituitary, AC—anterior commissure, AM—accessory magnocellular nuclei, AR—arcuate nucleus, MP—medial preoptic nucleus, OC—optic chiasm, P—posterior pituitary, PE—hypothalamic periventricular nucleus, PV—paraventricular nucleus, RCAL—lateral retrochiasmatic area, SO—supraoptic nucleus, VP—ventral premamillary nucleus.

hypothalamus, mainly on the two sides of the third ventricle. It is made up by five, rostro-caudally oriented subdivisions. The first subdivision is unpaired, lying in the midline under the third ventricle between the medial retrochiasmatic area and the rostral beginning of the median eminence. The two most caudal subdivisions of the arcute nucleus belong to the posterior hypothalamus (Fig. 2, Table 2). Cells in these subdivisions are located on the sides of the inframamillary recess. Arcuate neurons give rise to the tuberoinfundibular system projecting to the median eminence. This nucleus incorporates the A12 catecholaminergic (dopaminergic) cell group, and contains, besides several other neuropeptides, growth hormone-releasing hormone (GRH) and proopiomelanocortin (POMC)-synthesizing neurons.

The *ventromedial nucleus* occupies the major part of the medial-basal hypothalamus. It can be divided into five, easily recognized subdivisions. These subdivisions are consisted of morphologically and functionally distinct neurons with neuronal connections to various components of the limbic system. The nucleus is surrounded by a cell-free "shell" region with dense terminations of hippocampal and amygdaloid neurons.

The *dorsomedial nucleus* is made up of three subdivisions on the both side of the third ventricle, above the ventromedial, and just caudal to the paraventricu-

lar nucleus. Immediately caudal to the dorsomedial nucleus is a region containing loosely arranged cells which is frequently called dorsal or (together with more laterally located cells) dorsolateral hypothalamic area.

The *perifornical nucleus* is comprised of cells over, and partly lateral to the fornix, at the level of the dorsomedial nucleus (Fig. 2). Some of these cells synthesize angiotensin II and project to the paraventricular nucleus and the subfornical organ.

In this part of the hypothalamus, the *lateral hypothalamic area* is relatively rich in neuronal cells, which may relay signals between the medial and the lateral hypothalamus. The most ventral cells are referred to as subfornical nucleus (lateral tuberal nuclei, in humans).

2.1.4. Nuclei of the Posterior Hypothalamus

The *tuberomamillary nucleus* consists of one magnocellular (also called prelateral mammillary nucleus) and three parvocellular subdivisions in the most ventral and the medial part of the posterior hypothalamus. Some of the neurons of this nucleus are the only source of neuronal histamine in the brain.

The *ventral premamillary nucleus* is a small group of cells continuous with the ventromedial and, partially, the arcuate nuclei. At least half of its neurons synthesizes POMC.

The *dorsal premamillary nucleus* lies immediately caudal to the posteromedial part of the ventromedial nucleus at the sides of the inframamillary recess of the third ventricle. The nucleus extends as far lateral as the fornix and the mamillothalamic tract.

The *posterior hypothalamic nucleus* is a relatively large group of diffusely arranged cells (Table 2). The nucleus occupies the dorsal part of the posterior hypothalamus and is continuous with the periaqueductal central gray. It contains dopaminergic cells (A11 catecholaminergic cell group) with spinal cord projections.

The *supramamillary nucleus* is the most caudal nucleus of the hypothalamus immediately dorsal to the mammillary body. Neurons of this unpaired nucleus are horizontally arranged among the fibers of the supramamillary decussations. The nucleus projects to the dentate gyrus and the medial septal/diagonal band nuclei.

In the posterior hypothalamus, the lateral hypothalamic area is bordered by the fornix and the mamillothalamic tract, medially, and the subthalamic Forel's fields, dorsally. It gradually decreases in size and passes to the midbrain ventral tegmental area.

2.2. Principal Topographical Differences Between the Human and Rat Hypothalamus

Although, the general organization pattern of the hypothalamus is similar in mammals, substantial topographical differences can be recognized between the human and the rat hypothalamus.

1. The human hypothalamus can be divided only into three major parts: supraoptic (preoptic area and anterior hypothalamus, in rats), tuberal (middle), and mamillary (posterior) parts (Fig. 1). There is no distinction that can be made between the medial preoptic and anterior hypothalamic nuclei in the human.
2. The tuber cinereum with the funnel-shaped infundibulum is dominating as a protuberance of gray matter on the ventral surface of the middle (tuberal) hypothalamus, which is almost flat in the rat. The lateral tuberal nuclei produce a small eminence (called lateral eminence) on the basal surface of the hypothalamus. The lateral eminence does not exist, as such, in rats.
3. In humans, the posterior (postinfundibular) median eminence is better developed than the anterior one.
4. About 55,000 cells of the paraventricular nucleus are densely packed and arranged vertically along the third ventricle (therefore, it was referred as filiform nucleus in the early years). The nucleus practically incorporates the periventricular neurons, which do not form a separate nucleus, like in the rat.
5. The supraoptic nucleus with about 75,000 neurons lies above the rostral end of the optic tract in human. In rats, it occupies the lateral edge of the optic chiasm.
6. In human, the arcuate nucleus (also called infundibular nucleus in human) surrounds the ventral parts of the third ventricle, while it is almost completely separated by the ventricle, in rats.
7. The well-defined premamillary nuclei are poorly organized in the human hypothalamus. They are partly included in the lateral tuberal nuclei.

2.3. Fine Structure of the Median Eminence Pituitary Stalk

The *median eminence* has long been considered as a final common pathway for output of information from the central nervous system to the endocrine system, particularly to the anterior pituitary. It is a relatively small region in the medial-basal hypothalamus: about 1.7–2.0 mm long, 0.7–1.2 mm wide, and

0.2–0.3 mm thick, in rats. The internal layer contains 20–25,000 fibers in transit directed to the posterior pituitary and the vascular subependymal layer (*see* Section 3.4.3.). Nerve fibers and terminals comprise nearly 60% of the total volume of the external layer of the median eminence. Further constituents are vascular elements like the primary plexus, capillary loops and portal veins, a labyrinth-like pericapillary space, glial cells, and tanycytes. In rats, approximately 40–50,000 axons turn into the external layer. Although, nerve endings show no typical membrane specializations, they contain usual presynaptic structures including synaptic vesicles with a great variety of neuropeptides and neurotransmitters. The wall of the capillaries is fenestrated, no blood-brain barrier exists in the median eminence.

The median eminence is almost devoid of neuronal cell bodies. Neuropeptides and neurotransmitters here are present in fibers from different brain regions, mainly from the hypothalamus. Most of the extrahypothalamic fibers in the median eminence are of lower brainstem origin. They are biogenic amine-containing fibers: serotonergic fibers from the dorsal raphe nucleus, noradrenergic fibers from the A1 and A2, catecholaminergic cell groups from the medulla oblongata, and A6 (locus coeruleus) from the pons.

2.3.1.1. Internal Layer. Most of the fibers here are peptidergic. They are synthesized almost exclusively in hypothalamic neurons and terminate in the posterior pituitary. They arise from the supraoptic, magnocellular paraventricular and accessory magnocellular neurons. Beside oxytocin and vasopressin, fibers in the internal layer contain CRF, somatostatin, prolactin, enkephalins, dynorphins, cholecystokinin, neurotensin, angiotensin II, VIP, galanin, and substance P. They may colocalize with either vasopressin or oxytocin. Proopiomelanocortin (POMC)-containing fibers do not project to the posterior lobe, but terminate around the subependymal plexus in the internal layer. These axons are of arcuate nucleus origin.

2.3.1.2. External Layer. Fibers terminating in the external layer arise in various hypothalamic nuclei (Fig. 3).

1. Tubero-infundibular fibers arise mainly in the arcuate, and in small numbers, in the ventral premamillary and ventromedial nuclei. These neurons may synthesize POMC, growth hormone-releasing hormone, neurotensin, substance P, galanin, dopamine, and probably GABA. In certain conditions, arcuate neurons are able to express somatostatin mRNA.
2. Various types of peptidergic fibers arise from parvicellular paraventricular neurons. They may contain TRH, CRF, vasopressin, oxytocin, enkephalins, cholecystokinin, VIP, neurotensin, angiotensin II, and atrial natriuretic polypeptides.
3. Somatostatin-containing fibers in the external layer arise in the hypothalamic periventricular nucleus.
4. LH-RH-containing neurons project axons to the external layer after a long run in the lateral hypothalamus.

Fibers in groups 2–4 all enter the median eminence from a rostro-lateral direction passing through a delicate gate called *lateral retrochiasmatic area.*

Neuropeptides in the median eminence may have two major physiological roles:

1. *Neurohormonal role:*
   a. After their release from nerve terminals into the portal circulation, neuropeptides reach the anterior pituitary and exert their effects on pituitary cells.
   b. Neuropeptides transported by the paraventriculo-neurohypophysial tract through the internal layer to the posterior pituitary where they stored. From here, they are released through the pituitary veins into the general circulation and exert their hormonal effects in the periphery.
2. *Neuromodulatory Role:* Neuropeptides may act locally in the median eminence stimulating or inhibiting the release of other neuropeptides and transmitters from the nerve terminals and their uptake into the portal circulation. This action may be treated as a presynaptic action on neurohormonal neurons.

2.4. Structure of the Magnocellular Hypothalamic System

The magnocellular neurosecretory system consists of the *supraoptic, paraventricular,* and *accessory magnocellular nuclei.* The main hormones of these nuclei are oxytocin and vasopressin. At least 8 additional neuropeptides (putative neurotransmitters) have been identified in the magnocellular neurons coexpressed within oxytocin or vasopressin neurons (*see* Section 2.3.). There is a spatial distribution of vasopressinergic and oxytocinergic neurons within the supraoptic and paraventricular nuclei. In the supraoptic nucleus, vasopressin-synthesizing neurons occupy the ventral and the oxytocin-synthesizing neurons occupy the dorsal half of the nucleus. In the

paraventricular nucleus, oxytocin cells are concentrated in the anterior and medial parts of the magnocellular portion, whereas vasopressin cells tend to be concentrated posteriorly and laterally. Vasopressin- and oxytocin-synthesizing neurons form dense clusters in the anterior hypothalamus between the supraoptic and paraventricular nuclei and such accessory magnocellular nuclei occur in the preoptic area and the lateral hypothalamus. Fibers of these cells join the *hypothalamo-neurohypophyseal tract.* The tract arises is the paraventricular nucleus, the fibers run laterally and arch over the fornix to enter the lateral hypothalamus (Fig. 3A). (Until here, it may be called paraventriculo-hypophyseal tract). Then, they turn ventrally and dorsal to the supraoptic nucleus course medially to enter the median eminence through the lateral retrochiasmatic area. Here, fibers from the supraoptic nucleus and the accessory magnocellular cells join the tract. In the median eminence, the hypothalamo-neurohypophysial tract occupies the internal layer, runs through the pituitary stalk to enter and terminate in the posterior pituitary. In addition to this, vasopressin- and oxytocin-containing fibers arise in other subdivisions of the paraventricular nucleus which project to several brain areas (*see* Sections 4.3.2. and 4.3.4.).

Afferent fibers to magnocellular supraoptic and paraventricular neurons arise mainly in the lower brain stem, preoptic, and hypothalamic nuclei, and areas (*see* Sections 4.2. and 4.4.). The subfornical organ-preoptic-hypothalamic neuronal circuit (Fig. 4B) seems to be the major neuronal organization in the central control of the body salt and water homeostasis.

2.5. Structure of the Parvicellular Hypothalamic System

Hypothalamic and preoptic neurons associated with the synthesis, transport, and release of hypophysiotrophic hormones are referred to the *parvicellular neurosecretory system.* These neurosecretory neurons possess the ability to express multiple biologically active molecules. These neurons synthesize peptides that are transported by the axons down to the external layer of the median eminence (Fig. 3). Here, these substances are released into the pericapillary space from where they enter the portal circulation and reach the anterior pituitary.

Considering their topographical distribution, the parvicellular neurosecretory system can be classified into two major groups: tubero-infundibular and others that enter the median eminence through the lateral retrochiasmatic area.

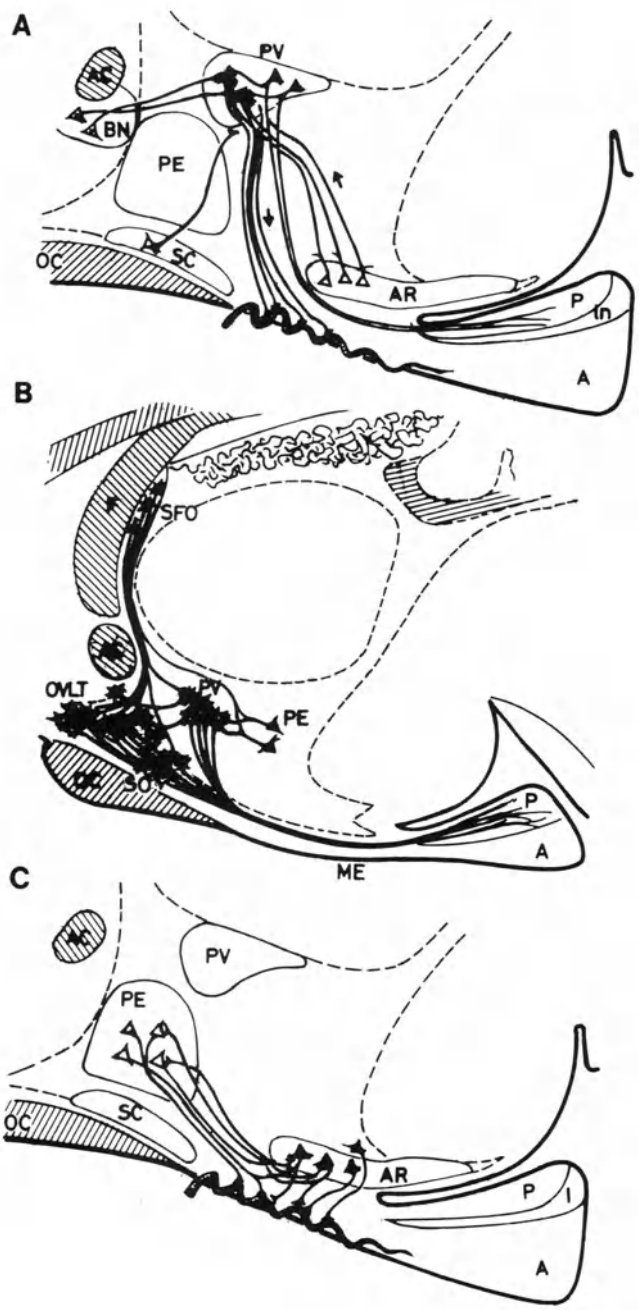

Fig. 4. Intrahypothalamic neuronal circuits. **(A)** Intrahypothalamic projections to the paraventricular CRF neurons. **(B)** Organization of the hypothalamic regulation of the salt and water homeostasis. **(C)** Somatostatin—growth hormone releasing hormone interaction in the hypothalamus. Abbr.: A—anterior pituitary, AC—anterior commissure, AR—arcuate nucleus, F—fornix, I—intermediate pituitary lobe, ME—median eminence, OC—optic chiasm, OVLT—organum vasculosum laminae terminalis, P—posterior pituitary, PE—hypothalamic periventricular nucleus, PF—perifornical nucleus, PP—preoptic periventricular (median) nucleus, PV—paraventricular nucleus, SC—suprachiasmatic nucleus, SFO—subfornical organ, SO—supraoptic nucleus.

2.5.1. Tubero-Infundibular Neurons

Perikarya of these neurons are mainly located in the arcuate nucleus, some of them are present in the ventral premamillary and ventromedial nuclei (just at the close vicinity of the arcuate nucleus). After a short run, the tubero-infundibular axons enter the median eminence, cross the paraventriculo-neurohypophyseal tract, and terminate mainly in the lateral portion of the external layer (Fig. 3B).

The major group of the tubero-infundibular neurons is peptidergic, whereas the others synthesize dopamine (A12 catecholaminergic cell group), acetylcholine and GABA. The peptidergic neurons synthesize POMC (ACTH, β-endorphin, and p-MSH), neurotensin, enkephalins, dynorphins, galanin, and NPY. Growth hormone-releasing hormone is produced by tubero-infundibular neurons located in the arcuate nucleus and between the arcuate and the ventromedial nuclei.

2.5.2. Neurons Project Through The Lateral Retrochiasmatic Area

The common feature of these neurons is that their axons enter the median eminence through the *lateral retrochiasmatic area* (RCAL). This gate is a discrete small area at the caudal edge of the optic chiasm, approx 1.0 mm lateral to the midline in adult rats. Numerous peptidergic fibers of various origin pass this gate to enter the median eminence (Fig. 3C): (1) Luteinizing hormone-releasing hormone (LH-RH)-containing fibers arise in the medial preoptic nucleus. From there, fibers enter the lateral preoptic area, and among the fibers of the medial forebrain bundle they run caudalwards until the RCAL. (2) Somatostatin-containing fibers leave their periventricular perikarya in the anterior hypothalamus and after a loop-like run they enter the RCAL. In the median eminence, somatostatin-containing fibers terminate ipsilateral to their periventricular origin. (3) The major component of these cells are present in the parvicellular subdivisions of the paraventricular nucleus. Thyrotropin releasing hormone (TRH), corticotropin releasing hormone (CRF), vasopressin, oxytocin, enkephalins, dynorphins, angiotensin II, and atrial natriuretic hormones (ANF) are synthesized in these neurons, some of them coexpressed in the same neuron. Fibers leave the nucleus in lateral direction arching over (a small portion of them below) the fornix, and after a short run in the lateral hypothalamus they enter the median eminence through the RCAL. The vast majority of these fibers terminate on only one side of the median eminence, ipsilateral to the origin of fibers in the paraventricular nucleus. A small portion of the paraventricular neurons (mainly in the periventricular subdivision) do not follow the lateral course, their axons run along the third ventricle in a ventral direction and terminate in the both side of the median eminence.

In addition to peptidergic neurons, noradrenergic axons from the lower brain stem reach the median eminence through the RCAL (*see* Section 4.2.3.).

2.6. Third Ventricle, Ependym, and Periventricular Structures

The *third ventricle* comprises the ventricle system of the diencephalon. Its dorsal part belongs to the thalamus, whereas the ventral part is located in the hypothalamus. It has three recesses: optic, infundibular, and inframamillary.

The rostral end of the third ventricle is formed by the *organum vasculosum laminae terminalis* (OVLT). This circumventricular organ is composed of ependymal cells and a dense capillary network. The capillaries in the OVLT, like in the median eminence, are fenestrated, the organ is outside of the blood-brain barrier. The lateral wall of the OVLT is bordered by tanycyte-like columnar ependymal cells, whereas the floor is covered by flat ependymal cells immediately on the dorsal surface of the optic chiasm.

The wall of the third ventricle is covered by regular, one- or two-layered cuboidal or columnar ependymal cells which possess microvilli and cilia. In the medial-basal hypothalamus, a part of the wall of the third ventricle is covered by tanycyte ependyma. The tanycytes are modified ependymal cells with long, ramifying basal processes. Some of these processes are arching over the arcuate nucleus and reach the ventral surface of the hypothalamus. The apical surface of the tanycytes is devoid of cilia. Their functional role, possible barrier or transport activities are still a subject of debates.

The inframamillary recess is covered by multilayered tanycyte ependyma.

3. VASCULATURE OF THE HYPOTHALAMUS

The angioarchitecture of the hypothalamus is characteristic for several points:

1. The hypothalamus has its own blood supply independent of thalamic and forebrain arteries. All of the hypothalamic arteries derived from the *circle of Willis* (circulus arteriosus Willisi), lying at the base of the diencephalon.

2. The arteries cover each other like shells in a mediolateral direction. The vessels entering at the midline supply the medial and basal parts of the hypothalamus, whereas those entering laterally supply the lateral and dorsal hypothalamus.
3. None of the hypothalamic nuclei is supplied by a single artery, several branches form fine arterial networks in the hypothalamus.
4. The hypothalamo-hypophysial vascular system (*see* Section 3.4.) is a unique feature which constitutes the final common pathway in the neurohormonal regulation of the anterior pituitary.

The blood supply of the individual hypothalamic nuclei in the rat has been described in details (Ambach and Palkovits, 1979).

3.1. Hypothalamic Arteries

The hypothalamic arteries originate directly from the circle of Willis. (The branches forming the circle in human are different from those in the rat: the posterior cerebral artery arises from the internal carotid in the rat, but from basilar artery in human.) They can be classified according to their origin from the different components of the circle. Six major groups of arteries supply the hypothalamus (Table 3):

1. Branches from the *anterior communicating artery* supply the preoptic area.
2. The *anterior cerebral artery* gives numerous branches to the preoptic area and the anterior hypothalamus.
3. The supraoptic nucleus receive blood through branches from the *medial cerebral artery.*
4. The majority of the arteries supplying the hypothalamus arise from the internal carotid artery (from its segment in the circulus arteriosus). These are the suprachiasmatic (to the anterior hypothalamic and the supraoptic nuclei), tuberal (to the ventromedial and dorsomedial nuclei), and hypophyseal arteries. Beside the pituitary gland, the hypophyseal arteries supply the median eminence, the pituitary stalk, and the arcuate nucleus.
5. Branches from the *posterior cerebral artery* are the main vessels of the dorsomedial and premamillary nuclei and the caudal part of the lateral hypothalamus.
6. The posterior hypothalamus and the mamillary body receive the bulk of their supply from the *posterior communicating* artery.

The blood supply of the individual hypothalamic nuclei are summarized in Table 3.

3.2. Hypothalamic Veins

Most of the hypothalamic veins run into the anterior cerebral, basilar, and anterior interpeduncular veins. The anterior cerebral vein follows the identical artery on its lateral side and drains into the basilar vein. The basilar vein is the largest one in the basilar region of the diencephalon, it runs backward on the lateral side of the circle of Willis. Posteriorly, the basal vein passes round the midbrain to enter the great cerebral vein of Galen.

Major venous branches:

1. The *anterior cerebral vein* collects the vessels from the preoptic area and the anterior hypothalamus (preoptic, perioptic, infrachiasmatic veins).
2. The *basilar vein* collects the veins of a large part of the hypothalamus (anterior hypothalamic, retrochiasmatic, tuberal, posterolateral, premamillary veins). The hypophyseal veins are also collected by the basilar vein.
3. Branches of the *anterior interpeduncular vein.* It collects the veins of the posterior hypothalamus and the mamillary body and drains into the basilar vein.
4. From some dorsal regions of the hypothalamus, including the dorsal portions of the paraventricular nucleus, the blood is collected by the thalamic veins, which run dorsally to drain into the vein of Galen.

The venous drainage of the individual hypothalamic nuclei is summarized in Table 3.

3.3. Capillary Density in Hypothalamic Nuclei and Areas

The vascular density of the hypothalamic nuclei is not different from that in other gray matters of the central nervous system except the supraoptic, paraventricular, arcuate, and mamillary nuclei. The capillary beds of the magnocellular cellgroups are exceptionally rich: the highest capillary density was measured in the supraoptic and paraventricular nuclei (twice as high as elsewhere in the brain) followed by the magnocellular tuberoinfundibular nucleus and the accessory magnocellular cell groups in the hypothalamus. The fine structure and the caliber of the capillaries in these nuclei do not differ from those in other parts of the hypothalamus.

The two hypothalamic circumventricular organs (OVLT and the median eminence) are extraordinarily rich in capillaries.

Table 3
Blood Supply of the Hypothalamic Nuclei

Nuclei	*Arteries*	*Veins*
Preoptic area		
medial preoptic nucleus	1 2	10
preoptic periventricular nucleus	1 2	10 11
lateral preoptic area	1 2	10
Anterior hypothalamus		
periventricular nucleus	2 3 6a	10 12 14
suprachiasmatic nucleus	3	10 12
anterior hypothalamic nucleus	1 3	10 11 12
supraoptic nucleus	2 3 4 5	10 11 12
paraventricular nucleus	1 3	10 11 12 13
medial retrochiasmatic area	6a	10 12
lateral hypothalamic area	2 3 4 5	12
Middle hypothalamus		
arcuate nucleus (I–III parts)	6m	15
ventromedial nucleus	4 6a 6m 7	12 15
dorsomedial nucleus	7	12 15
perifornical nucleus	7	15
median eminence	6a 6m 6p	15 17
lateral hypothalamic area	4 7 8	12 15
Posterior hypothalamus		
arcuate nucleus (IV–V parts)	6p 7	12
tuberomamillary nucleus	7 8	15 16
ventral premamillary nucleus	7 8 9	15 16
dorsal premamillary nucleus	7 8 9	15 16
posterior hypothalamic nucleus	8 9	15 16
supramamillary nucleus	9	16
lateral hypothalamic area	7 8 9	16

1 = anterior communicating artery
2 = anterior cerebral artery
3 = retrochiasmatic artery
4 = direct braches from the internal carotid
5 = middle cerebral artery
6 = hypophyseal arteries (a = ant, m = middle, p = post)
7 = tuberal arteries
8 = posterior cerebral artery
9 = posterior communicating artery
10 = anterior cerebral vein
11 = perioptic vein
12 = basal vein
13 = great cerebral vein (Galen)
14 = thalamic veins
15 = tuberal veins
16 = anterior interpeduncular vein
17 = portal veins

3.4. Blood Flow in the Hypothalamo-Hypophysial System

3.4.1. Hypophyseal Arteries, Primary Capillary Plexus

The pituitary, the median eminence, and the arcuate nucleus are supplied exclusively by the three hypophyseal arteries in the rat. The *anterior hypophyseal artery* runs medially to the rostral two-thirds of the median eminence and gives rise to 3–4 twisted branches. The *middle hypophyseal artery* runs 0.2–0.3 mm behind the anterior one and takes part with two branches in supplying a portion of the median eminence rostral to the pituitary stalk. Branches of these two arteries terminate in the anterior pituitary and the ventral surface of the pituitary stalk. The *posterior hypophyseal artery* supplies the post-infundibular median eminence and the posterior pituitary. In humans, vessels in the hypothalamo-hypophysial vascular system are made up from only two, superior and inferior hypophysial arteries.

The hypophyseal arteries entering the median eminence give small, twisted branches that form a superficial capillary network in the palisadic zone, which is called the *primary plexus* of the median eminence. From the primary plexus, capillary loops penetrate into the deeper layers of the median eminence. The walls of the capillaries and loops of the primary plexus

is fenestrated, the median eminence is outside of the blood-brain barrier, i.e., there is a relatively free communication between the capillary lumen and the pericapillary space. Neurohormones transported by axons of the hypothalamic neurons and released into the pericapillary space have free excess into the blood stream of the primary plexus. Close to the midline, especially in the caudal portion of the median eminence, the capillary loops become long and form a *subependymal capillary plexus* by multiple anastomoses along the basal surface of the third ventricle. Some of the loops move laterally and enter the arcuate nucleus.

From the primary plexus and the deep capillary loops the blood is collected by different venous circuits: *portal, subependymal, and arcuate nucleus circuits.*

3.4.2. The Portal Circuit

Six to eight large portal veins run parallel to each other on the basal surface of the median eminence and the pituitary stalk toward the pars distalis of the pituitary gland where they form a network of large capillaries, called pituitary sinusoids. The blood of the sinusoids drain into two *hypophyseal veins* located in the lateral and posterior surfaces of the pituitary gland. These veins pass into the posterior intercavernous sinus.

The portal veins arise in the top of the capillary loops of the primary plexus, forming secondary loops. They are collected into wider branches and finally pour into the portal veins. Apart from the relatively strong portal veins, four to six thinner ones run on the lateral part of the median eminence. These veins receive no branches from the primary plexus and the capillary loops and they may serve as shunts in the portal circulation.

3.4.3. The Subependymal Circuit

Along the ventral surface of the third ventricle, the long capillary loops form an abundant anastomosis under the ventricular ependyma. This subependymal plexus extends from the retrochiasmatic area caudally at the beginning of the pituitary stalk where it is connected with the capillary network of the pituitary gland. In lateral direction, the subependymal plexus is strengthened by several small arteries from the anterior and middle hypophyseal arteries and by some other arterial branches which supply the arcuate nucleus.

No portal veins arise from the subependymal plexus. It drains into the basilar vein through the medial tuberal veins.

3.4.4. The Arcuate Nucleus Circuit

In contrast to other hypothalamic nuclei, the arcuate nucleus is supplied exclusively by the hypophyseal arteries. Two groups of arteries can be recognized as the direct branches from all three hypophyseal arteries, and the fine branches from the primary plexus and capillary loops pass through the median eminence. These represent a vascular link between the arcuate nucleus and the median eminence, as well as through anastomoses with the subependymal plexus between the arcuate nucleus and the pituitary gland (*see* Section 3.4.5.).

The veins of the arcuate nucleus display no connection with either the portal veins or the veins of the adjacent hypothalamic nuclei. The venous branches run to the basal surface of the hypothalamus and pour into the tuberal veins.

3.4.5. "Back-Flow" in the Hypothalamo-Hypophyseal Vessels

Morphological and biochemical examinations of the vascular structure in the median eminence have shown that beside the portal vein there are vessels in the pituitary stalk and the median eminence where the blood flow was directed from the pituitary toward the hypothalamus. The terminal branches of neurohypophyseal arteries are connected with the subependymal plexus that terminates at the ventromedial territory of the arcuate nucleus. The retrograde flow in these vessels has been demonstrated by direct inspection of the floor of the third ventricle in living animals. All branches of the subependymal plexus consist of fenestrated capillaries. This direct vascular connection provides a morphological basis for the short loop feedback between the pituitary and the hypothalamus, which is one of the key signal routes in the neuroendocrine regulatory mechanism.

3.5. Blood-Brain Barrier in the Hypothalamus

There are two hypothalamic structures that lack the *blood-brain barrier:* the *median eminence* and the OVLT. Electron microscopic studies have verified the existence of fenestrated capillaries in these areas. The only structure between the capillaries and the pericapillary space is a highly permeable basement membrane. Both areas contain axons and axon varicosities derived from various hypothalamic and pre-

optic nuclei. These axons are particularly rich in neuropeptides that enter through the pericapillary space into the capillaries. The blood from the capillaries of the median eminence flows down to the pituitary and establish the hypothalamo-hypophyseal portal system (*see* Section 3.4.1). Vessels of the OVLT have an intimate topographical connection with preoptic neurons, but the blood from here is drained by the basilar vein without any direct communication with the hypothalamo-hypophyseal portal system.

Another circumventricular organ, the *subfornical organ,* which has direct neuronal and significant functional connections with the hypothalamus (*see* Section 4.4 and Fig. 4B) is located dorsal to the anterior hypothalamus.

4. INNERVATION OF THE HYPOTHALAMUS

The neuronal inputs and outputs (projections) of the hypothalamus show various appearance. Some fibers run together in compact bundles, they may be called bundles, tracts, or fascicles. Other fibers may run individually or consist of loosely arranged pathways and all of these are summarized below. Large tracts bordering the hypothalamus, like anterior commissure, internal capsule, optic tract, and chiasm, are not discussed except the retinohypothalamic fibers in the optic chiasm.

4.1. Major Neuronal Bundles in the Hypothalamus

4.1.1. Medial Forebrain Bundle

The *medial forebrain bundle* (MFB) is the largest fiber system connecting the hypothalamus to extrahypothalamic brain areas. The longitudinally running fibers occupy almost the entire lateral hypothalamus. This bundle collects a fairly high number of fibers with various destinations from the hypothalamic nuclei, as well as fibers from cortical, limbic, and brain stem areas directed to the hypothalamic nuclei. The MFB contains several fibers of passage connecting the limbic and brain stem areas without any synaptic contact with hypothalamic neurons. In addition to fibers, the lateral hypothalamus contains several thousand neuronal perikarya. Many of them may serve as relay neurons transferring signals to the medially located hypothalamic nuclei.

The MFB starts rostrally from the olfactory bulb, and some fibers join it from forebrain areas, like septum, accumbens nucleus, and the piriform cortex (lateral corticohypothalamic tract). The bundle occupies the lateral part of the preoptic region. The medial half of this area is rich in neuronal perikarya, and is also called lateral preoptic nucleus. The MFB extends in the anterior hypothalamus, and through the entire hypothalamus and terminates in the ventral tegmental area of the midbrain.

4.1.2. Supraoptic Decussations

The *supraoptic decussations* constitute a loop-shaped fiber system with ill-defined origin and destination. On the basis of their topography inside the hypothalamus, three pathways can be distinguished. All three are localized at the posterior edge of the optic chiasm where they run horizontally and cross over in the medial retrochiasmatic area. They arise in the lower brain stem and may terminate in the hypothalamus, subthalamus, lateral geniculate body, and tectum. They contain myelinated fibers mixed with unmyelinated, most probably aminergic, nerve fibers.

4.1.3. Fornix

Although the *fornix* is a characteristic bundle inside the hypothalamus, it constitutes a major neuronal link in the limbic system interconnecting the hippocampus with the septum and the mamillary body. Fibers leave the main bundle of the fornix (postcommissural fornix) along its course of the hypothalamus and terminate in the cell-free zone surrounding the ventromedial nucleus. The smaller portion of the fornix (precommissural fornix) has also fibers with hypothalamic (preoptic) terminations.

4.1.4. Medial Corticohypothalamic Tract

The *medial corticohypothalamic tract* arises in the hippocampus (mainly from the ventral subiculum). The fibers leave the dorsal hippocampus in rostral direction, run with the fornix. After entering the hypothalamus, the medial corticohypothalamic tract emerges the postcommissural fornix and their fibers turn ventrally and run periventricularly down to the medial retrochiasmatic area and the rostral subdivision of the arcuate nucleus. The tract may contain fibers from the hippocampus (subiculum) to the arcuate, ventromedial, and ventral premamillary nuclei.

4.1.5. Stria Terminalis

The *stria terminalis* serves as a major bidirectional pathway between the amygdala and the hypothalamus. Most of the fibers in the stria terminalis are

amygdalofugal: they arise principally from the medial and central amygdaloid nuclei. On the basis of origin and course of the fibers, the stria terminalis has been divided into dorsal, ventral, and commissural components. Fibers in the dorsal component turn caudally below the anterior commissure, pass the preoptic and anterior hypothalamic areas, and terminate around the ventromedial nucleus. Fibers of the ventral component enter the bed nucleus of the stria terminalis from a dorsolateral direction. A portion of the fibers terminate here, whereas others can be followed until the medial preoptic, anterior hypothalamic, ventromedial, and dorsomedial nuclei. The bed nucleus of the stria terminalis relays amygdaloid signals to the hypothalamus and further down to the lower brain stem and the spinal cord. Their hypothalamic fibers terminate mainly in the paraventricular nucleus. A fairly high percentage of the fibers in the stria terminalis are peptidergic (CRF, somatostatin, neurotensin, and enkephalin).

The stria terminalis also contains fibers that are directed to the amygdala. Most of them are of paraventricular nucleus origin and these terminate mainly in the central and medial nuclei.

4.1.6. Stria Medullaris

This is a large bundle within the limbic system having mainly topographical relation to the hypothalamus. Fibers of the *stria terminalis* pass the anterior hypothalamus from the lateral preoptic area in a dorsomedial direction crossing both the stria terminalis and the postcommissural fornix up to the dorsal surface of the thalamus. From here, fibers run caudally and terminate in the habenula.

4.2. Hypothalamic Afferents (neuronal inputs)

By introducing powerful anterograde, retrograde, and more recently transneuronal tract tracing techniques, information about the existence and the fine topography of afferent fibers to the hypothalamus have been increased substantially.

4.2.1. Cortical Afferents

Pathways from the cerebral cortex to the hypothalamus are classified as *cortico-hypothalamic fiber connections.* Pathways with names of medial or lateral cortico-hypothalamic tracts represent limbic rather than neocortical hypothalamic inputs. The existence of direct neocortical-hypothalamic fibers, as suggested by several studies, can not be excluded, but they need further support by using more appropriate techniques.

4.2.2. Limbic Afferents

These can be classified as olfactory, hippocampal, amygdaloid, and septal afferents:

1. Fibers arising from the olfactory bulb form the rostral beginning of the medial forebrain bundle. Apart from these, signals from the olfactory region may reach the hypothalamus through the amygdala.
2. There are direct (*medial corticohypothalamic tract* and *fornix*) and indirect (through a fimbria hippocampi-septal transfer) hippocampal, mainly ventral subicular neuronal inputs to the medial hypothalamus and the preoptic area.
3. The amygdala has close neuronal connections with the hypothalamus. Fibers reach the hypothalamus through two major pathways: the *stria terminalis* (*see* Section 4.1.5.) and the *ventral amygdalofugal pathway.* Fibers of the ventral amygdalofugal pathway are collected in the anterior, the central, and basolateral amygdaloid nuclei. From here, they run rostromedially, enter the lateral hypothalamus, and terminate there at the preoptic area/anterior hypothalamus level.
4. The *septohypothalamic fibers* run in the medial forebrain bundle as far caudal as the supramamillary nucleus. On their way down, collaterals may turn medially to terminate in the middle hypothalamic nuclei. A certain percentage of the supraoptic and paraventricular afferents are of septal origin.

4.2.3. Brainstem Afferents

The majority of the hypothalamic afferents arise in the lower brainstem. These projections are both aminergic and peptidergic. Most of them run upwards either among the fibers of the medial forebrain bundle or through the periventricular system.

1. Ascending *noradrenaline-containing pathways.* Noradrenergic neurons in the lower brainstem innervate the hypothalamic nuclei. Cells are located in the ventrolateral and the dorsomedial medulla (A1 and A2 catecholaminergic cell groups, respectively) and in the locus coeruleus. Fibers from these nuclei form two ascending pathways, the dorsal and ventral noradrenergic bundles. The *dorsal noradrenergic bundle* arise mainly in the locus coeruleus (with some axons of A2 neurons), ascends in the lateral part of the periaqueductal central gray and enters the hypothalamus between

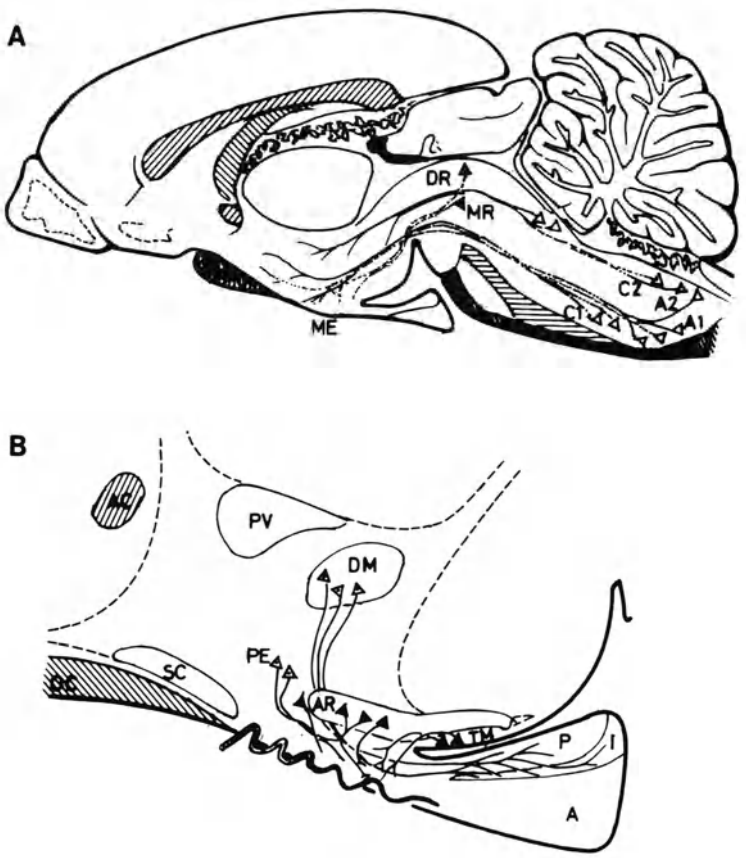

Fig. 5. Biogenic amines in the hypothalamo-pituitary system. Localization of biogenic amine-containing neurons that innervate the median eminence and the pituitary gland. **(A)** *Extra-hypothalamic projections:* Noradrenergic and adrenergic projections from the medulla oblongata and the locus coeruleus; serotoninergic fibers from the midbrain and dorsal raphe nuclei. **(B)** *Intrahypothalamic projections:* projections from the dorsomedial (serotonin), arcuate (dopamine), and tuberomamillary (histamine) nuclei. Abbr.: A—anterior pituitary, A1—A1 noradrenergic cell group in the ventrolateral medulla, A2—A2 noradrenergic cell group in the dorsomedial medulla, AR—arcuate nucleus (A12 catecholaminergic cell group), C1—C1 adrenergic cell group in the rostral ventrolateral medulla, C2—C2 adrenergic cell group in the dorsomedial medulla, DM—hypothalamic dorsomedial nucleus, DR—dorsal raphe nucleus, I—intermediate pituitary lobe, LC—locus coeruleus, ME—median eminence, MR—midbrain raphe nucleus, P—posterior pituitary, PV—paraventricular nucleus, SC—suprachiasmatic nucleus, TM—tuberomamillary nucleus.

the zona incerta and the posterior hypothalamic nucleus. Fibers of this bundle may innervate the posterior, dorsomedial and partly the paraventricular nuclei. The *ventral noradrenergic bundle* is larger than the dorsal one, it is consisted of noradrenergic fibers from the A1 and A2 cell groups. The fibers travel upward in the ventrolateral medulla and in the pontine reticular formation. They enter the territory of the ventral tegmental area and proceed within the medial forebrain bundle. High density of noradrenergic nerve terminals are found in the paraventricular, dorsomedial and periventricular hypothalamic nuclei. Fibers of the ventral bundle give rise to the noradrenergic innervation of the median eminence (Fig. 5).

2. *Ascending adrenaline-containing pathway.* Adrenaline-synthesizing neurons are present only in the medulla oblongata, in close vicinity of the A1 and A2 noradrenergic cell groups, and are called *C1* and *C2 adrenergic cell groups.* Ascending fibers may run together with the ventral noradrenergic bundle up to the hypothalamus and the amygdala.
3. *Ascending serotonergic pathway.* Except for the dorsomedial nucleus, all hypothalamic cell groups contain serotonin exclusively in axons and nerve terminals. They arise from the dorsal and midbrain raphe nuclei. Fibers in the midbrain proceed ventrally then turn rostrally and enter the lateral hypothalamus immediately below the ventral surface of the diencephalon.
4. *Dorsal longitudinal fascicle.* This fascicle serves as a reciprocal avenue for ascending and descending fibers innerconnecting the dorsal and posterior hypothalamus and the pariaqueductal central gray. Some ascending cholinergic fibers from the pedunculopontine nucleus enter and ascend in this bundle up to the dorsomedial hypothalamic nucleus.
5. *Ascending peptidergic fibers.* These fibers arise in the primary (nucleus of the solitary tract) and the secondary (parabrachial nuclei) autonomic centers and innervate the major neuroendocrine cell groups in the hypothalamus. At least eight different neuropeptides have been reported as neurotransmitters in these systems. The fibers travel along the catecholaminergic bundle through the lower brainstem and enter the lateral hypothalamus without any strict topographical organization. These fibers may carry viscerosensory signals to the neuroendocrine hypothalamus.

4.2.4. Spinohypothalamic Connections

The existence of a direct *spinohypothalamic tract* with nociceptive fibers has been demonstrated by tract-tracing techniques. Cells of origin are located in layer IV and V of the dorsal horn all along the spinal cord. Their axons cross over the midline at the spinal cord and ascend together with the spinothalamic tract up to the lateral hypothalamus where they terminate. Relay neurons in the lateral hypothalamus transfer nociceptive signals to the paraventricular nucleus.

4.2.5. Retinohypothalamic Tract

Ganglion cells in the retina project to the suprachiasmatic nucleus, which is involved in generating light-dark cycles (biological clock). Fibers leave the optic chiasm and enter the nucleus without any cross over in the midline. A dipeptide, N-acetyl-aspartylglutamate appears to be the major neurotransmitter in this pathway. The suprachiasmatic nucleus receives indirect neuronal input from the retina via the geniculo-hypothalamic pathway (from the lateral geniculate body). This pathway exerts a GABA-mediated inhibitory effect.

4.3. Hypothalamic Efferents (Neuronal Projections)

Except the hypothalamo-hypophyseal system and the retinohypothalamic tract, the major hypothalamic efferent pathways are bidirectional.

4.3.1. Hypothalamo-Pituitary Connections

The neuronal (hypothalamo-neurohypophysial tract, or also referred to as supraoptico-hypophysial or paraventriculo-hypophyseal tract) and neurovascular connections between the hypothalamus and the pituitary have been detailed above (*see* Sections 2.3. and 2.4.). Reports about direct neuronal inputs from the hypothalamus to the anterior pituitary need further proof.

A small percentage of the hypothalamo-hypophyseal connections is aminergic (Fig. 5). The tuberoinfundibular dopaminergic fibers arise from the A12 (arcuate) and A14 (periventricular) dopaminergic neurons. They run through the pituitary stalk with a destination in the intermediate and posterior pituitary lobes. Neurons of the dorsomedial nucleus give rise to serotonergic fibers in the pituitary gland.

4.3.2. Hypothalamo-Autonomic Connections

The hypothalamus is extensively interconnected with the autonomic centers. Descending fibers from the hypothalamus terminate on medullary and spinal preganglionic neurons (Fig. 6). The parvicellular paraventricular and the arcuate nuclei provide input to these areas. The paraventricular fibers may contain oxytocin, vasopressin, and CRF, whereas POMC innervation of the lower brain stem is partly of arcuate nucleus origin. These peptidergic projections are well suited for coordinating neuroendocrine and autonomic responses.

Pressor and depressor neurons with brain-stem projections have been localized in the lateral hypothalamus, the dorsomedial, and perifornical nuclei. These neurons appear to have a wide terminal pattern that includes the central gray, serotoninergic raphe nuclei, the cholinergic pedunculopontine nucleus, the parabrachial nuclei, and cells in the pontine and medullary reticular formation.

Noradrenergic neurons in the locus coeruleus, the A1, A2, and A5 catecholaminergic cell groups receive bilateral innervations from hypothalamic, mainly from paraventricular neurons.

Several descending projections arise from the medial preoptic nucleus. Preoptic efferents may terminate in the midbrain (median and dorsal raphe nuclei, periaqueductal central gray), pons (dorsolateral tegmentum, locus coeruleus), and the lateral column of the sacral spinal cord.

The endocrine hypothalamus is involved in the regulation of several homeostatic functions. Some of these regulations might be mediated by neuronal projections to the circumventricular organs. The subfornical organ (Fig. 4B), the OVLT and the area postrema receive inputs from the parvicellular paraventricular nucleus.

4.3.3. Dorsal Longitudinal Fascicle

This is the caudal extension of the hypothalamic periventricular system with several fiber components, which interconnect the posterior hypothalamus and the periaqueductal central gray in both directions. Among others, fibers from the dorsal and the posterior hypothalamus may use this avenue towards the midbrain. The neurotransmitter character of these fibers is unknown.

4.3.4. Hypothalamolimbic Connections

1. Strong neuronal input to the hippocampus, especially to the dentate gyrus arise from supramamillary neurons. Their axons terminate on the proximal dendrites of granule cells. A large group of neurons in the various parts of the lateral hypothalamic area project to the hippocampal formation.

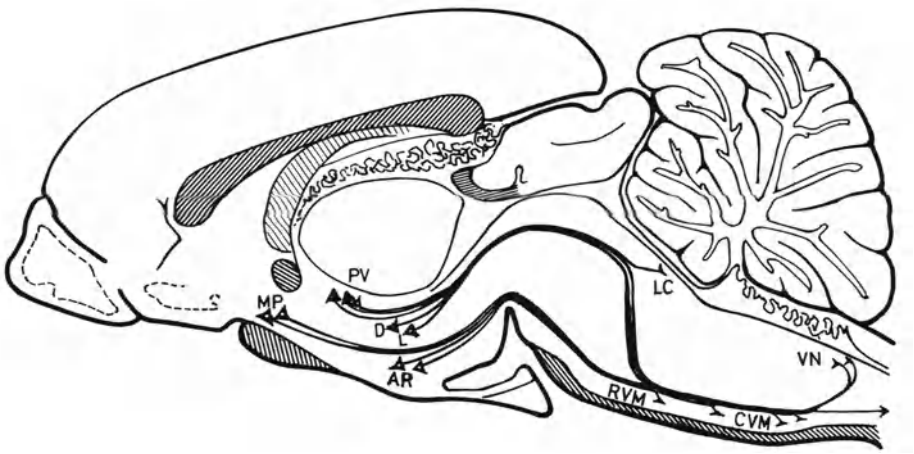

Fig. 6. Descending pathways from the hypothalamus to the brainstem and spinal cord. Fibers arise mainly from the paraventricular (PV), arcuate (AR), perifornical (PF), and preoptic nuclei (MP) nuclei, as well as from the dorsal (D) and lateral (L) hypothalamic areas. Descending fibers leave the hypothalamus through a ventral (medial forebrain bundle) and a dorsal route (periventricular-central gray). CVLM—caudal ventrolateral medulla, LC—locus coeruleus, RVLM—rostral ventrolateral medulla, VN—vagal nuclei.

In addition, cells of the medial preoptic and the dorsomedial nuclei have indirect projections (through septodiagonal band neurons) to the hippocampus. The hippocampus may receive histaminergic inputs from the tuberomamillary, POMC inputs from the arcuate nucleus.

2. Paraventricular, supramamillary, and lateral hypothalamic neurons project to the amygdala. Ascending projections from the medial preoptic nucleus reach the medial amygdaloid nucleus, which in turn projects to the accessory olfactory bulb and to the bed nucleus of the stria terminalis.

4.3.4. Hypothalamospinal-Cord Connections

The paraventricular nucleus projects directly to the intermediolateral column in the thoracolumbar spinal cord. Fibers (*paraventriculo-spinal tract*) descend through the medial forebrain bundle, then through the ventrolateral medulla to the dorsolateral funiculus. Several neuropeptides and dopamine have been identified in these paraventriculo-spinal neurons. In addition to the sympathetic preganglionic neurons, paraventricular neurons may innervate nociceptive neurons in the dorsal horn. Spinal projections from the retrochiasmatic area, perifornical, and dorsomedial nuclei and the lateral hypothalamus have also been demonstrated by tract-tracing techniques and autoradiography.

4.4. Intrahypothalamic Neuronal Connections

A high degree of intrinsic organization of hypothalamic nuclei has been reported. Several hundred or thousands of synaptic terminals can be revealed on the surface of a single hypothalamic neuron. A large percentage of synaptic connections represent local (intranuclear) or intrahypothalamic connections. Most of the hypothalamic cells receive inputs from more than one other hypothalamic nucleus (Fig. 4A).

4.4.1. Intranuclear Connections

The local network of fibers seems to be a common feature of the hypothalamic nuclei. These connections may be established by recurrent (initial) collaterals of projecting axons, or by axon terminals of neighboring neurons. Local GRH-GRH, ACTH-ACTH, POMC-POMC, and substance P-substance P synapses have been demonstrated in the arcuate nucleus. LHRH-LHRH synapses were found in the medial preoptic, CRF-CRF in the paraventricular, and oxytocin-oxytocin in the supraoptic nuclei. Local circuit neurons may synchronize the activities of peptidergic neurons in a hypothalamic nucleus to integrate or coordinate them as a functional unit. Recurrent axon collaterals of hormone-producing peptidergic neurons may provide the morphological basis for an ultrashort feedback mechanism.

Intranuclear connections exist between neurons that synthesize different peptides. Synaptic contacts between magnocellular and parvicellular (CRF) neurons have been shown in the paraventricular nucleus, or NPY-ACTH synapses in the arcuate nucleus of rats.

4.4.2. Intrahypothalamic Connections

The intrahypothalamic connections can be classified as intrahypothalamic innervations and intrahypothalamic neuronal circuits:

1. There is morphological evidence that most of the hypothalamic nuclei have extensive neuronal interconnections. Certain neurotransmitters are expressed only in one or two cell groups in the hypothalamus; they innervate the others:
   a. Histamine-synthesizing neurons exist only in the tuberomamillary nucleus. From here, fibers

spread in all directions, mainly to the forebrain and the hypothalamus. Almost all of the hypothalamic nuclei receive a moderate or delicate histaminergic innervation.

b. POMC-expressing neurons form a single, discrete group of cells in the arcuate and ventral premamillary nuclei. These cells innervate other hypothalamic neurons besides POMC-neuronal inputs to almost every other brain region.

c. Intra- and extrahypothalamic oxytocin immunoreactive fibers originate exclusively in the paraventricular and some accessory magnocellular nuclei.

d. The lateral hypothalamus contains a great number of neuronal perikarya. These cells probably form a relay system between medial hypothalamic cell groups and extrahypothalamic brain areas.

2. There are functionally well-defined neuronal circuits in the hypothalamus:

a. Arcuate-paraventricular circuit (Fig. 4A). POMC neurons of the arcuate nucleus innervate magno- and parvicellular paraventricular cells, from where they receive oxytocinergic inputs.

b. Preoptic and periventricular ANF cells receive inputs from angiotensin II-immunoreactive cells in the subfornical organ, and send fibers to the supraoptic and paraventricular nuclei to influence vasopressin synthesis (Fig. 4B). Neurons in the subfornical organ project directly to the supraoptic and paraventricular nuclei. Vice versa, paraventricular neurons project to the subfornical organ.

c. There is a reciprocal neuronal connection between GHRH cells in the arcuate and somatostatin neurons in the periventricular nuclei (Fig. 4C).

Both cell types project to the median eminence, but their axon collaterals terminate on either arcuate or periventricular perikarya giving a morphological base of the intrahypothalamic balance of growth hormone release from the pituitary.

5. SUMMARY

The complexity of the hypothalamic neuronal inputs and outputs should not be surprising in view of the role of the hypothalamus in neuroendocrine, behavioral, and autonomic responses. A great deal also remains to be learned about the chemical nature, connections, and functional significance of individual hypothalamic neurons.

SELECTED READINGS

Ambach G, Palkovits M. The blood supply of the hypothalamus in the rat. In: Morgane PJ, Panksepp J, eds. Handbook of the Hypothalamus, vol. 1. Anatomy of the hypothalamus. New York-Basel: Marcel Dekker, 1979:267.

Bleier R, Cohn P, Siggelkow IR. A cytoarchitectonic atlas of the hypothalamus and hypothalamic third ventricle of the rat. In: Morgane PJ, Panksepp J, eds. Handbook of the Hypothalamus, vol. 1. Anatomy of the Hypothalamus. New York-Basel: Marcel Dekker, 1979:137.

Crosby EC, Showers MJC. Comparative anatomy of the preoptic and hypothalamic areas. In: Haymaker W, Anderson E, Nauta HJW, eds. The Hypothalamus. Springfield, Ill: CC Thomas, 1969:61.

Diepen R. Der Hypothalamus. In: Bargmann W, ed. Handbuch der Mikroskopischen Anatomie des Menschen, vol IV/7. Berlin: Springer, 1962.

Gurdjian ES. The diencephalon of the albino rat. Studies of the brain of the rat. II. J Comp Neurol 1927;43:1.

Haymaker W, Anderson E, Nauta WJH. The Hypothalamus. Springfield, IL: CC Thomas, 1969.

Hökfelt T, Meister B, Everitt B, Staines W, Melander T, Schalling M, Mutt V, Hulting A-L, Werner S, Bartfai T, Nordström O, Fahrenkrug J, Goldstein M. Chemical neuroanatomy of the hypothalamo-pituitary axis: focus on multimessenger systems. In: McCann SM, Weiner RI, eds. Integrative Neuroendocrinology: Molecular, Cellular and Clinical Aspects. Basel: Karger, 1987:1.

Mezey E, Palkovits M. Two way transport in the hypothalamo-hypophyseal system. In: Martini L, Ganong WF, eds. Frontiers in Neuroendocrinology, vol 7. New York: Raven, 1982;1.

Krieg WJS. The hypothalamus of the albino rat. J Comp Neurol 1932;55:19.

Mitro A, Palkovits M. Morphology of the rat brain ventricles, ependyma and periventricular structures. Basel: Karger, 1981.

Palkovits M. Neuropeptides in the median eminence of the rat. In: McCann SM, Weiner RI, eds. Integrative Neuroendocrinology: Molecular, Cellular and Clinical Aspects. Basel: Karger, 1987;36.

Palkovits M. Neuropeptides in the brain. In: Martini L, Ganong WF, eds. Frontiers in Neuroendocrinology, vol 10. New York: Raven, 1988;1.

Palkovits M. Stereotaxic map, cytoarchitectonic and neurochemical summary of the hypothalamic nuclei, rat. In: Jones TC, Capen CC, Mohr U, eds. Endocrine System, 2nd ed. Berlin: Springer, 1996:121.

Palkovits M, Záborszky L. Neural connections of the hypothalamus. In: Morgane PJ, Panksepp J, eds. Handbook of the Hypothalamus, vol 1. Anatomy of the Hypothalamus. New York-Basel: Marcel Dekker, 1979:379.

Parent A. Carpenter's Human Neuroanatomy, 9th ed., ch. 16: The hypothalamus. Baltimore, MD: Williams and Wilkins, 1996.

Swanson LW. The hypothalamus. In: Björklund A, Hökfelt T, Swanson LW, eds. Handbook of Chemical Neuroanatomy, vol. 5. Integrated systems of the CNS, Part 1, Hypothalamus, hippocampus, amygdala, retine. Amsterdam: Elsevier, 1987:1.

Swanson LW. Brain maps: Structure of the rat brain. Amsterdam: Elsevier, 1992.

Szentágothai J, Flerkó B, Mess B, Halász B. Hypothalamic control of the anterior pituitary. Budapest, Hungary: Akadémiai Kiadó, 1962.

3 Hypothalamic Hormones

Akira Arimura, MD, PhD

Contents

1. INTRODUCTION

The hypothalamus regulates the secretion of both the anterior and posterior pituitary hormones. The regulation of the anterior pituitary is mediated by the hypothalamic hormones. These hormones are synthesized in the hypothalamic neurons, transported along the axons, and released into the hypophysial portal vessels. Eventually, they reach the sinusoid of the anterior pituitary where they interact with their respective receptors on the glandular cells. The neurohypophysial hormones, vasopressin, and oxytocin, are also considered hypothalamic hormones. These two hormones are synthesized in the hypothalamic neurons and transported to the axonal termini in the posterior lobe where they are stored. In response to appropriate stimuli, they are released into general circulation where they regulate distal target tissues.

This chapter will provide a basic overview of the hypothalamic hormones. It will describe their chemistry, physiological actions as hypophysiotropic hormones and neurohypophysial hormones, mechanisms of action, and receptors. Most of the hypothalamic hormones are also expressed or distributed in the extrahypothalamic areas of the brain and peripheral tissues. Within the brain, they function as neurotransmitters and neuromodulators, a topic which will be discussed in Chapter 6. The hypothalamic hormones are also expressed in peripheral tissues, and are involved in the regulation of peripheral organs (such as somatostatin in the gut and the pancreas), but these issues will be discussed only briefly.

From: *Neuroendocrinology in Physiology and Medicine*, (P. M. Conn and M. E. Freeman, eds.),

2. HYPOTHALAMIC HORMONES

2.1. Overview

Direct evidence for the existence of hypothalamic hormones was first presented in the 1950s. Saffran and Schally (1955), and Guillemin et al. (1957), reported that crude or partially purified neurohypophysial or hypothalamic extracts of rat, ovine, or

bovine origin stimulated release of ACTH by the pituitary in vitro. Although vasopressin, which had previously been identified and synthesized, was reported to evoke ACTH release and was considered a *corticotropin releasing factor* (CRF), the CRF prepared by Saffran and Schally and Guillemin et al. was different from vasopressin. Factors with pituitary hormone releasing or inhibiting activity, which remain chemically unidentified, and whose physiological role is yet to be established, are called releasing or inhibiting factors. Factors that have been chemically identified and have established physiological roles are termed releasing or inhibiting hormones. Exhaustive efforts to isolate CRF in a pure form for chemical identification had not met with success, partly due to an inaccurate assay method for CRF. However, a new sensitive and simple method using in vivo release of [^{131}I]-labelled thyroid hormone by *thyrotropin releasing factor* (TRF) was developed by McKenzie, facilitating screening of TRF in the hypothalamic extracts. Subsequently, Guillemin and Schally's groups began to focus on isolating *thyrotropin releasing hormone* (TRH). In 1969, both teams nearly simultaneously reported the isolation and identification of TRH. Schally's team reported the isolation and chemical characterization of *luteinizing hormone releasing hormone* (LHRH) in 1971. In 1973, *GH release inhibiting hormone,* also known as *somatostatin,* was identified by Guillemin's group. *Corticotropin releasing hormone* (CRH) was isolated and characterized by Vale et al. in 1981. *Growth hormone releasing hormone* (GHRH) was isolated from an ectopic pancreatic tumor from a patient with acromegaly by Vale and Guillemin's groups independently in 1982. Evidence has accumulated that *dopamine* tonically inhibits prolactin secretion, thus dopamine has been considered a *prolactin inhibiting factor* (PIF). These peptides and two neurohypophysial hormones, vasopressin and oxytocin, are now considered to be the classical hypothalamic hormones. Table 1 shows the primary structures of these hypothalamic hormones in humans. Whether the function of the agranular cells of the adenohypophysis or folliculostellate cells is also regulated by a hypothalamic neurohormone has remained unknown.

2.2. Requirements for Hypothalamic Hypophysiotrophic Hormones

Among hypothalamic hormones, the regulatory roles of TRH, LHRH, GHRH, CRH, and somatostatin on the glandular cells of the adenohypophysis have been well established. All of these hypothalamic hormones are produced by hypothalamic neurons, but each hormone is distinctively distributed. The hypothalamic hormones are transported through the tuberoinfudibulum tract and eventually reach the axonal terminals in the external layer of the median eminence, where they are released into the primary capillary plexus in response to appropriate stimuli.

To be considered physiological *hypothalamic hypophysiotrophic hormones*, hormones must fulfill the following five requirements.

1. They must be immunohistochemically demonstrated in the axonal termini in the external layer of the median eminence, adjacent to the primary plexus of the hypophysial portal vessels (Fig. 1.). The neurons that produce the hypothalamic hormones are called parvocellular neurons, and are smaller than the neurons (magnocellular neurons) in the hypothalamo-neurohypophysial system, such as those which produce vasopressin and oxytocin. The supraoptic nucleus (SON) of the hypothalamus contains mostly magnocellular neurons, whereas the paraventricular nucleus (PVN) contains both magnocellular and parvocellular neurons.
2. Because the hypothalamic hormones are released in the hypophysial portal vessels, their concentrations in the portal blood must be considerably greater than the levels in the general circulation.
3. The hypothalamic hormones must directly regulate the anterior pituitary cells through the specific receptors for their respective hormones. Thus, in concentrations found in the portal blood, they stimulate or inhibit the activities of dispersed pituitary cells in vitro.
4. The specific receptors of these hormones must be expressed in the glandular cells of the pituitary.
5. Finally, when the biological activity of the endogenous hypothalamic hormones is abolished, such as through immunoneutralization by administration of the antiserum against a hypothalamic hormone, pituitary function under the control of that hypothalamic hormone should be altered.

3. PROHORMONES AND PROCESSING ENZYMES

Like other bioactive peptides and proteins, hypothalamic hormones are expressed in the form of large preprohormones in neurons. After the signal peptide is cleaved, the prohormones undergo extensive processing by various processing enzymes, such as peptidylglycine α-amidating monooxygenase, to become

Table 1
Amino Acid Sequences of Hypothalamic Hormones

Vasopressin (AVP)

Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2 (MW 1084.4)

Oxytocin

Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2 (MW 1007.4)

Thyrotropin Releasing Hormone (TRH)

Glp-His-Pro-NH_2 (MW 362.4)

Luteinizing Hormone Releasing Hormone (LHRH)

Glp-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (MW 1182.4)

Corticotropin Releasing Hormone (CRH)

Ser-Glu-Glu-Pro-Pro-Ile-Ser-Leu-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Met-Ala-Arg-Ala-Glu-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser-Asn-Arg-Lys-Leu-Met-Glu-Ile-Ile-NH_2 (MW 4757.5)

Growth Hormone Releasing Hormone (GHRH)

Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-Gln-Gln-Gly-Glu-Ser-Asn-Gln-Glu-Arg-Gly-Ala-Arg-Ala-Arg-Leu-NH_2 (MW 5040.4)

Somatostatin

Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys (MW 1638.1)

Somatostatin 28

Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys (MW 3149.0)

Pituitary Adenylate Cyclase Activating Polypeptide (PACAP38)

His-Ser-Asp-Gly-Ile-Phe-Thr-Asp-Ser-Tyr-Ser-Arg-Tyr-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Ala-Ala-Val-Leu-Gly-Lys-Arg-Tyr-Lys-Gln-Arg-Val-Lys-Asn-Lys-NH_2 (MW 4534.7)

PACAP27

His-Ser-Asp-Gly-Ile-Phe-Thr-Asp-Ser-Tyr-Ser-Arg-Tyr-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Ala-Ala-Val-Leu-NH_2 (MW 3148.0)

Vasoactive Intestinal Peptide (VIP)

His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Leu-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH_2 (MW 3325.7)

bioactive, matured hormones (Fig. 2). In recent years, a family of prohormone convertases (PCs) that is evolutionarily related to the serine proteases of bacterial subtilisin and the yeast dibasic-specific endoprotease Kex2 has been characterized in mammalian tissues (Fig. 3). Among these PCs, PC1 and PC2 mRNAs were detected in endocrine and neuronal tissues. PCs cleave paired basic amino acids. In the rat hypothalamus, PC1 mRNA is strongly expressed in the PVN and SON of magnocellular neurons, whereas PC2 mRNA is expressed in both magnocellular and parvocellular neurons in the PVN as well as in magnocellu-

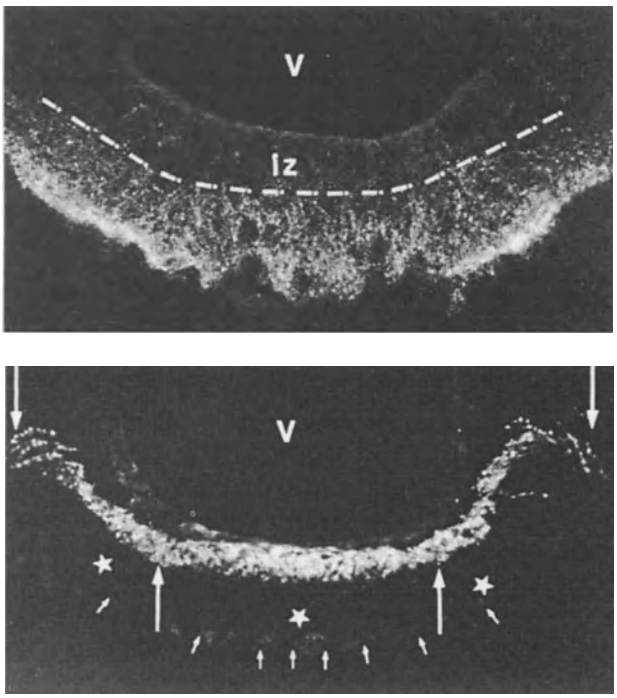

Fig. 1. The characteristic location of hypothalamic hypophysiotropic hormones (TRH in this photomicrograph) and neurohypophysial hormones (AVP) in the rat median eminence (ME). Whereas the TRH-containing nerve terminals occupy the external zone of the ME (upper panel, immunoreactivity below the dotted line), AVP immunoreactivity is present in axons running in the internal zone (iz) of the ME (arrow in lower panel) toward the neurohypophysis. The external zone (white star) is free of AVP immunoreactivity. The hypothalamic hypophysiotropic hormones are released into hypophysial portal circulation within the ME; the neurohypophysial hormones are transported along the axons that run through the internal zone and released into general circulation within the neural lobe. (Drawn from Merchenthaler. In Principals of Med. Biol. 1997 10A, 119.)

lar neurons in the SON, suggesting that PC1 and PC2 process the precursors of neuropeptides expressed in these hypothalamic nuclei.

4. TRH

4.1. Chemistry, Precursor, and Processing

TRH was the first hypothalamic hypophysiotropic hormone to be identified. TRH is a tripeptide: (pyro)-Gly-His-Pro-NH_2. The rat TRH precursor, with a m.w. of 29,247, was characterized by cloned cDNA. The nucleotide sequence of the protein contains five copies of the sequence Gin-His-Pro-Gly flanked by paired basic amino acids, and can therefore generate five TRH molecules. Human preproTRH protein contains six copies of the TRH sequence (Fig. 4).

In situ hybridization histochemistry of rat brain sections has demonstrated preproTRH mRNA expressing neurons concentrated in the parvocellular division of the PVN, in the same location as cells detected by immunohistochemistry for TRH prohormone. PC1 and PC2 cleave pro-TRH. Double *in situ* hybridization showed that PC2 mRNA is present in 60–70% of TRH neurons, whereas PC1 mRNA is found in 37–46%. TRH neurons expressing either PC1 or PC2 mRNA are found throughout the areas containing TRH cells.

4.2. Physiological Actions

Injection of TRH promptly induces release of TSH, resulting in a prompt rise in blood TSH levels. A surge of TSH leads to a rise in circulating thyroid hormones, T3 and T4. TRH's action on the pituitary is blocked by pretreatment with thyroid hormone. This direct negative effect of thyroid hormone on the pituitary level is the major basis of the integrated neuroendocrine control of TSH secretion. TRH also stimulates release of prolactin dose-dependently and, as on TSH, pretreatment with thyroid hormone, suppresses TRH action on prolactin release. The prolactin releasing activity of TRH may be responsible for the occasional occurrence of hyperprolactinemia in patients with hypothyroidism. However, the physiological role of TRH as a prolactin releasing hormone has not been established.

4.3. TRH Receptors and the Mechanism of Action on the Pituitary

The TSH stimulatory action of TRH is initiated by the interaction of TRH with its receptors on the membranes of the pituitary cells. The structure of mouse pituitary TRH receptors was characterized by cloned cDNA. The 3.8-kbase mouse TRH receptor cDNA encodes a protein of 393 amino acids that shows similarities to other G-protein-coupled receptors. Human TRH receptors have also been cloned, confirming that the TRH receptor is a G-protein-coupled receptor with seven transmembrane domains. The TRH receptor couples principally to the inositol phosphate second-messenger pathway. The regulatory G-proteins associated with TRH receptor signal transduction are identified as Gq and G_{11}.

Although TRH increases intracellular cAMP, and cAMP increases TSH secretion, cAMP response is not always associated with the increased TSH secretion induced by TRH. Therefore, the increase in intracellular cAMP may not be the major biochemical event

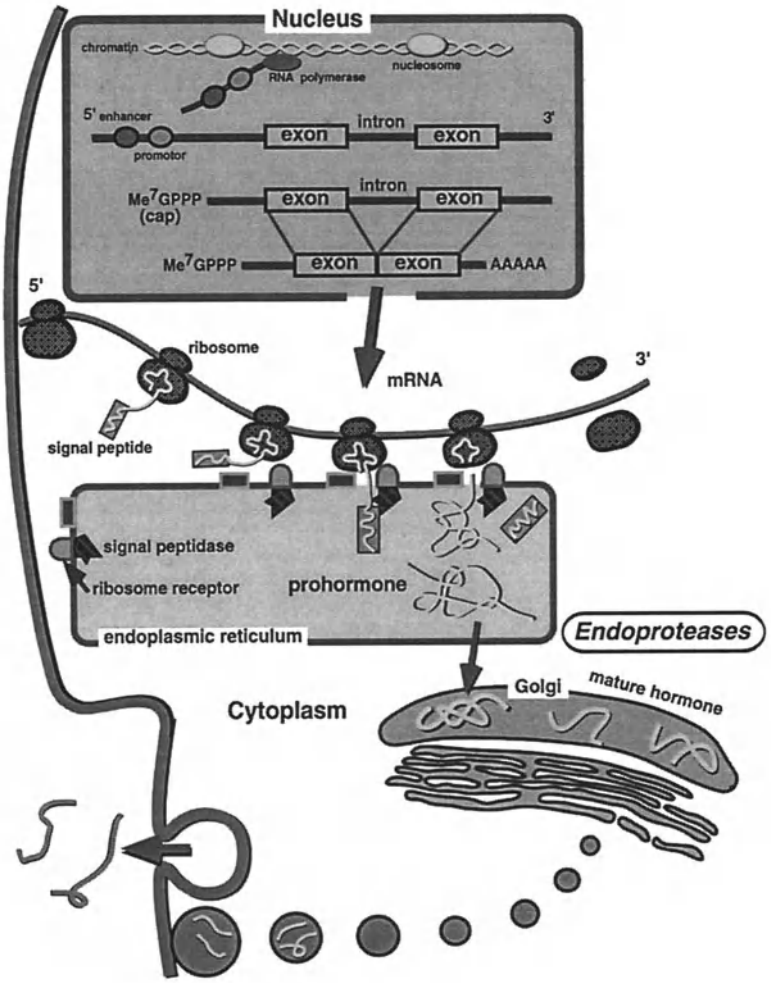

Fig. 2. Schematic illustration of the synthesis and processing of the preprohormone in the cell. The signal peptide in the preprohormone is removed in the endoplasmic reticulum, forming the prohormone, which undergoes extensive processing by various processing enzymes during packing and transport and becomes matured and bioactive hormone.

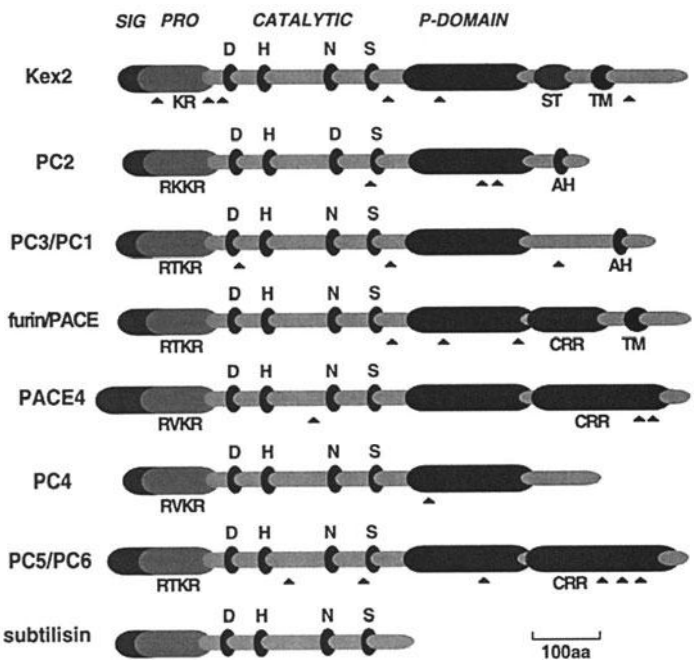

Fig. 3. Domain structure of eukaryotic subtilisin-related endoproteases. PC2 and PC3/PC1 are expressed in endocrine cells and neurons, and PC4 is expressed in testicular germ cells. PC2 and PC3/PC1 participate in the processing of all hypothalamic hormones in the hypothalamus. Cleavage by these proteases generally occurs at paired basic residues. The signal peptide (Sig), pro-domain (Pro), catalytic domain and P-domain are shown at the top of the figure. The Asp, His, Asn/Asp, and Ser residues of the catalytic domain and the sequence of basic residues at the pro-domain cleavage site of the eukaryotic proteases are indicated by their single letter codes. Triangles indicate sites of potential N-linked glycosylation. Other domains are as follows: ST, serine/threonine-rich region; CRR, cysteine-rich region; TM, transmembrane domain; and AH, amphipathic helical region. (Drawn and modified from S. P. Smeekens, Bio/Technol 1993, 11:182.)

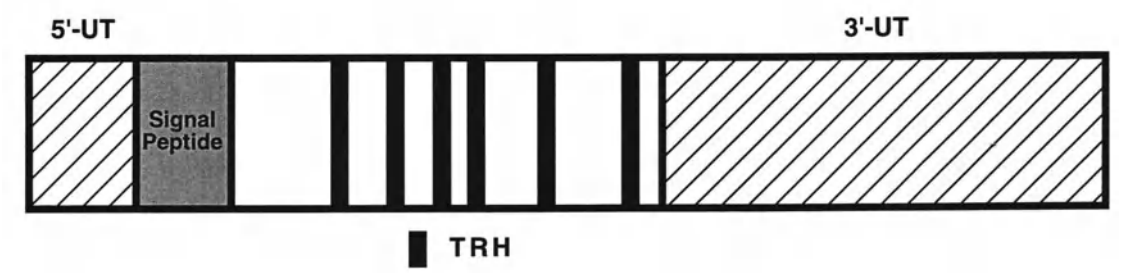

Fig. 4. Structure of human preproTRH. This molecule shares six repeated regions coding for TRH (solid black block), each of which is preceded by dibasic amino acids and followed by Gly, which is exchanged for NH2 during posttranslational processing. The shaded area indicates untranslated regions of mRNA.

required for TRH-induced TSH secretion. Ca^{2+}-dependent hydrolysis of phosphatidylinositol with phosphorylation of protein kinase C has been thought to be linked with TRH-induced TSH secretion. A number of studies have provided compelling evidence that the interaction of TRH with its receptors coupled to G-protein activates a phospholipase C that hydrolyzes phosphatidylinositol 4,5-biphosphate to form two second messenger molecules, inositol 1,4,5-triphosphate and 1,2-diacylglycerol. Activation of TRH receptor in membranes isolated from pituitary tumor cells causes stimulation of adenylate cyclase and formation of cAMP.

5. LHRH

5.1. Chemistry, Precursor, and Processing

LHRH is a decapeptide with pyroGlu in the N-terminus and Gly-amide at the C-terminus and it is formed in the tissue as a large precursor protein. The PC2 expressed in the hypothalamus is at least one of the processing enzymes for LHRH precursor. The cDNA of human prepro-LHRH has been cloned. The cDNA sequences code for a protein of 921 amino acids in which the LHRH decapeptide is preceded by a signal peptide of 23 amino acids and followed by a Gly-Lys-Arg sequence, as expected for enzymatic cleavage of the decapeptide from its precursor and amidation of the carboxy-terminal of LHRH (Fig. 5). Immunochemical studies have indicated that mammalian and amphibian LHRH are similar, but both differ from the LHRH of birds, reptiles, fish, and elasmobranch. Even within the same animal, more than one form of LHRH can be found in different sites. It appears that the structures of all releasing hormones larger than the TRH display species differences, and in some instances, are coded for by more than one gene in the same species.

5.2. Distribution of LHRH in the Hypothalamus

Immunohistochemical studies have shown that two distinct areas of the hypothalamus are particularly rich in LHRH neurons. One is the arcuate and ventromedial nuclei. The axons of the neurons, mostly from the arcuate nucleus, which make up the majority of the tuberoinfundibular tract, terminate in the primary plexus of the hypophysial portal vessels. Lesion of this area in female rats results in atrophy of the ovaries, and reduction of estrogen secretion. The second LHRH neuron-rich area is the preoptic area, which regulates cyclic release of gonadotropin. In estrous rats, electrical stimulation of this area induces ovulation. Lesion of this region causes cessation of ovulation and persistent estrus. This area is readily affected by environmental stimuli such as light, by steroid hormones and by the higher centers of the nervous system.

5.3. Physiological Actions

A bolus intravenous injection of LHRH induces a prompt rise in LH and follicle stimulating hormone (FSH) in blood. However, the onset of increase in FSH levels is delayed in comparison with LH secretion. The ratio of LH/FSH release is influenced by various factors, such as the gonadal steroid milieu, and the pattern of secretion or the method of administration of LHRH. It is thus, generally accepted that both LH and FSH secretions are regulated by one hypothalamic hormone, LHRH, which is called *gonadotropin releasing hormone* (GnRH). LHRH can stimulate testosterone production and spermatogenesis by releasing LH and FSH in animals and men with hypothalamic hypogonadotropic hypogonadism.

Immunoneutralization of endogenous LHRH results in the complete absence of spermatogenesis and atrophy of the testis in rabbits. LHRH induces

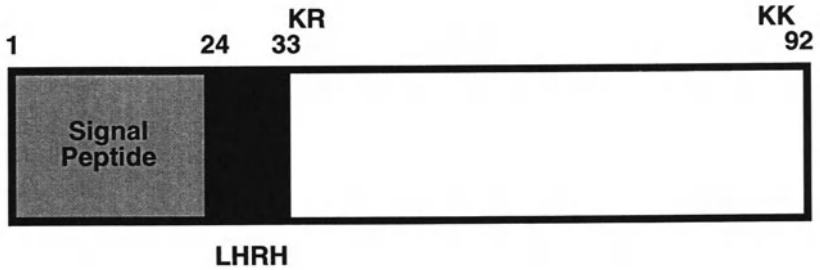

Fig. 5. Scheme of human preproLHRH. The N-terminal amino acid of preproLHRH is numbered 1, and the other amino acid in preproLHRH is numbered accordingly. A protein of 92 amino acids in which the LHRH decapeptide is preceded by a 23-amino acid signal peptide and followed by a processing site, Gly-Lys-Arg.

ovulation in women with hypothalamic amenorrhea. However, LHRH in a high dose given over an extended period suppresses gonadal function and decreases testosterone secretion. This effect could be owing to desensitization of pituitary gonadotrophs caused by prolonged elevation of LHRH levels in circulation. However, a continuous administration of LHRH reduces plasma testosterone levels in hypophysectomized rats receiving exogenous gonadotropins to maintain normal gonadal function. This indicates a direct inhibitory action of LHRH on testicular activity. Administration of a potent LHRH agonist is now widely used to reduce testosterone in place of surgical castration in the treatment of prostate cancer, thus, LHRH has biphasic effects. Stimulation and suppression of the gonadal functions depend on the dose and method of administration. Potent LHRH antagonists have also been developed that suppress gonadotropin secretion by antagonizing endogenous LHRH at the pituitary.

5.4. The Mechanism of Action and Receptors of LHRH

The action of LHRH on the gonadotrophs is initiated by the interaction of LHRH with its receptors on the plasma membrane. Although LHRH increases intracellular cAMP by activation of adenylate cyclase, this does not appear to be essential for hormone release. LHRH and TRH regulate pituitary hormone secretion by similar postreceptor mechanisms involving inositol phosphate generation with Ca^{2+} mobilization, and diacylglycerol formation with protein kinase C activation. The initial response in pituitary cells results from the activation of a phosphoinositide-specific phospholipase C, as demonstrated for TRH. Phospholipase C activation is mediated through heterotrimeric GTP-binding (G) proteins, in particular, by Gq and G11, as for TRH.

A cDNA of mouse LHRH receptors was cloned, and its identity was confirmed using transfection of the Xenopus oocyte with the receptor cDNA. The cDNA sequence encodes a 327 amino acid protein that has the seven transmembrane domains characteristic of G protein-coupled receptors, but which lacks a typical intracellular C-terminal cytoplasmic domain. *In situ* hybridization histochemistry in the rat anterior pituitary showed a characteristic LHRH receptor distribution. Subsequently, human, sheep and rat LHRH receptor cDNAs have been cloned. Human LHRH receptors shows 85% similarity to mouse LHRH receptor. The expression of LHRH receptors in COS cells results in enhanced formation of 1,4,5-trisphosphate in response to LHRH, indicating that the cloned receptors can couple to phospholipase C, causing enhanced phosphatidylinositol turnover, as shown for the LHRH receptor of rat pituitary cells.

6. GHRH

6.1. Chemistry, Precursor, and Processing

Early efforts to isolate GH-releasing factor and to identify its chemical structure were unsuccessful, mainly due to lack of a reliable and sensitive assay system for GH release. Despite rapid progress of purification technology for peptides and the development of a sensitive RIA method for GH, isolation of GHRH was not successful until the discovery of the paraneoplastic syndromes of ectopic GHRH secretion by pancreatic adenomas in humans. The difficulty of the assay had mainly been owing to the existence of somatostatin in crude hypothalamic extracts that masked the activity of GHRH. Ectopic GHRH producing tumors were rich in GHRH, and served as an ideal source for isolating this hormone. Using these tumors, both Guillemin and Rivier's groups independently isolated and characterized GHRH. Three

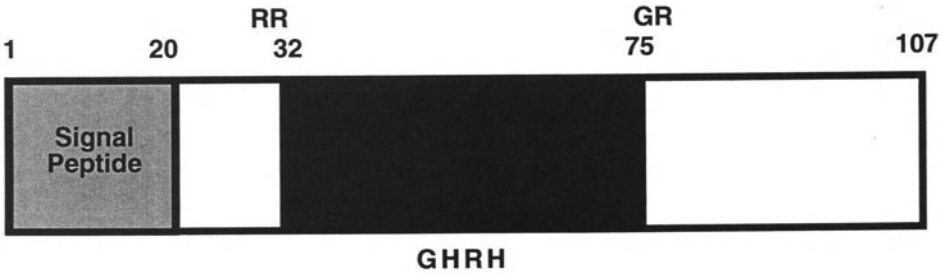

Fig. 6. Human preproGHRH consists of 107 amino acids. After the signal peptide is removed, proGHRH undergoes extensive processing by various endoproteases which cleave at dibasic amino acid sites preceding amino acid 32 and following amino acid 75, yielding GHRH-44-NH2 and a carboxyl terminal peptide of unknown function.

molecular forms of human GHRH (GHRH1-44 NH_2, GHRH1-40-OH, and GHRH1-37-OH) were identified. Subsequently, identical peptides have been isolated directly from the human hypothalamus. The N-terminal of GHRH is essential for biological action, and all three forms of GHRH are active. There are species differences among GHRHs. GHRH isolated from rat hypothalamic tissues contained 43 amino acids. The physiological relevance of GHRH for GH release was established by the finding that immunoneutralization of endogenous GHRH resulted in the absence of GH secretory response to several GH-releasing stimuli.

The cDNA for preproGHRH has been cloned from a pancreatic tumor. Encoded preproGHRH protein consists of 107 amino acids. After the signal peptide is cleaved, proGHRH protein is processed by proprotein convertases, generating two equipotent forms of GHRHs, hGHRH44-NH2 and hGHRH40-OH, and a carboxyl terminal peptide of unknown function (Fig. 6). Restriction analysis of genomic DNA indicates that there is probably a single human GHRH gene, and suggests that the pancreatic tumor and hypothalamic proteins are encoded by an identical mRNA.

6.2. Distribution in the Hypothalamus

Immunohistochemical studies in rats indicate that the majority of GHRH immunoreactive cells are found in the arcuate nucleus and the medial perifornical region of the lateral hypothalamus. Studies with unilateral hypothalamic deafferentation show that the arcuate nucleus is the major source of GHRH in the median eminence. The highest concentration of GHRH as determined by RIA in the human hypothalamus has been demonstrated in the tuberoinfundibular nucleus. Somatostatin, the second neuropeptide involved in the regulation of GH secretion from the anterior pituitary, has a pattern of distribution along the pituitary stalk that is very similar to that of GHRH.

6.3. Physiological Action of GHRH

It is generally accepted that the episodic pattern of GH secretion from the pituitary is generated by an interplay between two hypothalamic hormones: stimulatory GHRH, and inhibitory somatostatin. In rats, both GHRH and somatostatin are secreted rhythmically from the hypothalamus into hypophysial portal circulation at regular 3- to 4-h intervals, about 180° out of phase. An ultradian rhythm of GH secretion is also observed in peripheral blood. In anesthetized male rats, it has been shown that GHRH and somatostatin, as determined by respective RIA, are rhythmically released from the median eminence into the hypophysial portal circulation. Immunoneutralization of endogenous GHRH abolishes the spontaneous GH pulses in rats. The administration of monosodium glutamate (MSG) to neonatal rodents produces permanent lesion of the arcuate nucleolus that secretes GHRH. Compared to normal rats, neonatal treatment with MSG produces a marked inhibition of GH secretion.

A bolus injection of GHRH in humans and animals induces a prompt rise in blood GH, followed by a rapid return to basal levels, indicating the short life of its action. Fluctuation of blood GH levels in normal humans supports the view that GHRH is released in a pulsatile manner. Sustained infusion of GHRH over several hours causes a decrease in blood GH levels. Most men over 40 yr of age show either low or absent GH responses to GHRH. This finding is compatible with the observation that older individuals have lower 24-h secretion of GH.

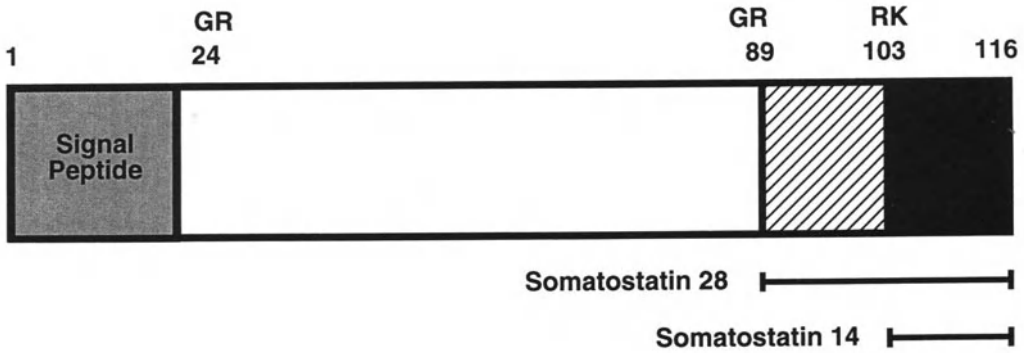

Fig. 7. Structure of rat prepro-somatostatin. Somatostatin 14 is located at the carboxy-terminal region (solid black block), and somatostatin 28 is amino-terminally extended (shaded block and solid black block.

6.4. Mechanism of Action and Receptors

GHRH binds to its receptors on the plasma membranes of pituitary somatotrophs and stimulates GH secretion by activation of adenylate cyclase, increasing intracellular cAMP levels and activating protein kinase A. The stimulation of GHRH-induced GH release is Ca^{2+} dependent. It also activates the phosphatidylinositol turnover and increases expression of GH by stimulating its transcription.

The cDNA encoding the human GHRH receptor has been cloned from an acromegalic pituitary cDNA library. The isolated cDNA encodes a 423 amino acid protein that has a seven putative transmembrane domain characteristic of G-protein-coupled receptors. Transient expression of this cDNA in COS cells induces saturable, high affinity, GHRH-specific binding, and stimulates intracellular cAMP accumulation in response to physiological concentrations of GHRH. Northern blot analysis shows that GHRH receptor mRNA is most abundant in extracts of the pituitary and has not been detected in other tissues.

7. SOMATOSTATIN

7.1. Chemistry, Precursor, and Processing

The GH release inhibiting activity in hypothalamic extracts was first demonstrated in vitro by Krulich and associates. It was postulated that GH secretion is regulated by dual control of GH releasing factor and GH release inhibiting factor in the hypothalamus. In 1973, GH release inhibiting factor was isolated from hypothalamic tissues and its amino acids sequence was characterized by Guillemin et al., and renamed *somatostatin.* Somatostatin is a cyclic peptide with 14 amino acid residues, as shown in Table 1. In addition, an amino-terminal-extended somatostatin with 28 amino acids was isolated and designated somatostatin 28. The cDNAs of the preprosomatostatin of humans, rats, anglerfish and catfish have been cloned. The rat somatostatin gene encodes preprosomatostatin with 116 amino acids that is processed cotranslationally within the endoplasmic reticulum to yield prosomatostatin, a peptide of 92 amino acids. Prosomatostatin is subsequently cleaved posttranslationally to produce somatostatin 28 and somatostatin 14, that correspond to the carboxyl terminal region of prosomatostatin (Fig. 7).

The sequence of somatostatin 14 in humans and one of the two somatostatins of anglerfish are identical and the other is highly homologous, suggesting that this molecule was well conserved during evolution. Somatostatin has even been demonstrated in the single cell protozoan *Tetrahymena pyriformis.*

7.2. Distribution

In the hypothalamus, the majority of somatostatin containing neurons are concentrated in the periventricular region. These cells are observed in the parvocellular part of the paraventricular nucleus, anterior hypothalamic nucleus, perifornical region, and lateral hypothalamus. Somatostatin-positive cell bodies are also detected in the preoptic nucleus, as well as in the lateral region of the lateral preoptic nucleus. Somatostatin-immunoreactive fibers and terminals are more widely distributed in the median eminence and pituitary stalk than other hypothalamic hormones. Studies with knife cuts and electrolytic lesions showed that most of the somatostatin neurons with terminals in the median eminence are in the preoptic-anterior hypothalamic region. Electrical stimulation of the preoptic area enhanced somatostatin release into hypophysial portal blood in rats. Subsequently,

somatostatin has been found in the rat stomach and pancreas in a concentration similar to that in the hypothalamus as measured by RIA. D-cells of the pancreas have also been found to express somatostatin.

7.3. Physiological Actions

In the pituitary, somatostatin suppresses secretion of GH and TSH under physiological conditions. Immunoneutralization of endogenous somatostatin increases basal levels of blood GH and TSH, and enhances their response to administration of GHRH and TRH, respectively (Fig. 8). In rats, stress reduces plasma GH levels. Pretreatment with antisomatostatin serum blocks the reduction of GH levels, indicating that stress-induced decrease in plasma GH results from increased release of somatostatin. Somatostatin is also an important pancreatic and gastrointestinal hormone. In the gut and pancreas, somatostatin decreases nearly all secretions, such as gastrin, gastric acid, pancreatic glucagon, and insulin, as well as exocrine secretion, and suppresses the absorption of nutrients from the intestinal tract. In experimental diabetic animals and diabetic patients, hyperplasia of somatostatin-producing D-cells in the pancreas is observed. Somatostatin is also secreted in the pancreatic duct, and the levels of somatostatin in pancreatic juice increase in diabetic patients, suggesting that somatostatin plays a compensatory role in the control of blood glucose levels when the secretion of insulin decreases. In addition, somatostatin suppresses hormone release from many endocrine-secreting tumors, including VIPomas, carcinoid tumors, insulinomas and glucagonomas, and is used for the treatment of these tumors.

7.4. Mechanism of Action and Receptors

Somatostatin interacts with its specific receptors in the plasma membrane of somatotrophs and thyrotrophs of the pituitary and other target cells. Five somatostatin receptors—$SSTR_1$ to $SSTR_5$—of humans, rats and mice, have been cloned. $SSTR_2$ has two subtypes, $SSTR_{2A}$ and $SSTR_{2B}$, which result from alternate splicing. All of these somatostatin receptors are G-protein associated and have seven transmembrane domains. Moreover, all these receptors bind to somatostatin with a high affinity, but SSTR5 has the unique property of having a higher affinity for somatostatin-28 than somatostatin-14.

Although all five somatostatin receptors have about 45 to 50% amino acid sequence similarity, the amino acid sequences of these five somatostatin receptors have no similarity to any other receptors, except the

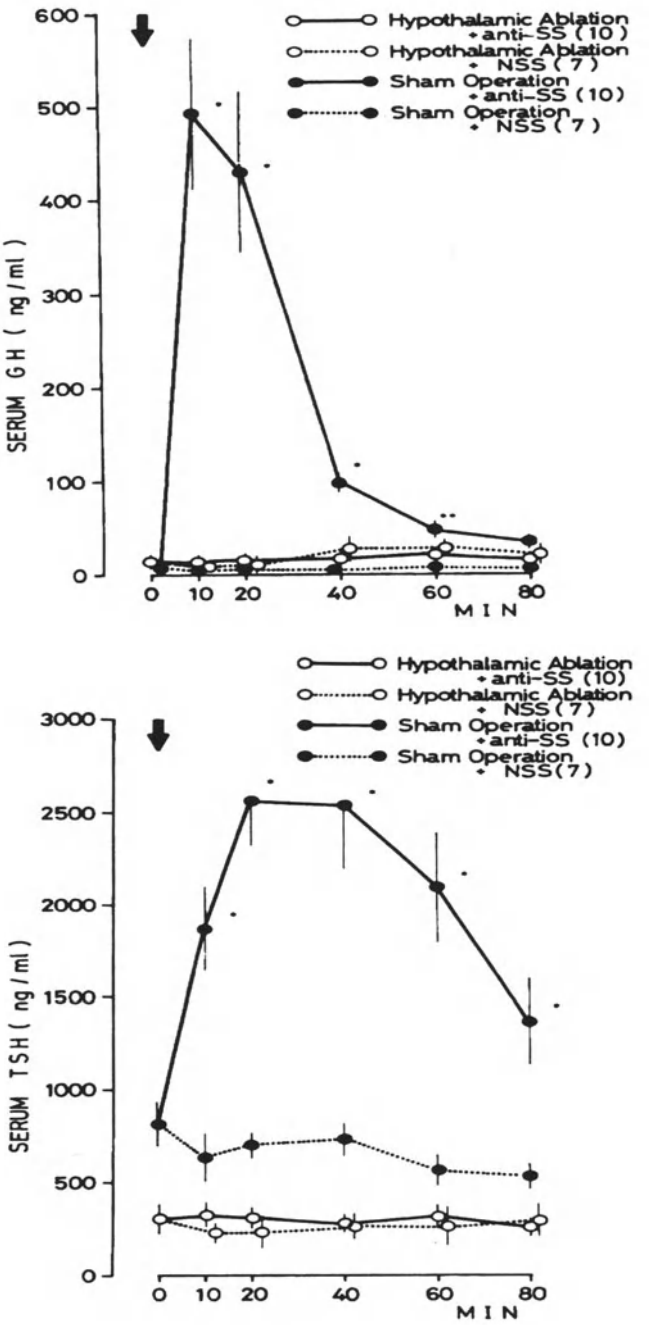

Fig. 8. Effect of an intravenous injection of sheep antiserum to somatostatin on serum GH and TSH levels in urethane-anesthetized control male rats and rats with ablation of the basal medio-hypothalamus. The mean basal level of serum TSH in rats with hypothalamic ablation was significantly lower than those in sham-operated rats. Serum GH and TSH levels in rats with hypothalamic ablation did not change significantly after an iv injection of antiserum to somatostatin or normal sheep serum. The injection of somatostatin antiserum in sham-operated rats resulted in a significant increase in serum GH and TSH, as compared to those in normal sheep serum-injected rats. (Drawn from Chihara et al. *Endocrinology* 1978; 103:1916.)

opioid receptor family, with which they share about 40% similarity.

Although somatostatin binds to somatostatin receptors with high affinity, their selectivity to varying synthetic somatostatin agonists, such as cyclic hexa- and octapeptides, varies among the receptor subtypes. Availability of selective somatostatin agonists for rodent somatostatin receptors has permitted the identification of selective functions of the different somatostatin receptor subtypes. These receptor subtypes are expressed in a tissue-specific manner and in various tissues they couple to different signal transduction pathways. The pituitary expresses $SSTR_2$ and $SSTR_5$, with $SSTR_5$ being more abundant. $SSTR_2$ appears to selectively mediate the inhibition of growth hormone release by somatostatin.

Somatostatin receptors associate with Gai-a, Gai-3 or Ga0, which couple the receptor to adenylate cyclase. All five sets are functionally coupled to inhibition of adenylate cyclase via pertussis toxin-sensitive G-proteins. Although somatostatin reduces the accumulation of intracellular cAMP induced by GHRH, this inhibitory effect may not be the sole inhibitory mechanism, since the GH stimulatory effect of cAMP is also suppressed by somatostatin, and hence may act downstream from cAMP. Some of the subtypes of SSTRs are also coupled to tyrosine phosphatase ($SSTR_{1,2}$), Ca^{2+} channels ($SSTR_2$), Na^+/H^+ exchanger ($SSTR_1$), phospholipase A ($SSTR_4$) and MAP kinase ($SSTR_4$). Ongoing structure-activity studies will hopefully yield selective analogs for each receptor subtype. This will greatly aid in clarifying the physiological role of the receptor subtypes and help define their signal transduction pathways.

8. CORTICOTROPIN RELEASING HORMONE (CRH)

8.1. Chemistry, Precursor, and Processing

A 41 residue CRH with an amidated C-terminus was isolated and characterized from ovine hypothalami in 1981 by Vale et al. Subsequently, the cDNA of ovine and rat preproCRH as well as the human CRH gene were cloned. Considerable similarity was demonstrated in the sequence of CRH precursor molecules among species. Rat and human CRH are identical, whereas ovine CRH sequence varies by 7 amino acids from human CRH. The nucleotide sequence of ovine CRH precursor cDNA revealed that the prepro-CRH consists of 190 amino acid residues (Fig. 9). The rat CRH gene is also quite similar to the human CRH gene. As with other bioactive peptides and proteins, prepro-CRH is processed by processing enzymes to generate mature, amidated CRH. PC2, which is expressed in neurons in the parvocellular part of the PVN, appears to participate in the cleavage of a large pro-CRH molecule. Mammalian CRH has homologies with two peptides found in lower animal forms, the peptide sauvagine (isolated from the skin of a species of frog) and urotensin (a secretion of the caudal gland of the fish). Both have potent CRF activity. A mammalian CRH-related peptide, urocortin, with close sequence homology to fish urotensin, interacts with CRH receptors and elicits a potent ACTH releasing activity.

8.2. Distribution in the Hypothalamus

The cell group most rich in CRH containing cells is the PVN, mainly the parvocellular part. The CRH containing fibers from the PVN project to the median eminence, forming a medial, an intermediate, and a lateral pathway. In addition to the PVN, clusters of CRH containing cell bodies are demonstrated in the supraoptic, medial, and periventricular preoptic and premammillary nuclei of the hypothalamus. CRH containing cell bodies are also found in several extrahypothalamic regions, such as the bed nuclei of the stria terminalis and anterior commissure, nucleus accumbens, raphe nuclei, locus ceruleus, dorsal vagal complex, and other areas.

8.3. Physiological Action on the Pituitary

CRH stimulates pituitary corticotrophs and releases ACTH and β-endorphin into the blood, followed by the secretion of cortisol or corticosterone. The effect of CRH is specific to ACTH release and is inhibited by glucocorticoids. The transcription of mRNA for ACTH precursor (proopiomelanocorticotropin or proPOMC) is also enhanced by CRH, suggesting that CRH is a trophic factor.

Hypothalamic CRH is rapidly released in response to a variety of stressors, regardless of the nature of the stress. CRH also stimulates the release of hypothalamic CRH, thereby upregulating its own secretion, and suppresses the release of GHRH and LHRH.

In the median eminence, the content of CRH shows diurnal rhythm in rats; low in the morning, gradually increasing in the afternoon, and reaching a peak level in the evening. This rhythm is correlated with the diurnal rhythm of plasma corticosterone concentrations in rats. Because lesion of the suprachiasmatic nucleus abolishes the diurnal rhythm of corticosterone

Fig. 9. Schematic illustration of ovine preproCRH. Carboxyl end represents the CRH sequence (solid black block) preceded by the tetrapeptide, Arg-Lys-Arg-Arg, and followed by the dipeptide, Gly-Lys. The signal peptide is indicated by a shaded block.

secretion, this nucleus may regulate the rhythm of CRH content in the median eminence.

8.4. Mechanism of Action and Receptors

CRH is indispensable for the full ACTH secretory response and for the concomitant stimulation of POMC transcription. CRH interacts with its receptors on the plasma membrane of the pituitary corticotrophs that activate adenylate cyclase and increase intracellular cAMP concentration and transmembrane Ca^{2+} flux. This signal transduction is thought to be linked to the stimulation of ACTH secretion and POMC synthesis. During stress, vasopressin is also coreleased into the hypophysial portal vessels from the parvocellular neurons of PVN where it enhances CRH-induced ACTH release in a synergistic manner. This synergistic interaction appears to result from an interplay between the stimulation of protein kinase A by CRH and protein kinase C by vasopressin.

Two types of CRH receptors, CRH_1 and CRH_2, have been cloned. The CRH_2 receptor, which was cloned from the rat brain, has two subtypes, CRH_{2a} and CRH_{2b}, resulting from alternate splicing. The CRH receptor is membrane-bound, G-protein-coupled and has seven transmembrane domains. All these receptors transduce signals via the stimulation of intracellular cAMP production. The CRH_1 receptor is mainly expressed in the pituitary and brain, whereas the CRH_{2a} receptor is mainly expressed in the brain, and the CRH_{2b} receptor is expressed predominantly in the peripheral tissues such as the heart and skeletal muscles. However, in humans CRH_{2a} is the predominant CRH_2 receptor, and a human equivalent of CRH_{2b} has yet to be found. The CRH_1 receptor is not expressed in the heart. Expressions of CRH_1 and CRH_2 receptors appear to be differently regulated.

8.5. CRH Binding Protein

It has been suggested that a peptide-binding glycoprotein is present in human plasma, inactivating CRH and thus preventing pituitary-adrenal stimulation. The *CRH binding protein* (CRH-BP) was purified from human plasma in a pure form. Subsequently, human and rat CRH-BP cDNA were cloned. Both cDNAs encoded proteins of 322 amino acids with one putative N-linked glycosylation site and 11 conserved cysteine residues. Mouse and sheep CRH binding proteins were subsequently cloned. The structure of CRH-BP is distinct from the CRH receptor. Expressed CRH-BP in mammalian cells bind human CRH as well as alpha-helical (9–41) CRH (CRH antagonist) with high affinity. Recombinant murine CRH-BP also blocks CRH-induced ACTH release from cultured pituitary cells, but CRH-BP and CRH receptor utilize different molecular interaction to bind CRH.

9. PROLACTIN SECRETION REGULATORY FACTORS

9.1. Prolactin Release Inhibiting Factor (PIF)

Prolactin secretion is tonically inhibited by a hypothalamic factor(s) under normal conditions. When the hypothalamic extract is added to rat pituitary cultures, prolactin release into the medium is suppressed, indicating the presence of a prolactin release inhibiting factor (PIF) in the extract. The efforts of several laboratories to isolate the hypothalamic prolactin release inhibiting factor revealed that the major PIF activity is dopamine. Administration of dopamine, L-dopa (which is converted to dopamine in both peripheral tissues and the brain), or dopamine agonists such as bromocriptine, decrease blood prolactin levels sharply in normal individual and in persons with hyperprolactinemia.

Dopamine directly inhibits prolactin secretion from pituitary lactotrophs through interaction with its receptors. Dopamine also suppresses cell growth and DNA synthesis, and results in a loss of stored prolactin in the secretory granules. Dopamine inhibits cAMP formation and inhibits synthesis of phosphoinositol,

which is involved in the secretory process of prolactin. Gamma butyric amino acid (GABA) also suppresses prolactin release, but its inhibitory effect is much weaker than dopamine's.

9.2. Prolactin-Releasing Factor (PRF)

Prolactin secretion is not always regulated by the inhibitory action of dopamine. Suckling-induced prolactin release cannot be accounted for by the observed change in dopamine levels in hypophysial portal blood. Moreover, hypothalamic extracts contain several substances that stimulate release of prolactin. These prolactin-releasing factors (PRFs) include TRH, vasopressin, oxytocin, VIP, PHI, and PACAP. TRH stimulates prolactin release dose-dependently. TRH secretion into the hypophysial portal blood is increased by nipple manipulation in rats, which induces prolactin release. In humans, however, suckling does not increase TSH secretion. Oxytocin secretion, which is increased by suckling, also stimulates prolactin release in vitro. However, the PRF activity of these neurohypophysial hormone is weak. VIP stimulates prolactin release from the pituitary in vitro. VIP is also present in the hypophysial portal blood in concentrations sufficient to stimulate prolactin release, and its release is stimulated by serotonin, which increases prolactin secretion. Immunoneutralization of endogenous VIP by administration of its antiserum suppresses stress-induced prolactin release and reduces the elevated blood prolactin levels in suckling mothers. However, immunohistochemical studies have shown that VIP makes only a minimum contribution to the tuberoinfundibular system. Moreover, expression of VIP receptors in the pituitary is low. PHI, a peptide that is contained in the VIP prohormone and has a similar structure to VIP, also stimulates prolactin. PHI is colocalized with CRH in the same neurons of the tuberoinfundibulum system and probably coreleased with CRH during stress. Thus, there is a possibility that PHI contributes to stress-induced prolactin release.

10. MSH SECRETION REGULATORY FACTORS

Like prolactin secretion, MSH secretion is tonically inhibited by the hypothalamus. A tripeptide, Pro-Leu-Gly-NH2, an enzymatic degradation product of oxytocin, was originally considered to be a *MSH inhibiting factor* (MIF), but its MIF activity was not consistently demonstrated in various species of animals. MSH activity in rats can be attributed to βMSH, which is encoded by a part of the sequence of POMC gene. Secretion of POMC-derived peptides from the intermediate lobe is tonically suppressed by a direct dopaminergic nerve supply.

11. NEUROHYPOPHYSIAL HORMONES

11.1. Overview

Two neurohypophysial hormones, *vasopressin* (VP) and *oxytocin* (OXT), were the first chemically characterized neurosecretions. VP and OXT are mainly produced by magnocellullar neurons in the SON and PVN, packed in secretory granules, and transported through the axon of the supraoptico- or paraventriculo-hypophysial tract running toward the neural lobe via the internal layer of the median eminence (Fig. 1). When the pituitary stalk is cut, the secretory granules in the neural lobe disappear and are accumulated in the proximal cut end of the stalk.

11.2. Chemistry, Precursor, and Processing

Extensive efforts to isolate the active principle of the neural lobe extracts led to elucidation of structure of oxytocin (OXT) in 1950 and vasopressin (VP) in 1954. Both VP and OXT contain nine amino acids and a Cys-Cys bridge in the 1-6 position (*see* Table 1). VP differs from OXT by having Phe in place of Ile at amino acid position 3, and Arg or Lys in place of Leu at position 8. Among mammals, VP has identical amino acids (arginine vasopressin or AVP), with the exception of such animals as the pig, hippopotamus, and wild boar, in which Arg in position 9 is replaced by Lys, giving rise to lysine vasopressin (LVP). The amidated carboxyterminus is important for the bioactivity of both VP and OXT.

In lower vertebrates, such as *Cyclostomata,* a hypothalamic neurosecretory system is present and the neurosecretion exhibits vasopressor, antidiuretic, and oxytocic activities. The chemical component of these activities is argine vasotocin, perhaps the most primitive, and only neurohypophysial hormone in lower animals. Oxytocin is not present. Various neurohypophysal hormones have been isolated from fish, amphibia, reptiles, and birds (Table 2). These hormones differ from VP or OXT by only one or two amino acids.

VP and OXT are each associated and co-released with distinct peptides, VP with neurophysin II and OXT with neurophysin I. VP and OXT, with their respective neurophysins, are synthesized as prohormones mainly in the magnocellular part of the paraventricular nucleus and the supraoptic nucleus. These

Table 2
Natural neurohypophysial hormones. Amino acid sequences of lysine vasopressin, oxytocin, and nonmammalian neurohypophysial hormones are compared with arginine vasopressin. The amino acids differing from the corresponding amino acids in arginine vasopressin are shown.

Arg-vasopressin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2								
	1	2	3	4	5	6	7	8	9
Lys-vasopressin								Lys	
Phenypressin		Phe							
Oxytocin			Ile						
Arg-vasotocin			Ile						
Lys-vasotocin			Ile					Lys	
Mesotocin			Ile					Ile	
Valitocin			Ile					Val	
Ichthyotocin			Ile	Ser				Ile	
Glumitocin			Ile	Ser				Gln	
Aspargtocin			Ile	Asn				Leu	

prohormones are processed during transport to yield VP or OXT and their respective neurophysins. Prepro-AVP consists of the putative signal peptide, AVP, neurophysin II, and the glycoprotein domain, and is encoded by the AVP gene on chromosome 20 (Fig. 10). Neurophysin II is assumed to act as a carrier protein for AVP during axonal transport.

The AVP gene is mainly expressed in the supraoptic nucleus and paraventricular nucleus, but also in the suprachiasmatic nucleus of the hypothalamus and the bed nucleus of the stria terminals. AVP gene expression is induced in response to osmotic stimulation, and associated with the expression of carboxypeptidase H and *c-fos* mRNA. Carboxypeptidase H participates in the posttranslational processing of the precursors of many neuropeptides, including AVP and OXT. Expression of carboxypeptidase H mRNA is significantly increased in AVP-producing magnocellular neurons during chronic osmotic stimulation. In addition to carboxypeptidase H, four other processing enzymes are involved in processing the neurohypophysial hormone precursors. These include PC1/PC2, peptidylglycine monooxygenase giving peptidylhydroxyglycine, and an α-amidating ligase, which

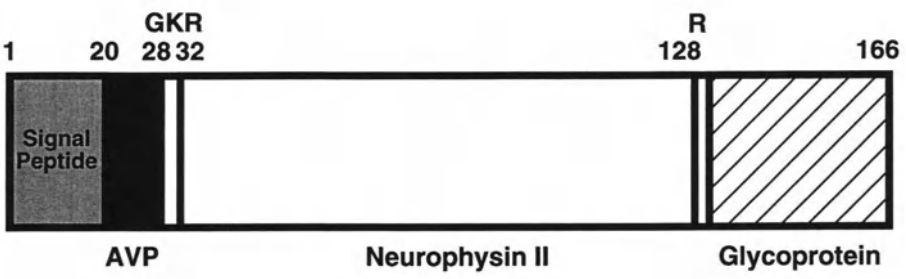

Fig. 10. Schematic illustration of pro-arginine vasopressin. The prohormone consists of three domains: arginine vasopressin, neurophysin II, and a 37/39 residue glycopeptide or copeptin. This organization is preserved in the nonmammalian tetrapod provasotocin. The genes of mammalian neurohypophysial hormones have the three-exon organization: exon 1, encoding the signal peptide, the hormonal nonapeptide, the processing tripeptide sequence and the first 9 residues of neurophysin; exon II, encoding the central part of neurophysin; and exon III, encoding the C-terminal part of neurophysin. The arginine processing site links to copeptin.

splits hydroxyglycine into an amide group and glyoxylic acid.

The precursor of OXT is similar to that of AVP. Prepro-OXT is composed of the putative signal peptide, OXT, and neurophysin I, but lacks a glycoprotein domain in contrast to prepro-AVP. The OXT gene encoding prepro-OXT is linked to the AVP gene with a 12 kb intervening in humans. The structure of 5′ promoter region of OXT is different from that of AVP, indicating that AVP and OXT may require distinct signals for their gene regulation. The gene expression of AVP and OXT is regulated by several physiological factors. The major factor regulating AVP synthesis is osmotic stimulation. A continuous increase in AVP mRNA and OXT mRNA has been reported in the magnocellular neurons of the SON and PVN of rats chronically given hypertonic saline as a drinking solution. Estrogen influences OXT gene expression in the hypothalamic nuclei. OXT mRNA levels in the SON are increased nearly twofold at estrus relative to the other periods of the estrous cycle. In contrast, no significant cyclic variation is observed in AVP mRNA levels.

11.3. Regulation of Vasopressin and Oxytocin Release

Cholinergic stimulation elicits release of VP, as observed in antidiuresis resulting from smoking, which stimulates nicotinic acid receptors. Noradrenergic stimulation is inhibitory on both VP and OXT. This inhibitory response is mediated by β-adrenergic stimulation. Stress-suppressed "milk let-down" reflex may result from β-adrenergic inhibition of OXT release. Similar inhibition of VP release may account for stress-induced diuresis.

Among the neuropeptides, which modify secretion of the neurohypophysial hormones, is angiotensin. An intracerebroventricular injection of a minute amount of angiotensin II suppresses water diuresis. Intracarotid injection, but not intravenous injection, of angiotensin II induces an increase in plasma VP levels.

Release of OXT is elicited by mechanical stimulation of the vagina, particularly stimulation of the cervix. This response is reduced during pregnancy, probably because of the effect of progesterone. OXT release, in response to vaginal stimulation, is reduced by pretreatment with progesterone. OXT is also released during sexual intercourse. Secretion of OXT is at its maximum during perturition, at the time of cervical dilation resulting from passage of the fetal head. OXT release is also evoked during infant suckling in women and milking in cows, when it induces milk ejection.

11.4. Physiological Actions of Vasopressin

The major physiological action of VP is to increase water absorption from the distal tubules and the collecting duct of the kidney to concentrate urine. Thus, VP is also called antidiuretic hormone (ADH). AVP has greater antidiuretic and vasopressor activities than LVP. The mechanism of the antidiuretic action of VP will be discussed in Chapter 14. OXT has uterus contractile activity, but neither antidiuretic nor vasopressor activity.

VP also constricts peripheral blood vessels, including capillary and increases blood pressure. Both VP and OXT are widely distributed in the brain and participate in various neurobehavioral functions. Other actions of VP include enhancement of CRH-induced ACTH release and stimulation of glycogenolysis.

11.5. Physiological Actions of Oxytocin

The major physiological actions of OXT are its effect on the mammary gland during lactation and on uterus contraction. When an infant begins to nurse, milk appears at the nipple after a delay of 30 s or so, a process known as "milk let-down." The stimulus induced by the suckling infant is initiated at the nerve endings of the nipple, and is transmitted through the spinal cord, midbrain, and finally the hypothalamic OXT producing neurons, where it triggers release of OXT from the pituitary neural lobe into circulation. OXT is transported to the breast and causes contraction of the myoepithelial cells that encircle the mammary acini, thereby expelling the milk. When the neural lobe is removed in mother rats, nursing pups cannot obtain milk, but they can do so after oxytocin injection. The milk let-down reflex is affected by changes in hypothalamic neural activity. Stressors such as pain block milk let-down in animals, possibly through β-adrenergic stimulation. In women, milk let-down can be conditioned by the crying of a hungry baby.

OXT induces contraction of the uterus in humans and animals. Just before the onset of labor, the uterine myometrium becomes extremely sensitive to OXT because of a dramatic increase in the number of OXT receptors. The magnitude of the uterine contractile effect varies during the estrous cycle, probably because of the effect of estrogen and progesterone. Estrogen generally sensitizes the uterine response to oxytocin, whereas progesterone decreases the sensitivity. When labor begins in normal women, maternal

oxytocin secretion takes place in spurts and increases to a maximum at the time of delivery.

11.6. Mechanism of Action and Receptors of Vasopressin

VP directly interacts with its receptors on the serosal side of the distal tubules and collecting ducts of the kidney, stimulates adenylate cyclase, and increases cAMP accumulation, which mediates enhancement of water absorption from the lumen. AVP receptors have been classified as V1a, V1b, and V2 receptors, usually based on their biologic actions. AVP induces antidiuretic action through V2 receptors, which are mainly located in the renal tubules and coupled to adenylate cyclase. The other biologic actions of AVP are mediated through V1 receptors, which are located in the vascular smooth muscle and hepatocytes (V1a), and the pituitary (V1b), and act through phosphatidylinositol hydrolysis to mobilize intracellular Ca^{2+}. The cDNAs encoding for AVP receptors have been cloned. The deduced amino acid sequence indicates that all these vasopressin receptors are proteins with putative seven transmembrane domains, coupled with G-protein.

11.7. Mechanism of Action and Receptors of Oxytocin

Oxytocin induces strong contractions of the uterus at term by increasing the cytoplasmic Ca^{2+} concentration. The hormone acts by activating phospholipase C to hydrolyze phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-triphosphate and diacylglycerol through a G_q/G11 protein. Oxytocin receptor-mediated stimulation of inositol phosphate has been demonstrated in the guinea pig, ovine, and human myometrium, human decidual cells, and the bovine mammary gland, indicating that oxytocin receptors, like AVP receptors of the V_1 subtype, are functionally coupled to a phospholipase C. In human amnion cells, oxytocin and AVP increase prostaglandin E_2 (PGE_2) production and may influence labor by stimulating PGE_2 in the amnion through the inositol phospholipid-protein kinase C system. Human oxytocin receptor cDNA has been cloned, and its deduced structure consists of 388 amino acids with seven transmembrane domains typical of G-protein-coupled receptors.

12. PITUITARY ADENYLATE CYCLASE ACTIVATING POLYPEPTIDE (PACAP)

Pituitary adenylate cyclase activating polypeptide (PACAP) was isolated and characterized from ovine hypothalamic tissues in 1989, in an attempt to discover a novel hypothalamic hormone with the ability to activate adenylate cyclase in rat pituitary cell cultures. PACAP exists as two amidated forms, with 38 (PACAP38) and 27 residues (PACAP27), PACAP38 being the major form found in tissues. PACAP is a new member of the secretin/glucagon/VIP family, with greatest homology to VIP (*see* Table 1). PACAP fulfills nearly all the requirements for a hypothalamic hormone. In the hypothalamus, PACAP is synthesized mainly in the magnocellular and parvocellular parts of the PVN, the SON and in the periventricular nucleus. PACAP-containing neurons contribute to both the tuberoinfundibular system and hypothalamo-neurohypophysial systems. Thus, a considerable amount of PACAP is present in the neural lobe, apparently coexisting with oxytocin. In sheep and primates, many nerve fibers containing PACAP terminate in the pituitary stalk adjacent to the capillaries of the hypophysial portal vessels (Fig. 11). In rats, the concentration of PACAP in the hypophysial portal blood is significantly higher than that in the systemic blood.

The cDNA of preproPACAP was cloned and the deduced amino acid sequence of the precursor of ovine PACAP contains 176 amino acids. Subsequently, human and rat PACAP preprohormone and human PACAP gene have been cloned. The PACAP precursor contains a GHRH-like peptide named PACAP-related peptide (PRP). PRP corresponds to PHI in the VIP precursor. The PACAP precursor is processed by PC1 and PC2, but with a greater efficacy by PC2, which is expressed in both the magnocellular and parvocellular parts of the PVN and SON.

The amino acid sequence of PACAP38 is identical among mammals. PACAP38 in lower vertebrates is also very similar to human PACAP38; frog and chicken PACAP38 differ from mammal PACAP38 by only one amino acid, and fish PACAP38 differs by 2–4 amino acids. Even the amino acid sequence of tunicate PACAP is identical to human PACAP in the N-terminal 27 residues, indicating strong conservation of the structure during an evolutionary period spanning over 700 million yr. In contrast to mammals, GHRH-like peptide in the PACAP precursor is the only GHRH-like molecule in lower vertebrates. That is, GHRH in lower animals is encoded by the same gene that encodes PACAP. Although, both the GHRH-like peptide in the PACAP precursor and PACAP38 of the eel stimulate GH release from the eel pituitary in vitro, PACAP38 is more potent. In this system, human GHRH is inactive. Therefore, it is likely that PACAP serves as the GHRH in lower

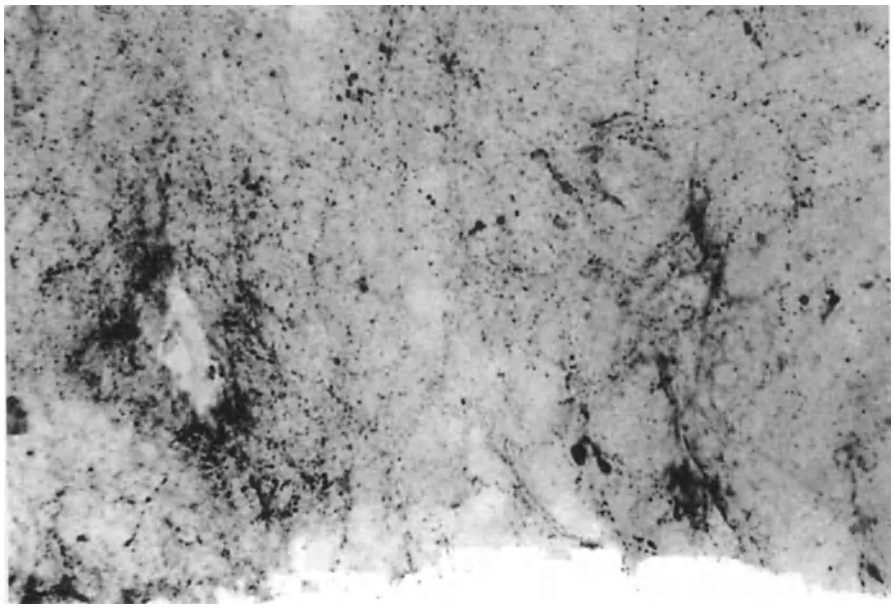

Fig. 11. Immunohistochemistry of PACAP-immunoreactive (ir) nerve terminals in the hypophysial stalk of sheep. Numerous PACAP-ir fibers are seen around the capillary of the hypophysial portal vessels. (Prepared by K. Köves).

animals. The GHRH gene in mammals may be generated by gene duplication.

12.1. Effects on Anterior Pituitary Cells

PACAP directly stimulates adenylate cyclase and increases cAMP accumulation, phospholipase C, and intracellular concentrations of Ca^{2+} in cultured normal and clonal pituitary cells. Abundant PACAP specific binding sites have been demonstrated in the anterior pituitary, but few in the neural lobe. Contrary to the classic hypothalamic hormones, all cell types of the anterior pituitary, including nongranular folliculo-stellate (FS) cells, express PACAP binding sites.

Although an intravenous administration of PACAP stimulates the release of pituitary hormones in animals and humans under certain conditions, it does not consistently stimulate hormone release from cultured normal rat pituitary cells, despite exhibiting stimulation of adenylate cyclase. PACAP alone only weakly stimulates LH and FSH release, whereas PACAP and LHRH, in combination, synergistically stimulate gonadotropin secretion in rat pituitary cell cultures. No significant changes in the secretion of either TSH, ACTH, or GH have been observed.

In anterior pituitary cells taken from castrated male rats, continuous treatment with 10 nM PACAP38 increased α-subunit and decreased FSH-β mRNA levels. This treatment had no effect on LH-β mRNA, but lengthened LH-β mRNA. In clonal GH_3 cells, a large dose of PACAP stimulated the expression of PRL mRNA over a 24-h incubation period. PACAP also stimulated transcription of the rat PRL in GH_3 cells. This effect was seen at 10 pM PACAP, which is ineffective in stimulating cAMP production in these cells. Long-term stimulation of AtT20 cells with PACAP led to a significant decrease in the rate of cell division concomitant with an increase in cell size and the formation of cell processes characteristic of cellular differentiation. Whether these stimulatory effects of PACAP on transcription and gene expression are seen in normal pituitary cells remains to be studied.

PACAP does not appear, at least in vitro, to be a potent secretagogue of the pituitary hormone, but it may exert a major action on the regulation of hormone synthesis. One exception is that PACAP enhances IL-6 release from pituitary folliculo-stellate (FS) cells in a dose-dependent fashion in vitro. PACAP-induced IL-6 release is mediated by activation of adenylate cyclase. FS cells and their processes are in close contact with pituitary glandular cells and may regulate the activities of the glandular cells in a paracrine fashion by IL-6 and possibly by other growth factors produced by FS cells (Fig. 12). The concentration of PACAP in the rat hypophysial portal blood is sufficient to stimulate IL-6 release from the FS cells in vitro. Thus, PACAP may regulate the pituitary glandular cells both directly and indirectly through FS cells.

12.2. PACAP Receptors and Signal Transduction

Human, rat and bovine PACAP receptors have been cloned and found to be proteins with seven

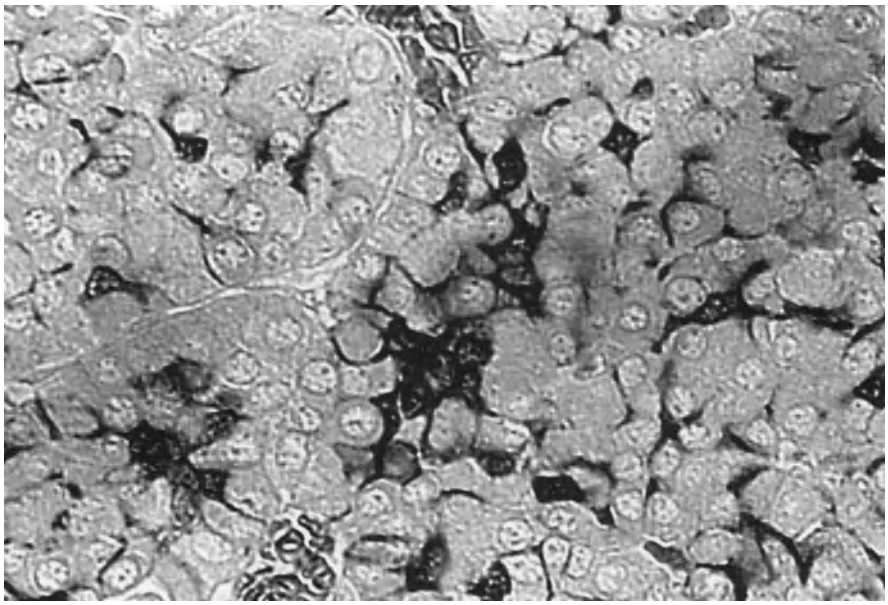

Fig. 12. Folliculo-stellate (FS) cells in the rat adenohypophysis stained with an antiserum against S100 protein, a marker for FS cells. Many processes of FS cells are seen between the glandular cells juxtaposed with these secretory cells. FS cells produce various growth factors, including IL-6, and PACAP stimulates IL-6 production by FS cells. PACAP may affect the pituitary glandular cells indirectly through FS cells by stimulating production of growth factors, which regulate activities of the glandular cells in a paracrine fashion. (Provided by K. Inone).

transmembrane domains, typical of the G-protein-coupled receptor. The PACAP specific receptor is called PAC_1 receptor. At least seven subtypes of PACAP specific receptors, resulting from alternate splicings, were cloned, each linked with distinct signal transduction pathways. PACAP also binds to VIP_1 and VIP_2 receptors with a high affinity similar or even greater than that of VIP. These receptors, which are shared between PACAP and VIP, are collectively known as $VPAC_1$ and $VPAC_2$ receptors. The anterior lobe of the pituitary expresses abundant PAC_1 receptors, but fewer $VPAC_1$ and $VPAC_2$ receptors.

The interaction of PACAP with PACAP receptors triggers at least three cascades of signal transduction: (1) stimulation of adenylate cyclase and protein kinase A; (2) stimulation of phospholipase C, an increase in inositol phosphate turnover, generation of diacylglycerol, and an increase in intracellular Ca^{2+}; and (3) stimulation of MAP kinases.

SELECTED READINGS

Acher R, Chauvet J. The neurohypophysial endocrine regulatory cascade: precursors, mediators, receptors, and effectors. Front Neuroendocrin 1995;16:237.

Arimura A. Perspectives on pituitary adenylate cyclase activating polypeptide (PACAP) in the neuroendocrine, endocrine, and nervous systems. Jpn J Physiol 1998;48:301.

Guillemin R, Brazeau P, Bohlen P et al. Growth hormone releasing factor from a human pancreatic tumor that caused acromegaly. Science 1982;218:585.

Harris GW. Neural Control of the Pituitary Gland. London, UK. Edward Arnold Ltd., 1955.

McCann SM, Mizunuma H, Samson WK. Differential hypothalamic control of FSH secretion: a review. Psychoneuroendocrin 1983;8:299.

Montiminy MR, Goodman RH, Horvitch SJ, Habener JF. Primary structure of the gene encoding rat preprosomatostatin. Proc Natl Acad Sci USA 1984;81:3337.

Reichlin S. Neuroendocrinology. In: Wilson JD, Foster DW eds. Textbook of Endocrinology, Philadelphia, PA, 9th ed., W. B. Saunders, 1998:165.

Seeburg PH, Adelman JP. Characterization of cDNA for precursor of human luteinizing hormone releasing hormone. Nature 1984;311:666.

4 Neurotransmitters as Regulators of Hypothalamic Function

Paul V. Malven, PhD

Contents

1. INTRODUCTION

Neurons of the hypothalamus, like those of the entire central nervous system (CNS), are organized into circuits and networks with the communication between neurons involving either *electrical synapses* (which are a form of specialized *gap junctions*) or *chemical synapses.* When a gap junction is open, it allows free bidirectional migration of molecules and ions from the cytoplasm of one neuron directly into the cytoplasm of the other neuron. In electrical synapses, alternating opening and closing of gap junctions between two neurons creates a regulatory situation similar to that observed for gated ion channels imbedded in the plasma membrane (PM) of individual neurons. Although such bidirectional interneuronal communication via electrical synapses is not fully understood, it presumably allows two or more neurons to act together as a functional unit with very rapid information transfer. In contrast to the incomplete understanding of electrical synapses, there is abundant information about the widespread interneuronal signaling via chemical synapses, and this chapter will present that information in detail. Neuron-to-neuron communication via chemical synapses involves the release of chemicals from one neuron and their specific action on an adjacent neuron to alter its excitability. Neuron-derived compounds that alter the excitability of another neuron or a muscle fiber are defined as a *neurotransmitter* (NT), and the molecule structures of the many different NT exhibit great diversity.

As stated earlier, interneuronal communication via neurotransmitters involves an asymmetric morphological specialization known as a chemical synapse (hereafter called a *synapse*) wherein part of the NT-producing neuron is called the *presynaptic terminal* and part of the NT-receptive neuron is called the *postsynaptic terminal.* The approximately

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Table 1
Names and Categories of Various Neurotransmitters

Nonpeptide Neurotransmitters	*Peptide Neurotransmitters*	
Aminergic compounds:	**Neural lobe neurohormones:**	**Opioidergic peptides:**
Dopamine	Oxytocin	Enkephalin (multiple forms)
Norepinephrine	Vasopressin	β-endorphin
Epinephrine	**Hypophysiotropic neurohormones**	Dynorphin-A (and -B)
Serotonin	(multiple compounds)	α / β-neo-endorphin
Histamine	**Brain-gut peptides:**	**Tachykinin peptides:**
Acetylcholine	Cholecystokinin	Substance P
Amino acid compounds:	Gastrin	Neurokinin-A
Glycine	Insulin	Neurokinin-B
Gamma-aminobutyric acid	Glucagon	Melanocortin (multiple forms)
Glutamic acid	Secretin	Angiotensin II
Gases:		Calcitonin gene-related peptide
Nitric oxide	Neuropeptide-Y	Endothelin (multiple forms)
Carbon monoxide	Galanin	Neurotensin

15-nm-wide space between these two elements of the synapse is termed the *synaptic cleft.* Because a synapse is morphologically asymmetrical, neuron-to-neuron communication is primarily unidirectional. Occasionally, pairs of two unidirectional synapses between two adjacent neuronal dendrites form what are called bidirectional *dendro-dendritic* synapses. Each synapse has a very small contact area (<0.2 μm^2) allowing each presynaptic terminal to communicate with multiple postsynaptic neurons and for each postsynaptic neuron to receive input from many different presynaptic terminals possibly derived from multiple neurons. In addition, synaptic contacts between neurons are not fixed. New synapses may form and existing synapses may be eliminated during the life of the organism.

Because there are many different types of NT chemicals that alter neuronal excitability in the hypothalamus and other CNS tissue (Table 1), the biosynthesis and intracellular migration of each type will be discussed separately. The NT designation was originally applied to a group of small molecules that are now referred to as *classical* NT and include the following: *acetylcholine,* selected amino acids such as *glutamic acid* (also known as glutamate in its ionized form), *glycine* and *gamma-aminobutyric acid,* and various biogenic amines such as *dopamine, norepinephrine, histamine,* and *serotonin.* Neuron-derived peptides, which are involved in interneuronal communication, constitute a second major class of *peptide* NT (*see* Table 1). Some of these NT peptides can also function as *neurohormones* when secreted into the blood for delivery to distant cells that possess the appropriate receptors. A third class of NT chemicals, only recently discovered, consists of the neuron-derived gases such as *nitric oxide* and *carbon monoxide* that can readily diffuse between adjacent neurons to alter excitability of the receptive neuron. Neither the unidirectional nature nor the morphological features of synapses using NT gases are well understood.

2. NEUROTRANSMITTERS EXHIBIT GREAT CHEMICAL DIVERSITY

2.1. Biosynthesis of Catecholamines and Serotonin Share Similarities

Catecholamines derive their name from having a catechol nucleus (benzene ring with two adjacent hydroxyl groups) plus one amine group. *Norepinephrine* (NE) and *dopamine* (DA) are two catecholamines that function as NT, and they share a common biosynthetic pathway (Fig. 1). Neurons take up the amino acid (aa) known as tyrosine, and using the enzyme tyrosine hydroxylase, they convert tyrosine into *d*ihydroxy-*p*henyl*a*lanine (abbreviated DOPA), which is

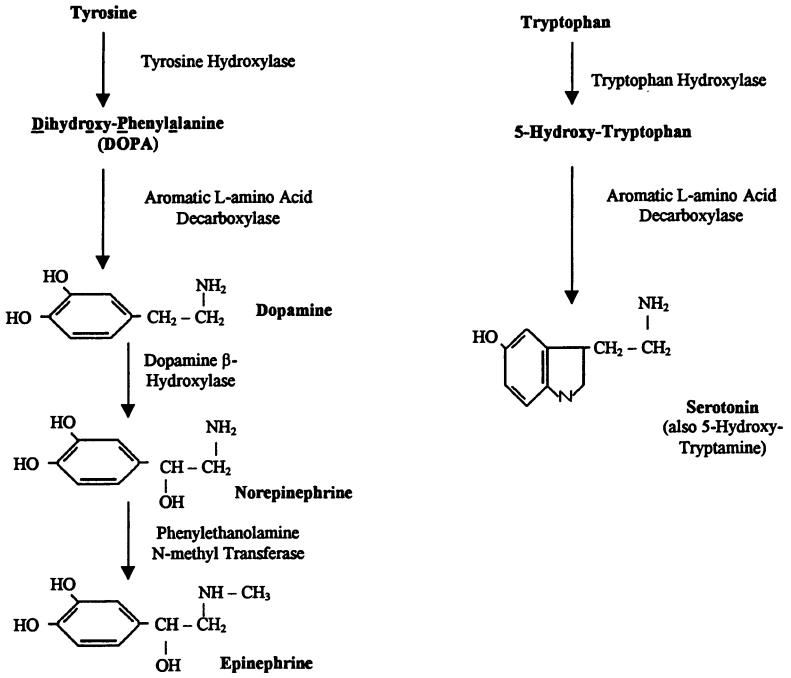

Fig. 1. Synthesis and structures of catecholamines and serotonin molecules as neurotransmitters.

the immediate precursor of DA. In those neurons that either utilize DA as their NT or secrete it as a neurohormone, biosynthesis continues no further. In other neurons that possess an enzyme known as dopamine-β-hydroxylase, the DA molecules serve as precursors for synthesis of NE, and it functions as a NT in these neurons. Those neurons that can convert DA into NE are classified as *noradrenergic* (noradrenaline is another name for NE) and are less abundant than the dopaminergic neurons in the CNS. Their neuronal perikarya are located primarily in the locus ceruleus, a small but important group of neurons located in the midbrain.

Epinephrine (Epi) is formed by the enzymatic actions of *p*henylethanolamine-*N*-*m*ethyl *t*ransferase (abbreviated as PNMT), which adds a methyl group to the amine group of NE (*see* Fig. 1). From a chemical point of view, Epi (also known as adrenaline) is not a catecholamine because its amine group has been blocked by the added methyl group, but this distinction is often ignored because Epi and NE share many of the same actions as well as common adrenergic receptors. The presence of the PNMT enzyme in neurons identifies them as either using Epi as their NT or secreting it as a neurohormone. PNMT was first discovered in a subset of modified postganglionic neurons in the adrenal medulla that secrete Epi into blood to produce its neurohormonal effects. The existence of central neurons that utilize Epi as their NT is controversial although some central Epi may be derived from NE after its release into the synaptic cleft, but clearly NE is more important than Epi as a NT the CNS.

Serotonin (also known as *5-h*ydroxy-*t*ryptamine and abbreviated 5-HT) is considered here with the catecholamines because its biosynthesis also begins with an ordinary aa, namely tryptophan (*see* Fig. 1), and because it shares many properties with the catecholamines. The benzene ring in the indole group of tryptophan is first hydroxylated by the enzyme tryptophan hydroxylase. The same enzyme (aromatic L-amino acid decarboxylase) that catalyzes the formation of DA removes the C-terminal carboxyl group, and after this decarboxylation, 5-HT can be classified as an *indolamine.* As in the case of catecholamines, the rate-limiting step in biosynthesis of 5-HT is the hydroxylation of its precursor amino acid tryptophan.

Catecholamines and 5-HT are synthesized throughout the neuron including its presynaptic terminals. In addition to precursor amino acid being obtained from blood and extracellular fluids, there are mechanisms for reuptake of released NT from the synaptic cleft and adjacent areas. This reuptake into the presynaptic terminal not only halts the action of the NT on the postsynaptic terminal, but it replenishes quantities of the precursor for synthesis of more NT. The mecha-

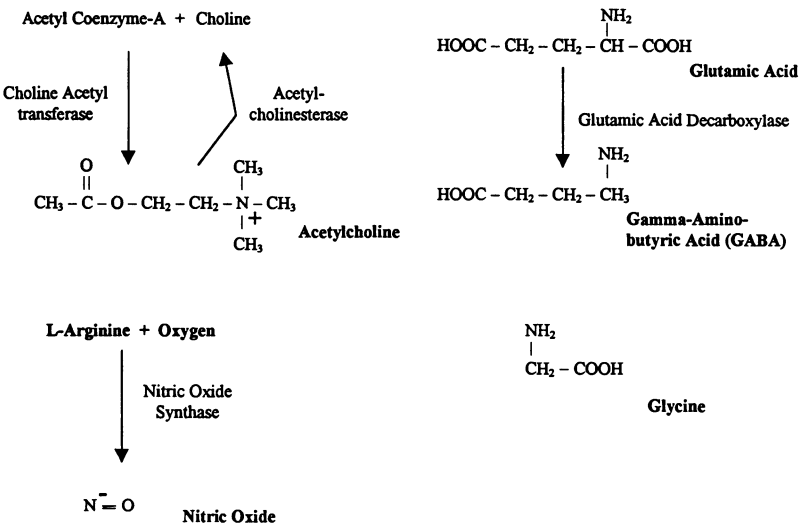

Fig. 2. Synthesis and structures of diverse molecules that function as neurotransmitters.

nisms of this reuptake via what are called *transporters* will be discussed in detail later in this chapter.

2.2. Acetylcholine Discovered as Activator of Muscle Contractions and an Important Neurotransmitter

The first classical NT to be discovered was *a*cetyl-*ch*oline (ACh), a compound that affects muscle fibers as well as neurons. The discovery of ACh established firmly the principle that neurons could secrete chemical compounds capable of exciting other neurons or muscle fibers. The intraneuronal synthesis of ACh involves the enzymatic combination of choline with an activated form of acetate, namely acetyl coenzyme-A using the enzyme choline acetyltransferase (*see* Fig. 2). After its release into the synaptic cleft, extracellular ACh is rapidly hydrolyzed by another enzyme known as *acetylcholinesterase* (Fig. 2), and the resulting choline is taken up by the presynaptic neuron to serve as a precursor for synthesis of more ACh.

2.3. Unmodified and Modified Amino Acids Function as Neurotransmitters

Although amino acids often serve as precursors for catecholamines and 5-HT, some of them do not require any structural modification to function as a neuroactive NT. Glycine (*see* Fig. 2 for structure) is an abundant aa that appears to act as a NT in the spinal cord and lower brain stem where it functions as an inhibitor of postsynaptic neurons. Glutamic acid (*see* Fig. 2 for structure) is another unmodified aa that functions throughout the brain as an excitatory NT. In addition to functioning as a NT, glutamic acid also serves as a precursor to *g*amma-*a*mino*b*utyric *a*cid (abbreviated GABA) in those neurons that possess the enzyme known as glutamic acid decarboxylase (*see* Fig. 2). The action of GABA on postsynaptic neurons is mainly as an inhibitor of their spontaneous or induced excitability. In addition, the amino acid histadine can be converted into the aminergic NT known as *histamine.*

2.4. Neuron-Derived Gases Represent Unique Neurotransmitter Molecules

Knowledge about labile gases that diffuse from one neuron to another and function as biological messengers within the nervous system has only recently become available. Whether or not labile gases such as nitric oxide (NO) and carbon monoxide should be considered as NT may still be controversial to some neuroscientists. However, it seems well established that NO is produced by neurons using L-arginine and oxygen as substrates and the enzyme known as nitric oxide synthase (*see* Fig. 2). The resulting molecules of NO can then diffuse into other neurons and modify their excitability, thereby qualifying as a NT molecule.

2.5. Neuron-Derived Peptides Function as Neurotransmitters

Neuron-to-neuron communication also occurs via chemical synapses that use various peptides as their

NT. Many of the peptides that function as NT were first discovered and identified as locally acting chemical messengers or in other cases as blood-borne hormones. The idea that neurons can produce and utilize peptides as chemical messengers was first proposed by the late Ernst and Berta Scharrer more than 50 years ago when they observed a few invertebrate neurons that had the histological staining characteristics previously ascribed only to peptide-producing cells in endocrine organs. These histological observations were subsequently verified by other techniques, and they provided the foundation for the field of *neuroendocrinology.* Those blood-borne peptides that were proved to also be neuron-derived were called *neurohormones* (blood-borne chemical messengers synthesized by neurons). *Vasopressin* and *oxytocin* produced by neurons in the hypothalamus, transported via axons to the neural lobe of the pituitary gland, and released from the axon terminals into blood constituted the first definitive neurohormones. Those other peptides that are produced by and released from neurons, but for which blood-borne delivery has not been proven, are called *neuropeptides* to denote their origin although their mode of action (hormone or NT) remains uncertain.

Many neuropeptides that function as neurohormones when released into blood for delivery to their target tissues can also function as NT when released from the presynaptic terminal of synapses to act upon postsynaptic receptors (*see* Table 1). These peptidergic NT include the neurohormones oxytocin and vasopressin released from the neurohypophysis as well as some of the neurohormones released from axons into the hypophysial portal veins for delivery to and regulation of anterior pituitary cells (defined as hypophysiotropic hormones; *see* Chapters 1 and 7).

Another category of peptides that function as NT were discovered first as hormones produced by tissues of the digestive tract for hormonal regulation of metabolism and digestion. It was subsequently found that these digestive and metabolic hormones could regulate neuronal excitability. Acceptance of these peptides as NT, however, did not occur until after their synthesis by neurons had been proven. *Cholecystokinin* is the best known of these peptidergic NT discovered first as a hormone of the gut, but others include *gastrin, insulin, glucagon, secretin,* and *vasoactive intestinal peptide.* The generic term *brain-gut peptide* is sometimes used to describe this category of gut-derived and brain-derived peptides that can function as both a hormone and a NT (*see* Table 1).

Those peptidergic NT derived from the known precursors of *endogenous opioids* (EO) constitute another category. Because each EO precursor can be cleaved into many different neuropeptides, there may be some uncertainty about which EO neuropeptide actually functions as the opioidergic NT at the postsynaptic terminal. However, the EO precursor known as proenkephalin yields *methionine-enkephalin, leucine-enkephalin,* and other structurally similar peptides that can function as an opioidergic NT. Proopiomelanocortin, another EO precursor, yields β-*endorphin* that acts as an opioidergic NT, and this same precursor yields *adrenocorticotropin* (ACTH) and several forms of *melanocortin* (*see* Chapter 15) that may act as a NT through nonopioidergic mechanisms. Cleavage of the EO precursor known as prodynorphin yields multiple NT molecules including *dynorphin-A, dynorphin-B,* and α / β-*neo-endorphin* (*see* Table 1).

In addition to the many categories of neuropeptide NT described above, there are also many peptidergic NT molecules that do not fit readily into any category. The first one of these NT molecules to be isolated and later sequenced was an 11-residue peptide named *substance P* (SP) that caused contractions of smooth muscle in arterioles and other tissues. It was discovered later that SP is produced by neurons and is a member of the tachykinin family of neuropeptides that also includes *neurokinin-A* and *neurokinin-B* each derived from the same precursor as SP (*see* Table 1). Other hypothalamic neuropeptides that function as NT molecules include *neuropeptide-Y* (NPY), *neurotensin,* and *galanin* each of which represents a different family of peptides. Other neuron-derived peptidergic NT include many unique compounds such as *angiotensin II* that can be enzymatically produced in blood or in neurons as well as the multiple forms of *atriopeptin* that were discovered first in cardiac tissue, but are also produced by neurons. *Calcitonin gene-related peptide* (CGRP) is also a neuron-derived NT that is a potent vasodilator and an alternative product of the gene for the thyroid-derived hormone known as calcitonin. Multiple forms of the vasoactive peptide known as *endothelin,* that was discovered first as a product of endothelial cells, have also been shown to function as a neuron-derived NT. Because of the great molecular diversity of NT peptides, there seems no valid reason to exclude from considering any peptidergic biosynthetic product as a potential NT (*see* extensive listing of peptidergic NT in Table 1).

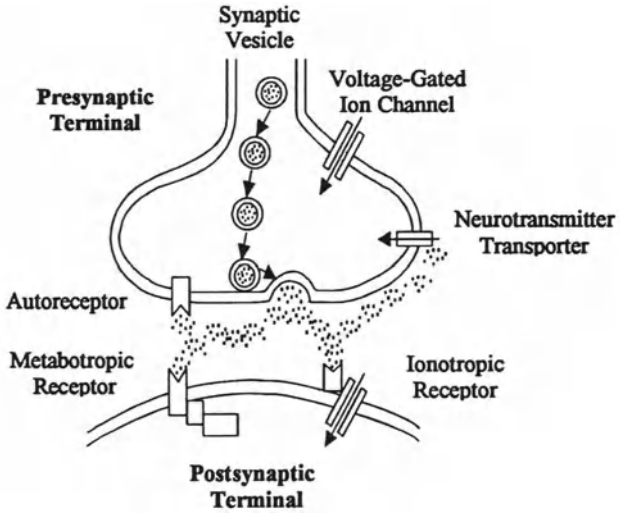

Fig. 3. Axonal transport and exocytosis of synaptic vesicles to release their contents of NT (denoted by dots) into the synaptic cleft to interact with transporters and autoreceptors on the presynaptic terminal as well as with metabotropic and ionotropic receptors on the postsynaptic terminal.

3. NEUROTRANSMITTERS ARE PACKAGED IN SYNAPTIC VESICLES AND RELEASED FROM PRESYNAPTIC NEURONS BY EXOCYTOSIS

3.1. Synaptic Vesicles Are Formed and Transported

Except for the NT gases, the many different compounds that function as NT are contained within *synaptic vesicles* (SV) prior to their release from presynaptic neurons. Those SV that contain peptidergic NT are formed from the *Golgi apparatus* in the neuronal *perikaryon* (also known as the cell body, soma, or nucleus-containing part of the neuron) where its peptide precursors are also synthesized. The peptide-filled SV are then transported down the axon to reach and become concentrated in each of the many axonal branches that function as presynaptic terminals (*see* Fig. 3). The process by which peptidergic SV and other nucleus-derived material migrate into the many axonal branches is called *axonal transport,* and the details of its dynamics are incompletely understood. However, certain toxic substances that disrupt cytoskeletal function can block axonal transport, and one of these substances, known as *colchicine,* is often used in experiments to increase the quantity of NT-containing SV in neuronal perikarya.

In addition to SV that contain perikaryon-derived neuropeptides, the SV in many neurons contain other NT molecules such as biogenic amines, acetylcholine, and modified aa compounds. Only a small proportion of these nonpeptidergic SV originate in the nucleus because most of them appear to be formed by the endosomes located some distance from the nucleus in the axon and its presynaptic terminals. After budding off the endosome, these SV appear to take up their respective NT from the cytoplasm using specific uptake mechanisms located in the membrane of the SV and to have their NT available for immediate release into the synaptic cleft.

The population of SV, whether found locally in the presynaptic terminal or delivered to the terminal by axonal transport from the perikaryon, must undergo preparation for release of their contents of NT into the synaptic cleft. This preparation of SV begins with a process called *docking* in which SV make physical contact with the PM in what is called an intracellular *active zone* adjacent to the synaptic cleft. After docking occurs, SV must go through an additional priming that makes them competent for very rapid exocytotic release of their NT when the appropriate signal is generated.

3.2. Neurotransmitters Released by Exocytosis Bind to Metabotropic or Ionotropic Receptors

When a propagated action potential reaches and depolarizes the PM of the presynaptic terminal, the *voltage-gated* Ca^{2+} channels located in the PM are opened. Thereafter, the population of SV that were docked and primed undergoes immediate *exocytosis* (fusion with PM and disruption of the PM at the site of fusion) releasing NT into the synaptic cleft (*see* Fig. 3). Each of the many different types of NT has one or more receptors that bind specifically to that extracellular NT. Many of the NT receptors are located on the PM of the adjacent postsynaptic terminal (*see* Fig. 3). Some of these NT receptors, also known as *metabotropic* receptors, are linked to G-proteins that mediate the effects of NT binding (i.e., they transduce the signal from the ligand). Other NT receptors, called *ionotropic* receptors, are combined with membrane-embedded channels that transport specific ions. These receptor-associated channels are also known as *ligand-gated ion channels* because binding of the receptor with its specific ligand can either open or close the channel to passage of cations or anions. Depending upon the specific nature of the NT and its receptor, binding of the NT can either promote or suppress depolarization of the postsynap-

tic membrane to either increase or decrease the probability of action potentials being generated in the postsynaptic neuron.

Because each exocytotic release of NT inserts membrane from the former SV into the PM of the presynaptic terminal, a method to recover and recycle the inserted PM is needed to prevent enlargement of the terminal. Membranes of the former SV inserted into the PM are rapidly internalized by *endocytosis* to form *coated vesicles* that are translocated intracellularly to the endosomes for fusion with them. These endosomes then become the source for formation of new SV thereby completing the recycling of the SV membranes.

The PM of the presynaptic terminal also has two elements that interact with NT molecules that have been released into the synaptic cleft, and these two elements are illustrated in Fig. 3. Some presynaptic terminals have what are called *autoreceptors* for their released NT. These autoreceptors may function to regulate the activity of the presynaptic neurons in ways that are probably inhibitory. Many presynaptic terminals as well as adjacent neuroglial cells also have mechanisms for selective reuptake of released NT (or their breakdown products), but these mechanisms involve *transporters* rather than receptors. Each transporter is presumed to be specific for reuptake of only one NT, and these transporters can also be targets for drugs. Pharmacological antagonism of a NT transporter will leave more NT molecules in the synaptic cleft to increase the functional interaction with receptors on the postsynaptic terminal. Other drugs can either enhance or delay the extracellular degradation of NT molecules after their release into the synaptic cleft and in this way alter the efficiency of neurotransmission.

4. PROPERTIES AND SIGNAL TRANSDUCTION PATHWAYS FOR IONOTROPIC AND METABOTROPIC RECEPTORS

The specific affinity of each receptor leads to selective binding with its NT for the purpose of *signal transduction* inside the receptor-containing cell. Some receptors are located in the interior of cells so that their ligands must diffuse through the PM to interact with them. Most receptors of this type belong to a superfamily of ligand-activated *transcription factors* that must reach the nucleus to produce their effects on gene transcription (*see* Chapter 6). Because most NT are not freely diffusible through the PM, the intracellular ligand-activated transcription factors are rarely involved in signal transduction by NT although they do mediate many effects of lipid-soluble hormones in the brain. Another general class of receptors is embedded in the PM of cells such that their ligands do not enter the cell to produce their effects. All PM-associated receptors are an integral part of the neuronal PM, which is a lipid bilayer, and therefore they must have at least one hydrophobic *trans*membrane (TM) domain in order to be stable in the hydrophobic local environment of the PM. As discussed in the previous section, PM-associated receptors for NT are classified as either *ionotropic* or *metabotropic*, and the characteristics of each category are presented in Table 2 and will be discussed in the following paragraphs.

4.1. Ionotropic Receptors Directly Produce Immediate Changes in Ion Flux

Ionotropic receptors mediate the rapid transmission of signals between neurons. Because they are an integral part of a ligand-gated ion channel (for passage of Ca^{2+}, Na^+, K^+, or Cl^-), the channel can be opened or closed within a few milliseconds after the ligand binds. Each ionotropic receptor contains between three and five TM segments (always fewer than seven) that consist of hydrophobic aa sequences. The entire ionotropic receptor is a macromolecular complex that contains the NT binding site plus the ion channel (*see* Fig. 3). Occupation of the receptor by its ligand leads to a change in the conformation of the ion channel to either open or close it. Because of the speed with which ligands bind and unbind, these mechanisms mediate synaptic transmission that has a very rapid onset and an equally rapid termination (*see* Table 3). The most thoroughly studied ionotropic receptor is the nicotinic ACh receptor that is coupled to a nonspecific cation channel (mainly Na^+/K^+, but some Ca^{2+}) and is activated by nicotine, one of the most widely used pharmacological agents in the world. Other ionotropic receptors include the $GABA_A$ receptor and the glycine receptor that are each coupled to an anionic channel for Cl^-. Another ionotropic receptor is the $5\text{-}HT_3$ receptor that is coupled to a Na^+/K^+ channel. Glutamate (Glu), the ionized form of the NT glutamic acid, is the ligand for at least three subtypes of ionotropic Glu receptors each named for the synthetic ligand (or its abbreviation) that activates the receptor most effectively: (1) kainic acid, (2) *a*mino-hydroxy-*m*ethyl-isoxazole-*p*ropionic *a*cid (AMPA), and (3) *N*-

Table 2
Categories, Characteristics, and Examples of Ionotropic and Metabotropic Receptors

IONOTROPIC	*METABOTROPIC*
Shared Characteristics	
Contains NT (ligand) binding site Is embedded in neuronal PM Alters ion fluxes across PM	
Distinguishing Characteristics	
Integral part of ion channel	Physically separate from ion channel
No association with G-proteins	Coupled to G-proteins
Contains less than seven TM domains	Contains exactly seven TM domains
Rapid onset and termination of ion fluxes across PM	Slower onset and delayed termination of ion fluxes across PM
Names of Receptors in Each Category (*endogenous ligand in italics*)	
Nicotinic—*acetylcholine*	Muscarinic—*acetylcholine*
5-HT_3—*serotonin*	Serotoninergic (multiple subtypes except for 5-HT_3)—*serotonin*
$GABA_A$—*gamma-aminobutyric acid*	$GABA_B$—*gamma-aminobutyric acid*
Kainic Acid—*glutamate*	Metabotropic Glutamate—*glutamate*
AMPA—*glutamate*	Dopaminergic—*dopamine*
NMDA—*glutamate*	Noradrenergic—(multiple forms of both α and β subtypes)—*norepinephrine* and *epinephrine*
Glycineric—*glycine*	Histaminergic—*histamine*
	Peptidergic (multiple forms and subtypes for each of the many neuropeptide families)

*m*ethyl-*D*-*a*spartate (NMDA). These three ionotropic Glu receptors are coupled to Na^+/K^+ channels, but only the channel associated with the NMDA subtype also allows Ca^{2+} to enter the cell along with Na^+. Because this NMDA receptor–ion channel complex accepts Ca^{2+}, it can be blocked by the divalent cation, Mg^{2+}, that binds to the exterior of the channel whenever the PM is near its resting potential. When the PM is fully depolarized by other means (usually voltage gating), Mg^{2+} is displaced out of the NMDA-activated ion channels allowing influx of both Ca^{2+} and Na^+. The NMDA receptor for Glu also tends to be activated by glycine binding to its receptor at another site on the PM. In addition, these three ionotropic receptors for Glu are much larger than the four other ionotropic receptors, namely nicotinic ACh, $GABA_A$, glycinergic, and 5-HT_3. The greater size of the ionotropic Glu receptors includes the TM domains as well as the extracellular portions that apparently contain the ligand recognition site.

4.2. Metabotropic Receptors Indirectly Produce Slower but More Sustained Changes in Ion Flux

Metabotropic receptors are also often called *G-protein-coupled receptors.* When transducing the signal generated by binding with their ligands, they produce changes in ways that are much slower to initiate and to terminate than the ionotropic receptors. Whereas NT binding to an ionotropic receptor can open or close an ion channel in a few milliseconds, the ligand binding to G-protein-coupled receptors will either enhance or dampen the excitability of the postsynaptic neuron for several seconds or perhaps minutes. In many cases, the metabolic changes produced by the ligand-activated metabotropic receptors modulate the excitability created initially by ligand-occupied ionotropic receptors.

Metabotropic receptors are an integral part of the neuronal PM, just like the ionotropic receptors. Their

N-terminus extends into the extracellular space, and their C-terminus resides within the cytoplasm. However, they always contain exactly seven groups of approximately 24 mostly hydrophobic aa that constitute the seven TM domains (*see* Table 2). Their designation as metabotropic receptors stems from the fact that eventual movement of ions depends on one or more metabolic steps. Current models for ligand-induced activation of metabotropic receptors propose that the receptor is converted from an inactive state to an active state by ligand binding. Metabotropic receptors are physically linked to G-proteins anchored in the adjacent PM, and when in the active state, they interact with the G-proteins in a productive fashion. The G-proteins are heterotrimeric protein complexes consisting of alpha, beta, and gamma subunits, and there are multiple forms of each subunit. Their designation as G-proteins is derived from their structural and functional association with two guanyl nucleotides known as *g*uanine *di*phosphate (GDP) and *g*uanine *tri*phosphate (GTP). This chapter will omit specific details about how ligand-activated G-proteins either activate or inhibit neuronal excitability. However, these ligand-induced changes mediated by metabotropic receptors can involve either direct modulation of an ion channel by G-proteins or more commonly an indirect modulation through what is called a *second messenger.* Although there are multiple types of second messengers, they are each formed by the NT (designated as the first messenger) binding to its metabotropic receptor, and the activated G-protein complex then stimulating an enzyme that catalyzes the formation of one of the various second messengers. The following molecules have been shown to function as second messengers: Ca^{2+}, cyclic *a*denosine *m*ono*p*hosphate (cyclic AMP), cyclic *g*uanosine *m*ono*p*hosphate (cyclic GMP), inositol trisphosphate (IP_3), *dia*cyl*g*lycerol (DAG), and arachidonic acid. The entire system consisting of NT ligand, metabotropic receptor, G-protein complex, and enzymatic production of a second messenger greatly amplifies and prolongs the NT-induced changes in neuronal excitability.

Table 2 lists a few of the many metabotropic receptors that have been identified. A family of muscarinic receptors for ACh has been well characterized and shown to occur on both presynaptic and postsynaptic PM and to mediate both excitatory and inhibitory effects. There are also metabotropic receptors for each of the aminergic NT (5-HT, NE, DA, and histamine). Although there are ionotropic receptors for Glu and for GABA, each of these NT also activates a metabotropic receptor located on both presynaptic and postsynaptic PM. Among the families of receptors for the numerous peptidergic NT, there appear to be no ionotropic receptors since only metabotropic receptors have been identified.

Desensitization (also called down-regulation) of G-protein-coupled metabotropic receptors for various NT can also influence interneuronal communication. The quantity of receptors available to the ligand may be decreased when the receptor-containing section of the PM is internalized by endocytosis. Another type of receptor desensitization involves phosphorylation of specific proteins making ligand binding and/or signal transduction less efficient.

The amount of free cytoplasmic Ca^{2+} also determines excitability of neurons. As stated earlier, Ca^{2+} enters the cell via both voltage-gated and ligand-gated ion channels. However, levels of free intracellular Ca^{2+} are much lower than extracellular levels because free Ca^{2+} is rapidly taken up by various cytoplasmic binding proteins and sequestered into intracellular storage sites. The endoplasmic reticulum (ER) represents a major intracellular site for Ca^{2+} storage, and this stored Ca^{2+} can be released later to influence neuronal excitability. One of the second messengers described above, IP_3, can even act upon a Ca^{2+} channel in the ER membranes to release stored Ca^{2+}. Because intracellular Ca^{2+} can activate a number of processes, Ca^{2+} released from the ER by IP_3 could be considered as a third messenger. However, in most cases Ca^{2+} release or influx from outside the cell would not be the result of a second messenger but rather the opening of a ligand-gated ion channel.

4.3. Concurrent Release of Two Neurotransmitters into the Same Synaptic Cleft Increases Signaling Possibilities

The historical concept that an individual neuron operates with one and only one NT has been abandoned for a long time. Using histochemical and immunochemical techniques, the coexistence of multiple types of NT molecules in the same neuron has been repeatedly demonstrated. In many cases, one of the coexisting molecules is a classical nonpeptide NT (such as biogenic amines or modified aa) whereas the other coexisting molecule is a peptide NT. In some cases, there may be two or more different peptides coexisting with a classical NT.

When classical NT and peptide NT are found in the same neuron, they appear to be contained in different SV. However, it is not clear whether each type

of SV can release its contents independently of the other types. When there is more than one type of peptide produced by a given neuron, the evidence suggests that they coexist within the same SV and would therefore be released together.

Neurons that could release more than one type of NT from their presynaptic terminals would appear to have enhanced opportunities for interneuronal communication. As described previously, ionotropic receptors respond more rapidly than metabotropic receptors. If a presynaptic terminal could activate both classes of postsynaptic receptors, it would have enhanced signaling possibilities. Not all pairs of coexisting NT molecules, however, activate different classes of receptors. Nevertheless, the opportunity for a single presynaptic terminal to affect multiple types of ion channels in the postsynaptic terminal would also enrich the complexity of the transmitted message.

5. REGULATION OF NEURONS THAT PROJECT INTO THE NEURAL LOBE OF THE PITUITARY GLAND

The magnocellular hypothalamic neurons that project their axon and its branches into the neural lobe of the pituitary gland are the source of the posterior pituitary neurohormones *oxytocin* (OT) and *vasopressin* (VP). The precursors and carriers of these hormones are transported down the axon within large-diameter SV, and the cleaved OT and VP molecules are secreted from the axon terminals by the process of exocytosis as described for release of NT into the synaptic cleft (*see* Fig. 3). The exocytotic release is acutely regulated by the frequency of *action potentials* (AP) arriving in the axon terminal to open voltage-gated Ca^{2+} channels. This moment-to-moment regulation of OT and VP release by AP frequencies permits the precise and very immediate regulation of neurohormone secretion necessary for optimum homeostatic and reproductive processes (*see* chapters 10 and 14). Various hypothalamic NT molecules interact to regulate the AP frequency in the magnocellular hypothalamic neurons in a manner similar to the regulation of other ordinary hypothalamic neurons (also termed *parvocellular* neurons). Acting through both muscarinic and nicotinic receptors, ACh excites vasopressinergic as well as oxytocinergic neurons to increase their firing rates and thereby their release of hormones (*see* Table 3). Acting through β-adrenergic receptors, NE decreases the excitability of OT and VP neurons. Other NT molecules having effects on release of OT and VP include histamine that is stimulatory and GABA that is inhibitory. In addition to functioning as a neurohormone when released from the neural lobe into blood, OT can function as NT when released into a synapse (*see* Table 1). Such synaptic release of OT (whether derived from magnocellular or parvocellular presynaptic neurons) appears to increase excitability of magnocellular OT neurons (*see* Table 3). This mechanism may help to sustain AP frequencies (i.e., autostimulatory feedback) or to excite other OT neurons to fire in synchrony with a presumed pacemaker OT neuron. Synchronous bursts of AP have been observed in OT neurons found in different locations, but similar synchrony has not been observed in populations of VP neurons. In addition, opioidergic peptides (*see* Table 1) have been shown to inhibit the release of OT and VP from axonal terminals. Because VP neurons produce dynorphin neuropeptides and OT neurons produce enkephalin neuropeptides, the inhibitory actions of these endogenous opioids on neurohormone release may represent autoinhibitory mechanisms.

Table 3
Direct Stimulatory and Inhibitory Effects of Various NT Molecules on the Release of Hypothalamic Neurohormones

Classification of NT Based on Their Usual Effects		
Hypothalamic Neurohormone	*Stimulatory NT*	*Inhibitory NT*
VP and OT	ACh Histamine OT	NE GABA Opioid peptides
GnRH	Glu NO	GABA Opioid peptides (via NE)
CRH	ACh Epi 5-HT	GABA
TRH	NE Epi	NPY

6. NEUROTRANSMITTER REGULATION OF NEURONS THAT RELEASE HYPOPHYSIOTROPIC HORMONES

Hypophysiotropic hormones (HTH) are defined as those neurohormones produced in the hypothalamus for ultimate action (usually stimulatory or *trophic,* but sometimes inhibitory) on the anterior lobe of the pituitary gland (also known as the *hypophysis*). The many different parvocellular hypothalamic neurons

that secrete HTH into hypophysial portal blood for delivery to the anterior lobe are each excited or inhibited by a variety of NT molecules. In the following sections, the various NT known to influence each type of HTH-producing neuron will be discussed in separate sections.

6.1. Gonadotropin-Releasing Hormone (GnRH)

The perikarya of GnRH neurons are located throughout the rostral parts of the hypothalamus and even rostral to the hypothalamus in some species (*see* Chapter 8). Like other hypophysiotropic neurons, they project their axons to the *median eminence* where they secrete their neurohormones into the capillaries that give rise to *hypophysial portal* blood. Various NT molecules can act synaptically on the dendrites and perikaryon of each GnRH neuron as well as any place along the axon including its terminals in the median eminence. The regulation of GnRH neurons by any specific NT will depend on the nature of the NT receptors contained in the PM of GnRH neurons and the presynaptic elements releasing the appropriate NT in the vicinity of those receptors. For most NT molecules, the specific effect (to stimulate, inhibit, or have no effect) varies among species, sexes, and endocrine states. Therefore, general conclusions are very difficult to make for many NT. It can be concluded, however, that certain NT molecules will usually be either excitatory to GnRH neurons or have no effect depending on the situation. Glutamate is generally stimulatory to GnRH neurons as it is to most other neurons (*see* Table 3). Likewise, exposure of GnRH neurons to locally produced nitric oxide is stimulatory. Opioidergic neuropeptides usually inhibit GnRH release, but other NT (such as NE) may mediate some of these inhibitory effects because most GnRH neurons appear to lack opioidergic receptors. The various aminergic NT, such as DA, NE, and 5-HT, exert different effects on release of GnRH in different model systems, sometimes being stimulatory and other times being inhibitory. The type of feedback from blood-borne steroid hormones is often an important determinant of how each aminergic NT affects release of GnRH. Ionotropic receptors of the $GABA_A$ type have also been localized on GnRH perikarya suggesting that GABA may inhibit these neurons. GABA-producing neurons are known to have intracellular receptors for several gonadal steroids whereas GnRH neurons lack such hormone receptors. Therefore, some effects of gonadal steroids on GnRH release may be mediated by GABA neurons. The neuropeptide known as galanin is synthesized and stored within many GnRH neurons raising the possibility of galanin being released together with GnRH, but the functional significance of this colocalization is not known.

6.2. Corticotropin Releasing Hormone (CRH) and Parvocellular-Derived Vasopressin

This section deals with CRH as well as the VP that originates in parvocellular neurons because both neurohormones acutely increase the release of ACTH from the anterior lobe of the pituitary gland. Notably, the quantities of VP that are secreted into the general circulation from magnocellular-derived axons in the neural lobe appear insufficient to stimulate ACTH release after dilution and degradation in the systemic circulation. Under basal conditions of ACTH release, the parvocellular neurons in the *paraventricular nucleus* and adjacent areas that produce either CRH or VP are completely separate. When there is hypersecretion of ACTH, as occurs after removal of the adrenal glands, both CRH and VP may become colocalized in a population of neurons that formerly contained only CRH. The profile of ACTH secretion consists mainly of episodic discharges, only some of which are provoked whereas others probably represent the spontaneous discharges that comprise basal and circadian-related secretion. Release of either CRH or VP into the hypophysial portal blood can provoke release of ACTH, and the relative contributions of each neurohormone in specific in vivo situations is often difficult to determine.

Because it is difficult to study the regulation of parvocellular-derived VP independently of magnocellular-derived VP, most of the information about specific NT molecules that influence release of ACTH is based on those NT that either stimulate or inhibit release of CRH. In addition to releasing CRH into hypophysial portal blood, many other CRH neurons send axons to brain areas wherein CRH apparently acts as a NT to promote an integrated response to stressors including activation of the sympathetic nervous system. Regulation of CRH by various NT molecules is assumed to be the same for both types of CRH neurons, but current levels of technology do not usually allow this assumption to be validated experimentally. CRH neurons are activated by ACh acting through both nicotinic and muscarinic receptors that may or may not be located on the CRH neurons. Both Epi and 5-HT appear to also directly

stimulate the activity of CRH neurons through synaptic mechanisms (*see* Table 3). The actions of NE and DA on CRH neurons are difficult to generalize because there is evidence for both stimulatory and inhibitory effects. Perhaps this variability reflects the difficulty of determining whether specific NT agonists and antagonists act directly on the CRH neurons or indirectly on other neurons that are synaptically linked with CRH neurons. However, the actions of GABA are consistently inhibitory to CRH release. As stated above, ACTH secretion is regulated to yield a circadian profile, and neural input to CRH neurons from the *suprachiasmatic nucleus* is thought to mediate this effect. One peptidergic NT (vasoactive intestinal peptide) and one aminergic NT (5-HT) are thought to mediate these circadian signals.

The coexistence of multiple peptidergic NT within single neurons has been discussed previously, but the situation in CRH neurons represents an extreme example. In addition to colocalization with VP described above, CRH has been found to coexist together with oxytocin, neurotensin, and vasoactive intestinal peptide. It has not been determined whether these neuropeptides are released at the same time as CRH or whether they have any feedback effects on the CRH neurons.

6.3. Growth Hormone Releasing Hormone (GHRH) and Somatostatin

The secretion of *growth hormone* (GH) is regulated by two neurohormones, GHRH and *somatostatin* (SS), that have opposite actions on the release of GH from the anterior pituitary gland. With this dual control by separate neurons, it is difficult to determine the specific mechanisms of action by which various NT molecules modulate secretion of GH. For example, stimulation of GH release may result from excitation of GHRH neurons or from suppression of SS neurons. Despite this uncertainty, some generalizations are possible. The actions of NE, through one subtype of the α-adrenergic receptor, provoke the release of GH apparently mediating a variety of internal homeostatic signals for enhanced GH secretion. In contrast, a different subtype of the α-adrenergic receptor and the β-adrenergic receptor mediate suppression of GH secretion. Activation of muscarinic receptors by ACh promotes the secretion of GH, and there is evidence that suppression of SS release is involved. Other NT molecules, such as 5-HT and GABA, either stimulate or inhibit secretion of GH depending on the situation, and these differences may reflect their actions on neurons other than GHRH neurons and SS neurons as well as the complex synaptic interactions between GHRH neurons and SS neurons.

6.4. Thyrotropin Releasing Hormone (TRH)

The delivery of hypothalamus-derived TRH to the anterior pituitary stimulates the release of *thyroid-stimulating hormone* (TSH) and *prolactin* (PRL). TRH is also produced, however, in other areas of the CNS where it apparently functions as a NT. The present discussion will focus only on the NT regulation of neurons that release TRH into hypophysial portal blood. Therefore, the effects of any NT being studied are usually measured as the in vivo release of TSH or PRL. Each of these pituitary hormones, however, can be inhibited by the one or more inhibitory neurohormones. Secretion of TSH is inhibited by SS, whereas PRL secretion is inhibited by the aminergic NT dopamine. Therefore, inferences about TRH release based on secretory profiles of TSH and PRL can be problematic.

Both Epi and NE appear to stimulate release of TRH into hypophysial portal blood (*see* Table 3), and synaptic inputs from both Epi and NE axons have been observed on TRH neurons. Peptidergic inputs that contain NPY have also been observed on TRH neurons, and there is evidence for NPY-induced suppression of TSH secretion. The specific actions of DA cannot be generalized because both stimulatory and inhibitory effects on TRH release have been observed.

6.5. Dopamine

Dopamine-containing axons of the *t*ubero*i*nfundibular *d*op*a*minergic (abbreviated TIDA) tract that originate in the arcuate nucleus release DA from their axon terminals into hypophysial portal blood for delivery to the anterior lobe of the pituitary gland. Dopamine delivered to the anterior lobe inhibits secretion of PRL and may in some situations also inhibit the secretion of TSH. These neurons that produceDA for release into hypophysial portal blood constitute only a very small proportion of all DA neurons in the CNS, but their regulation by NT is important because of the indirect effects on anterior pituitary secretion. Another small subgroup of adjacent DA neurons form the *t*ubero*h*ypophysial *d*op*a*minergic (abbreviated THDA) tract, and send their axons into the posterior lobe of the pituitary gland. It is possible that DA released within the posterior lobe reaches the anterior lobe through the short hypophysial portal vessels that link the two lobes of the gland.

Dopamineric neurons of the TIDA tract and the

THDA tract receive synaptic input from NE neurons as well as Epi neurons with both types originating from outside of the hypothalamus. A variety of other NT, such as 5-HT, GABA, and ACh, also innervate the DA neurons involved in regulation of the anterior pituitary gland, but generalizations are difficult at the present time. DA neurons contain many different neuropeptides including neurotensin, enkephalin, and NPY. There is also some neuronal colocalization of DA with GHRH in the arcuate nucleus of the hypothalamus.

6.6. Vasoactive Intestinal Peptide (VIP)

Although VIP may function as a NT in the hypothalamus and other areas of the brain (*see* Table 1), it is considered here as a neurohormone in hypophysial portal blood because it is capable of stimulating the release of PRL from the anterior lobe of the pituitary gland. Those VIP neurons located in the hypothalamus and that may secrete into portal blood undoubtedly have synaptic inputs, but very little is known about which NT molecules affect them. Afferent suckling-related stimuli from the mammary gland appear to activate VIP neurons leading to suckling-induced release of PRL. In addition, profiles of PRL secretion are closely associated with circadian rhythms in rodent species, and VIP inputs from the suprachiasmatic nucleus to those neurosecretory neurons that regulate PRL (specifically TRH, DA, and VIP neurons) may participate in this circadian regulation of PRL.

7. PHARMACOLOGICAL ALTERATIONS OF NEUROTRANSMITTER SYSTEMS

The scientific discipline of *neuropharmacology* develops and investigates drugs that can selectively modify (increase or decrease) synaptic transmission in specific neural circuits to provide therapeutic benefits without undesirable side effects. Many of the earliest neuroactive drugs simulated the actions of certain NT molecules and were designated as *agonists* of these NT molecules. In contrast, other drugs appeared to oppose the actions of certain NT molecules and were designated as *antagonists* of that NT. The interaction of a drug with an ionotropic receptor or a metabotropic receptor for the NT could either activate the receptor (i.e., be a receptor agonist) or block receptor activation (i.e., be a receptor blocker or antagonist). Other more recent approaches to neuropharmacology have included drugs that regulate the quantity of precursors for NT synthesis or that alter the clearance of released NT from the synaptic cleft, either by metabolic degradation or removal via NT transporters. Each of the following sections will provide information about one or more prototypical drugs that alter synaptic neurotransmission using one of the above listed approaches.

7.1. Receptor Agonists Mimic the Effects of the Natural Ligand on the NT Receptor

Drugs that can activate a single subtype of one class of NT receptors are valuable tools for identifying which physiological events depend on that receptor. Selectivity of the drug for just one receptor subtype, however, must be verified before valid conclusions can be drawn. Delivery of the drug to the neural location of the receptor is also important. The actions of Glu at ionotropic receptors of the NMDA subtype are readily simulated by systemic administration of NMDA, a compound that readily penetrates into neural tissue and activates those receptors.

Opioidergic drugs, such as *morphine,* readily enter the neural tissue and are highly potent activators of opioidergic receptors although not all subtypes are equally responsive. Other drugs, such as *bromocriptine* and *haloperidol,* can activate DA receptors, but some of these DA agonists cannot enter the CNS and therefore must act only on the DA receptors in the pituitary gland and that portion of the median eminence outside the blood-brain barrier.

7.2. Receptor Antagonists Block Ligand-Induced Activation of the NT Receptor

Drugs that can prevent endogenous ligands from activating their usual receptor are highly effective therapeutic and investigational agents. In those cases where the NT ligand is a classical nonpeptide molecule, the agonist usually has a molecular structure that is similar to the natural ligand (i.e., to promote binding) but is lacking in some key structural element that activates the signal transduction pathway. If the binding affinity and/or local concentration of the antagonist is greater than those of the endogenous ligand, most physiological effects of the natural ligand are blocked. Antagonists that are able to block receptors for peptide NT may be modified peptides, but they usually have a completely different structure. Other molecular structures predominate as peptidergic receptor antagonists because peptidergic drugs are often degraded rapidly and/or fail to easily penetrate the CNS.

The opioidergic receptor antagonist *naloxone* is widely used to block the biological effects of exoge-

nous and endogenous opioids. Naloxone has a structure similar to that of the agonist morphine, but it does not activate any of the opioidergic receptors. Naloxone is a pure antagonist that does not partly activate the signal transduction pathway (i.e., does not have some agonist potency) in any of the numerous experimental paradigms. Various drugs that are selective for each of the many adrenergic subtypes can antagonize receptors for NE and Epi. *Propranolol* blocks β-adrenergic receptors whereas *phentolamine* blocks α-adrenergic receptors. Dopaminergic receptors can be antagonized by *pimozide.* A drug known as *dizocilpine* (usually designated MK-801) can selectively antagonize the NMDA subtype of ionotropic Glu receptors. *Atropine* is used to antagonize most forms of the muscarinic ACh receptor. When used for investigational purposes, these receptor antagonists may produce biological effects, but the neuronal cell type and anatomical location at which they act cannot always be determined. It can only be concluded that synapses that signal via those blocked receptors were indeed inhibited and that the biological effects resulted from this inhibition of neurotransmission.

7.3. Drugs Can Augment the Supply of Precursors for Synthesis of Neurotransmitters

In clinical syndromes resulting from deficiencies of a particular NT, it may be possible to increase the production of that NT by administering drugs that augment the supply of precursors for the NT. One example of this approach is the widespread use of DOPA (*see* Fig. 1) to treat people with Parkinson's disease, a disorder in which there is a deficiency of DA neurotransmission. Although used for investigational rather than clinical purposes, the production of the gaseous NT known as nitric oxide can be stimulated by increasing the quantities of its immediate precursor L-arginine (*see* Fig. 2).

7.4. Drugs Can Increase Quantities of Neurotransmitter in the Synaptic Cleft

In addition to interacting with its receptors, NT molecules released from the presynaptic terminal into the synaptic cleft are cleared from the area by enzymatic degradation and transporter-mediated uptake into neurons and neuroglial elements. Drugs that can decrease the efficiency of either of these NT clearance mechanisms may provide therapeutic benefits associated with increased actions of that particular NT on its postsynaptic receptors. Some of the earliest tranquilizing drugs, like *chlorpromazine* and *reserpine,* inhibited the enzymatic breakdown of catecholaminergic NT by antagonizing the NT-degrading enzyme known as monoamine oxidase. Another group of more recent and clinically popular drugs, of which *fluoxetine* is an example, stimulate serotonergic neurotransmission by increasing the quantities of 5-HT in the synapse through blocking its reuptake by presynaptic transporters.

In addition to producing therapeutic benefits, drug-induced increases of a particular NT within the synaptic cleft may alternatively produce harmful effects. A number of organophosphorous compounds (often designated as nerve gases or toxins) can irreversibly inhibit acetylcholinesterase, the enzyme that degrades ACh in the synaptic cleft (*see* Fig. 2). A chronic excess of extracellular ACh is toxic to neurotransmission, and the antidotes for such toxicity consist of drugs to decrease ACh production and/or receptor activation.

7.5. Drugs Can Delay the Degradation of Second Messengers Produced by Metabotropic Receptors

As knowledge about metabotropic receptors and the second messengers that they generate has increased, another approach to neuropharmacology has developed. Each intracellular second messenger generated as a result of NT activation of a metabotropic receptor is degraded within the cell in order to eventually terminate the excitatory or inhibitory actions of the NT. Drugs are being developed to delay this degradation in order to prolong the actions of the second messenger. *Sildenafil* represents one such drug that delays the degradation of cyclic GMP and thereby prolongs the vasodilation caused by nitric oxide in certain blood vessels.

8. CONCLUSIONS

Communication among CNS neurons via chemical synapses is mediated by a large number of NT molecules that differ widely in chemical structure (*see* Table 1). Presynaptic neurons produce both excitatory and inhibitory effects on postsynaptic neurons. The various receptors to which the released NT bind and which initiate the changes in neuronal excitability have some common features such as ligand recognition properties, location within the PM, and ability to alter ion fluxes (*see* Table 2). However, the various receptors differ widely in speed, duration, and mechanisms for altering ion fluxes.

An important function of the hypothalamus is to regulate the hormones secreted from the posterior and anterior lobes of the pituitary gland. The hypothalamic neurons that synthesize and release the neurohormones involved in this regulation are affected by NT molecules released from presynaptic axon terminals that originate from outside the hypothalamus as well as from inside the hypothalamus. Generalizations about NT-mediated effects on specific neurohormones produced in the hypothalamus are possible in a limited number of situations (*see* Table 3). However, the precise nature of a specific NT effect often depends on other interacting factors (e.g., environmental, hormonal, and other NT systems).

Effective neuroactive drugs usually alter some aspect of synaptic neurotransmission. Some drugs may promote signaling mediated by a specific NT through activating its receptor, increasing NT synthesis, decreasing extracellular clearance of the NT, or delaying degradation of its receptor-generated second messenger. Other drugs may decrease neurotransmission mediated by a specific NT through reducing synthesis of that NT or by blocking its receptors.

SELECTED READINGS

Cooper JR, Bloom FE, Roth RH. The Biochemical Basis of Neuropharmacology. New York: Oxford University Press, 1996:518.

Dawson TM, Snyder SH. Gases as biological messengers: nitric oxide and carbon monoxide in the brain. J Neurosci 1994;14:5147.

Jessell TM, Kandel ER. Synaptic transmission. A bidirectional and self-modifiable form of cell–cell communication. Neuron 1993;10 (Suppl):1.

Levitan IB, Kaczmarek LK. The Neuron, 2nd ed. New York: Oxford University Press, 1997:543.

Purves D, Augustine GJ, Fitzpatrick D, Katz LC, LaMantia AS. Neuroscience. Sunderland, MA: Sinauer & Associates, 1997:562.

Walmsley B, Alvarez FJ, Flyffe REW. Diversity of structure and function at mammalian central synapses. Trends in Neurosci. 1998;21:81.

5 The Hypothalamus as a Major Integrating Center

Jon E. Levine, PHD

CONTENTS

1. INTRODUCTION

The "constancy of the internal milieu," as described by Claude Bernard in 1859, is essential for the survival of any warm-blooded animal, and its maintenance requires strict control over behavioral, autonomic, and endocrine responses to the environment. The hypothalamus has clearly evolved as the major integrative center for regulating all of these homeostatic control systems. The location of the hypothalamus—superior to the hypophysis, and adjacent to subcortical "limbic" structures—suggests this role, as it is uniquely positioned to both send and receive endocrine signals, as well as neural signals from sensory organs, memory centers, and autonomic circuitries. Incoming information is registered, analyzed, and integrated in hypothalamic neurons. On the basis of these calculations, the hypothalamus effects the changes, if needed, in hormone secretions, behavioral state, and autonomic activity. The hypothalamus is thus responsible for monitoring the internal and external environment and coordinating adaptive physiological responses among several systems.

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A remarkably diverse set of physiological activities are regulated by the hypothalamus: energy homeostasis, water and electrolyte balance, reproduction, growth and development, stress responses, immune function, sleep and wakefulness, and thermoregulation. For each function, the appropriate neural and endocrine inputs must be processed and the appropriate outputs must be produced. This chapter will discuss the physiological, cellular, and molecular mechanisms by which the hypothalamus integrates relevant signals and thereafter formulates appropriate commands to effect changes in these vital processes.

2. BASIC PRINCIPLES OF NEUROENDOCRINE INTEGRATION

Hypothalamic regulatory systems can be generally described as either *homeostatic* or *reflexive* in their physiological features. Homeostatic systems usually function to restrict a physiological variable such that it is maintained about a *set-point* value. Neuroendocrine reflexes, on the other hand, resemble their neuromuscular reflex counterparts; they occur as transient, fixed-pattern, or graded reactions to an applied sen-

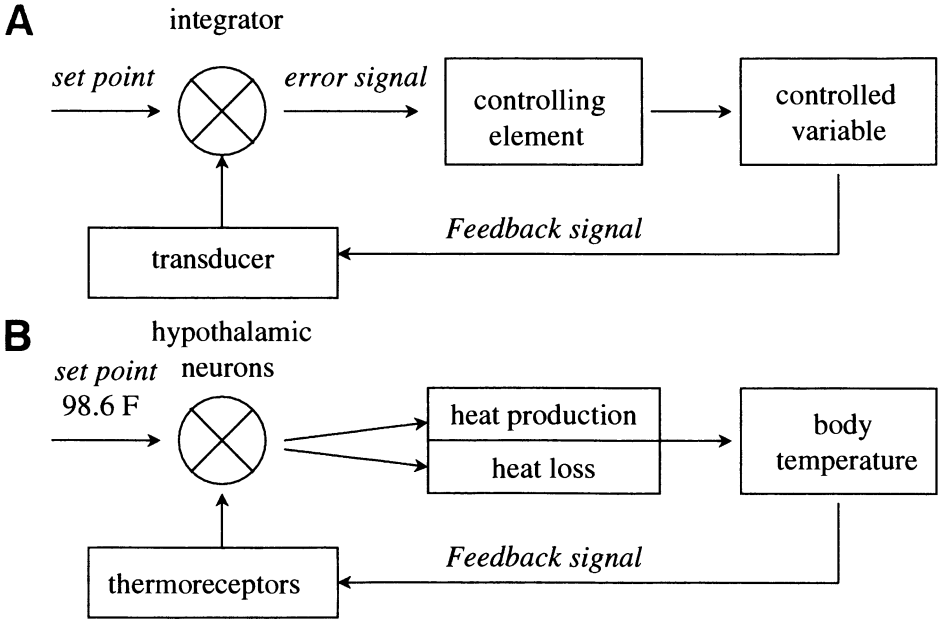

Fig. 1. Schematic representations of controlled systems. **(A)** Basic form of a controlled system, in which a controlling element regulates a controlled variable that provides a feedback signal that is registered and changed by a transducer; the transduced feedback signal is conveyed to an integrator, which compares the magnitude of the feedback signal with a preselected set-point value. Any discrepancy is calculated and an appropriate error signal is delivered to the controlling element, whose activity is increased or decreased accordingly. **(B)** Thermoregulatory homeostatic mechanisms represented as a controlled system. The controlling elements are those which produce and/or retain heat (e.g., shivering, metabolic and behavioral responses, venous constriction) and those that promote heat loss (e.g., sweating, increased respiration, behavioral responses, venous dilation). Core body temperature is the controlled variable and it is monitored by central nervous system and peripheral thermoreceptors. These transduce thermal information into neural signals, which are then conveyed to integrative hypothalamic neurons; neural signals for the body set-point value are compared with the transduced feedback signals, and appropriate error signals are sent to the controlling element for any needed adjustments. This controlled system thus makes use of a negative feedback mechanism to maintain core body temperature within a very narrow range of values about the set-point of 98.6°F.

sory stimulus. The basic components of homeostatic systems are first described and exemplified below.

2.1. Neuroendocrine Homeostatic Systems Use Negative Feedback to Maintain a Preselected State

Homeostatic regulation in any physiological system can often be better understood when described in terms of control system analysis. Borrowing principles and terms from engineers, Fig. 1A describes a generic neuroendocrine control system which features a *set point,* an *integrator* (also referred to as an error detector or a comparator), a *controlling element,* a *controlled variable,* a *feedback signal,* and a feedback signal *transducer.*

This simple system makes use of negative feedback to maintain the controlled variable within a narrow range of physiological values. The *controlling element* regulates the *controlled variable,* which in turn provides a *feedback signal* representing the real, momentary value of the variable. A feedback signal *transducer* registers the feedback signal, converts it to a "readable" signal (if necessary), and conveys that information to an *integrator;* here, a comparison is made between the ambient level of the variable and a desired set point value. If a discrepancy is calculated between the real and preselected state, then the integrator delivers an *error signal* to the controlling elements. In almost all physiological systems, this error signal is inverted in sign to produce an adjustment of the controlling element activity in the opposite direction of the original deviation from set point. This type of control system, in which an excess of the controlled variable provides a signal for a compensatory reduction in the value of the variable, is referred to as a *negative feedback* mechanism. Virtually all neuroendocrine homeostatic systems use some form of negative feedback control that can be described in these terms.

Thermoregulatory systems are particularly amenable to control system analysis, and a simplified repre-

sentation of such a system is given in Fig. 1B. The target body temperature of 98.6°F is defended within very narrow limits under normal conditions. This preselected point (which may vary among individuals by fractions of degrees, at most) is prescribed by the activity of sets of hypothalamic neurons, through mechanisms which remain largely unknown. The controlling elements include those that produce heat and compensate for low body temperature (e.g., shivering, metabolism, venous constriction) and those that facilitate heat loss and compensate for high body temperature (e.g., sweating, venous dilation, decreased metabolism). The controlled variable—body temperature—functions as a feedback signal. Thermosensitive cells in the hypothalamus monitor the temperature of the blood that perfuses the basal forebrain, and integrative neurons compare this information, along with signals from peripheral thermoceptors, with the desired set point. If a discrepancy is detected, error signals are formulated and commands for increased heat loss or heat production are conveyed to the appropriate effector systems. Much of the microanatomy of the system's components have yet to be characterized, and many of the cellular and molecular events that mediate these integrative and signalling mechanisms remain to be elucidated. Nevertheless, further studies will likely continue to be guided by control system representations of this type.

2.2. Neuroendocrine Systems Can Employ Positive Feedback to Produce Punctuated Physiological Events

Some physiological situations require that major deviations from a basal activity state be achieved. Parturition is one such circumstance in which labor produces intrauterine pressure, which in turn induces reflexive release of oxytocin from the posterior pituitary gland and leads to an intensification of the labor contractions. This circular stimulatory process can be described as a positive feedback process, in which "autocatalysis" proceeds until an end point is achieved. Attainment of the biological end point then breaks the cycle of stimulation. For childbirth, the process terminates with the expulsion of the infant from the birth canal. Another major example of positive feedback is the explosive release of the preovulatory gonadotropin surge during the ovulatory cycle in female rodents. In this case, ripened ovarian follicles release a surge of estrogen, which activates receptors in the hypothalamus and pituitary gland. The hypothalamus is thus prompted to release a surge of GnRH into the hypophysial portal vessels; whereas the pituitary gland is rendered exquisitely sensitive to the actions of GnRH. The net result is a massive, transient surge of gonadotropin secretion, which in turn triggers ovulation.

In both of the foregoing examples, major excursions from a basal activity state produce positive feedback signals, which in turn evoke *additional* departure from the original state. The lack of any inversion of the feedback signal enables this "snow-balling" effect. It is this unusual characteristic that ensures successful completion of a critically important, yet relatively infrequent physiological events, such as ovulation or childbirth.

3. NEUROENDOCRINE HOMEOSTATIC SYSTEMS

3.1. Homeostatic Systems Can Involve Hypothalamus, Pituitary, and End-Organ, Functioning Together as Neuroendocrine "Axis"

Neuroendocrine homeostatic systems often involve the coordinated activity of two or more organs. Many such systems are organized into distinct tiers of regulated activity, arranged so that signals are conveyed from hypothalamus to anterior pituitary, and from anterior pituitary to target tissues. In many of these *hypothalamic-hypophysial axes,* a third round of signaling occurs that completes the loop from the target tissue back to hypothalamic neurons and/or the anterior pituitary gland. In all of these axes, experimental analysis has revealed that feedback regulation—virtually always of the negative type—functions as the predominant feature of the controlled system. Feedback signals from anterior pituitary gland to hypothalamus comprise a *short-loop* feedback system and may either be conveyed through retrograde flow in the portal vasculature or via the peripheral circulation. A *long-loop* feedback mechanism is one in which feedback signals are conveyed from the end-organ (e.g., gonad, thyroid, adrenal cortex) to antecedent levels. Fig. 2 depicts schematically the organization of hypothalamic-hypophysial axes in which long-loop and short-loop feedback mechanisms operate.

The hypothalamic-pituitary-thyroidal (HPT) axis is shown in Fig. 3A as a representative "three-tiered" system. A population of hypothalamic neurons largely located in the paraventricular nucleus produces the tripeptide-releasing factor, thyrotropin-releasing hormone (TRH). The neuropeptide is secreted from neu-

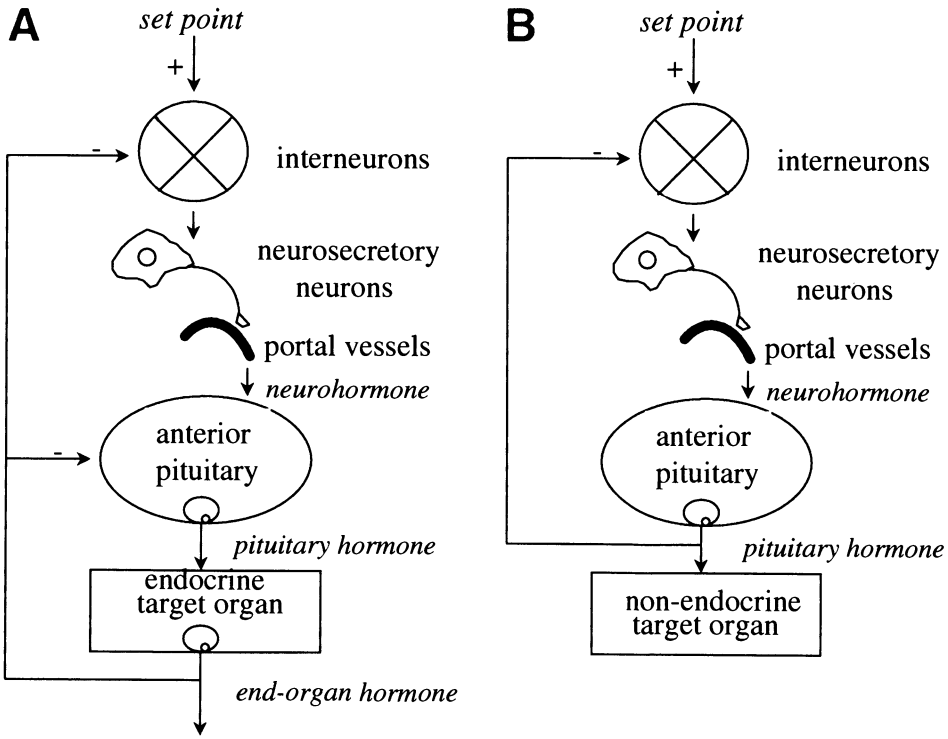

Fig. 2. Three- and two-tier hypothalamic-pituitary hormone axes, schematically depicted as controlled systems. Shown in **(A)** is the organization of a three-tier neuroendocrine axis in which "long-loop" negative feedback signals predominates. A set-point of activity determines the rate of neurosecretion of neurohormone into the hypophysial portal vessels; the actions of the neurohormone determines the secretion of the target adenohypophysial hormone, which in turn acts at the level of a target endocrine organ to determine the output of hormone from that end-organ. The end-organ hormone can exert negative feedback effects at the level of the hypothalamus and pituitary gland. Integration of negative feedback signals with feedforward signals can occur in interneurons that project to the neurosecretory neurons, as depicted, or they may alternatively be exerted directly on the neurosecretory neuron itself (not shown). **(B)** depicts a neuroendocrine axis in which the target organ is nonendocrine tissue and short-loop negative feedback signals are conveyed by the adenohypophysial hormone to hypothalamic interneurons or neurosecretory neurons.

rovascular terminals in the median eminence and is conveyed to the anterior pituitary gland through the hypophysial portal vessels. The TRH diffuses via a secondary, fenestrated capillary plexus into the interstitial fluid of the anterior pituitary gland, where it can bind membrane-bound TRH receptors on thyrotropes and activate signaling pathways, thus stimulating TSH synthesis and secretion. Through the peripheral circulation, TSH is conveyed to the thyroid where it binds the TSH receptors on follicular cells and promotes the production of T3 and thyroxine. Thyroid hormones exert their widespread actions on target tissues via their cognate intracellular receptors. Equally important, however, circulating thyroid hormones also provide the major feedback signal within the axis; elevations in T3 and thyroxine exert long-loop feedback actions at the hypothalamic level to suppress TRH neurosecretion, and at the pituitary level to suppress basal and/or TRH-stimulated TSH secretion. Conversely, reductions in thyroid hormone secretions are generally accompanied by elevations in TRH and TSH secretion. The HPT axis can thus be represented as a controlled system in which the *regulated variable* is "metabolism"; the major *controlling element* is thyroid hormone, which is primarily under the control of TRH via TSH; the major *feedback signal* is the ambient level of thyroid hormone in the peripheral circulation; the *integrator* is likely a set of hypothalamic neurons (and perhaps also thyrotropes). These neurons or thyrotropes register the magnitude of the feedback signal, compare it to a preselected set-point value, and dictate the degree of change (if any) that should be brought to bear on the TRH and TSH secretion rate. The physiochemical bases of the set-point, as well as the integrative process, are largely unknown and remain the subject of much research.

Similar feedforward and feedback relationships

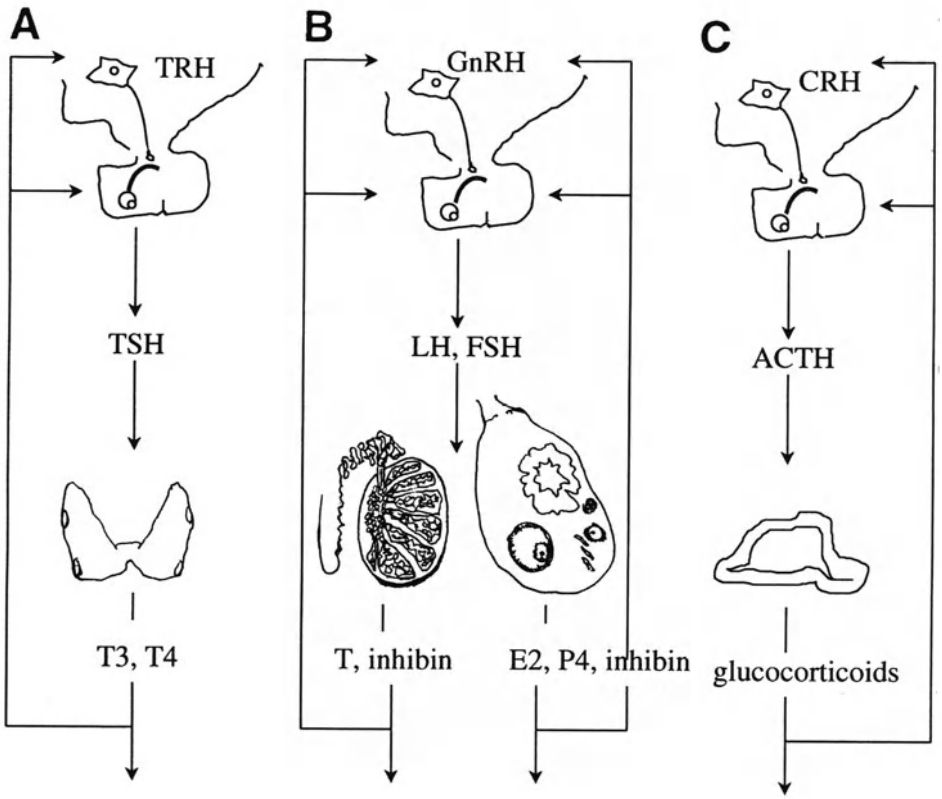

Fig. 3. Schematic representations of the major hypothalamic-pituitary axes in which long-loop negative feedback signals predominate. Shown are the respective hypothalamic neurohormones, anterior pituitary hormones, and end-organ hormones for the (**A**) hypothalamic-pituitary-thyroidal (HPT) axis, (**B**) hypothalamic-pituitary-gonadal (HPG) axis (both male and female axes depicted), and (**C**) hypothalamic-pituitary-adrenocortical (HPA) axis. Cellular targets of negative feedback are not specified, and may include hypothalamic interneurons, neurosecretory neurons, or both. TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone (thyrotropin); T3, triiodothyronine; T4, thyroxine; GnRH, gonadotropin-releasing hormone (GnRH); LH, luteinizing hormone; FSH, follicle-stimulating hormone; T, testosterone; E2, estrogen (estradiol 17-β); P4, progesterone; corticotropin-releasing hormone (CRH); ACTH, adrenocorticotropic hormone.

prevail in other hypothalamic-pituitary axes, albeit with different casts of neuroendocrine characters (Fig. 3B,C). As described fully in succeeding chapters, the hypothalamic-pituitary-gonadal (HPG) axis and the hypothalamic-pituitary adrenocortical (HPA) axis also function as three-tiered control systems in which long-loop feedback mechanisms predominate. Like the HPT axis, the HPG and HPA axes are organized so that primary releasing factors (GnRH and CRH) stimulate trophic pituitary hormones (gonadotropins and ACTH), which in turn stimulate end-organ hormones (gonadal steroid/peptide hormones, and corticosteroids), which exert long-loop feedback effects at preceding levels in the respective axes.

Prolactin and GH secretions are largely controlled by two-tier systems in which short-loop feedback functions as the major regulatory mechanism (Fig. 4). The deemphasis of end-organ feedback in these systems probably reflects the relatively distributed nature of GH and prolactin actions. Growth hormone is known to exert actions in bone, cartilage, liver, muscle, and other tissues, whereas prolactin's actions include those in mammary tissue, gonads, and accessory sex organs (e.g., prostate). Without a single target organ to provide feedback control, GH and prolactin appear to have evolved the ability to exert their own, direct feedback actions at target sites within the hypothalamus. Moreover, both stimulatory and inhibitory hypothalamic mechanisms appear to have evolved to control GH and prolactin in the absence of long-loop feedback; hypothalamic GHRH stimulates, and somatostatin inhibits, GH secretion, whereas dopamine inhibits, and one or more putative prolactin-releasing factors stimulate, prolactin release. It is likely that in both the hypothalamic-prolactin and hypothalamic-GH axis, the short-loop feedback control includes both a suppression of releasing-factor release and a stimulation of inhibitory-factor release.

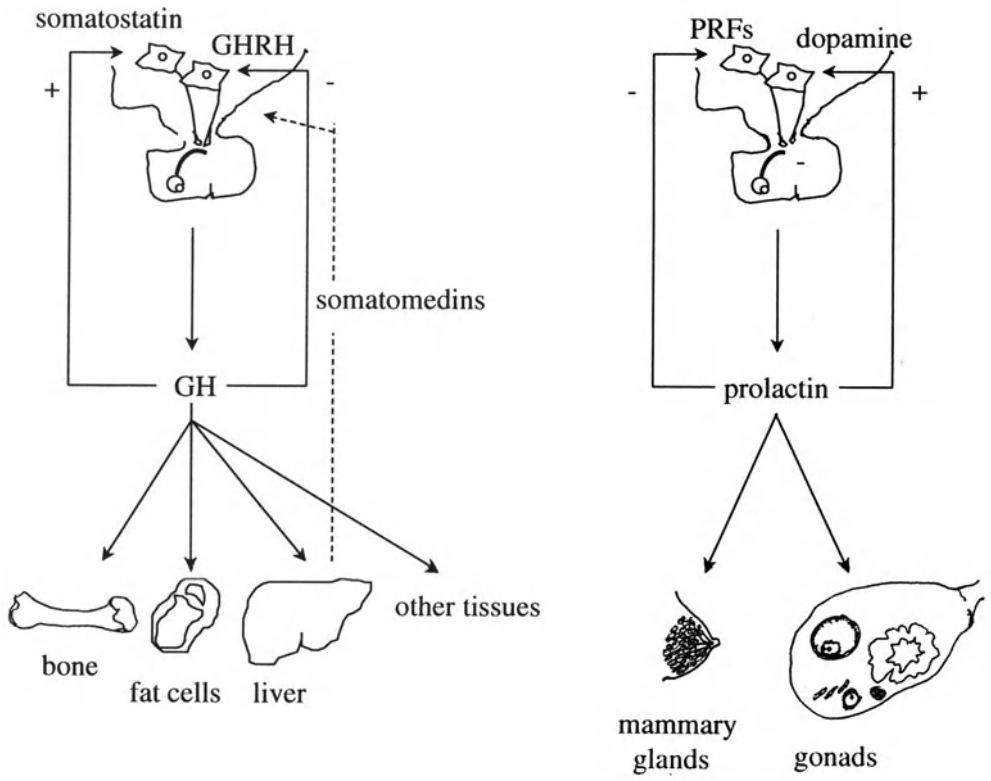

Fig. 4. Schematic representations of the major hypothalamic-pituitary axes in which short-loop negative feedback signals predominate. Shown are the respective stimulatory and inhibitory neurohormones for the regulation of **(A)** growth hormone (GH) secretion and **(B)** prolactin secretion. GHRH, growth hormone-releasing hormone; GH, growth hormone; PRFs, prolactin-releasing factors. Cellular targets of negative feedback are not specified, and may include hypothalamic interneurons, neurosecretory neurons, or both.

Thus, elevations in GH are generally accompanied by decreases in GHRH and increases in somatostatin neurosecretion; increases in prolactin secretion generally stimulate hypothalamic dopamine secretion and suppress prolactin-releasing activity.

One caveat is applied to the GH control systems. It is clear that the actions of GH in promoting cartilage formation (leading to bone growth) are mediated in large part by GH stimulation of somatomedins (IGF1, IGF2) from the liver. Evidence suggests that IGF1, apart from its intermediary role in bone growth, may also exert a negative feedback action within the hypothalamus, *viz.* inhibit GHRH release and stimulate somatostatin release. This action represents a potential long-loop feedback action, which may compliment the direct, short-loop feedback regulation mediated by GH itself.

3.2. Homeostatic Mechanisms Can Control Motivated Behavior

Early studies using lesions or electrical stimulation showed that particular hypothalamic loci play important roles in governing certain motivated behaviors. For example, lesions of the ventromedial nucleus or lateral hypothalamic areas were found to produce hyperphagia and hypophagia, respectively. Subsequent investigations demonstrated that drinking behavior can be elicited by chemical stimulation of the subfornical organ and that steroid hormones applied locally to the VMN can facilitate lordotic behavior in female rats. Results such as these have supported the concept that regulatory "centers" exist in the hypothalamus, from which signals originate that can modulate motivated behavioral patterns.

For many of these behaviors, it appears that negative-feedback signals from peripheral tissues are associated with the feedforward, stimulatory neural signals that regulate that activity. Such a combination of feedforward and feedback signals in the control of a behavior can be considered, of course, in terms of a homeostatic control system. An important example of this type of homeostatic system is one that governs feeding behavior. Much progress has been made in the last decade in characterizing components of this

homeostatic system, including the discovery of leptin, a hormone produced by adipocytes that clearly functions to restrain feeding behavior and likely acts to increase energy utilization. In addition, cell groups such as those producing neuropeptide Y in the mediobasal hypothalamus have been identified, which mediate at least some stimulatory control over food intake. One such cell population is the neuropeptide Y-producing neuronal group whose cell bodies reside in the arcuate nucleus. We can describe the regulation of feeding behavior in terms of (1) a body weight set-point determined by unknown cellular and molecular mechanisms in the mediobasal hypothalamus, (2) a controlling element consisting in part of neuropeptide Y neurons and likely other important neurotransmitter cell groups, (3) a controlled variable *viz.* stored potential metabolic energy represented as body fat (size and number of adipocytes), (4) leptin functioning as a feedback signal, and (5) leptin-sensitive neurons in the hypothalamus and/or their synaptic targets, functioning to integrate set-point and feedback signals. Thus, increases in feeding behavior are accompanied by increases in fat deposition, which in turn can lead to increases in circulating leptin levels, and hence, a restoration of lower levels of food intake. Conversely, prolonged fasting can reduce body fat stores, thereby reducing leptin levels and prompting a "disinhibition" of feeding behavior.

3.3. Homeostatic Systems Can Be Characterized Experimentally

The existence of homeostatic neuroendocrine systems has primarily been established through controlled, experimental manipulation of an axis, followed by careful measurement and analysis of system responses. Each component of the system is potentially subject to feedforward stimulation or inhibition and is also potentially subject to feedback regulation. Thus, a first step in understanding the physiological control system is to make straightforward predictions about the likely responses of a hormone in the axis to removal or application of a feedforward or feedback stimulus. For example, in the HPA axis, the feedforward (stimulatory) actions of CRH on ACTH secretions and ACTH on glucocorticoid secretions are easily demonstrated both in vivo and in vitro. Application of these hormones to experimental animals or to cultured pituitary or adrenocortical cells results in the expected secretory responses. Conversely, removal of these regulators in vivo, either by immunoneutralization, hypothalamic lesions, or by hypophysectomy results in a precipitous decline in glucocorticoid production.

The hallmark feature of most homeostatic systems—negative-feedback control—has also been characterized in virtually all of the neuroendocrine axes. The critical test invariably involves removal of the end-organ, where possible, and measurement of the hypothalamic or pituitary secretory response. As one would predict for systems featuring negative feedback, removal of the gonads in either sex results in an acceleration of GnRH release and a large increase in gonadotropin secretion; removal of the adrenal glands results in a robust increase in CRH and ACTH secretion; removal of the thyroid evokes substantial increases in thyrotropin (and likely TRH) secretion. In all cases, replacement with physiological concentrations of the appropriate end-organ hormones prevents the hypothalamic and pituitary-hormone secretions from deviating past their basal values.

The specific features of feedback control can differ, of course, between the homeostatic axes. In some axes, the principal feedback target may be the hypothalamus whereas in others, feedback control may be predominantly exerted at the level of the anterior pituitary. Moreover, in the two-tiered systems, short-loop feedback may be exerted through inhibition of a stimulatory feedforward component, stimulation of an inhibitory feedforward component, or both. Nevertheless demonstrating the existence of these control mechanisms is carried out according to the same general principles. An example is the control of GH secretion, which is stimulated by GHRH and inhibited by somatostatin. Removal of the anterior pituitary gland appears to be followed by an increase in GHRH expression and a decrease in somatostatin expression. Replacement with exogenous GH prevents or reverses both of these responses.

Whereas the logic of such experiments may seem straightforward, it should be noted that the exploration of these physiological relationships can often be exceedingly difficult, especially when measurements of neurohormones in portal vessels are required.

3.4. Sensory and Somatic Stimuli Evoke Physiological Changes in the Activity of an Axis

Neuroendocrine homeostatic systems function to maintain a preselected state, yet they must also be able to register an acute stimulus, permit an appropriate response to the stimulus, and return the system to the

preselected, basal state. In the HPA axis, for example, a stressful stimulus evokes CRH neurosecretion, which in turn stimulates ACTH and glucocorticoid release. The duration and form of the glucocorticoid response will be limited to some extent by an increase in feedback suppression of CRH and ACTH. With the termination of the stress, the original state of the system is restored. If the level of stress remains unabated, a somewhat higher basal state might be assumed by the system. Such a situation would not necessarily reflect a change in the set-point of the system; rather, it would only reflect the system's inability to mount sufficient and sustained feedback suppression to return the system to the real preselected state. In the case of the HPA axis, it is clear that prolonged excess of glucocorticoid secretion is deleterious to the longer term health of the individual and as such, not a desirable *status quo.*

The transient response of the HPA axis represents but one example of the many short-term responses by an axis to a physiological stimulus. In general, such perturbations arrive in the form of exteroceptive or interoceptive stimuli which are conveyed along sensory signaling pathways. Exteroceptive signals can be pheromonal, thermal, visual, auditory, tactile, olfactory, or gustatory. Through a multitude of neural pathways, such information is conveyed from sensory organs along the neuroaxis, usually via multiorder afferents to specific sets of hypothalamic neurons. In reflex ovulators, for example, the act of coitus produces specific stimulation of virtually all sensory modalities; these sensory stimuli are transduced and conveyed through spinal and brain stem synaptic pathways to the hypothalamus where they are integrated to produce a major ovulatory release of GnRH into the portal vasculature. The net result of this transient stimulation is a surge of LH and ovulation, the latter thus occurring at an optimum time relative to the presence of viable sperm in the reproductive tract. Following the ovulatory release of hormone, the HPG axis essentially returns to its basal homeostatic state. Another example is the response of the HPT axis to an acute cold stress. Such an environmental stimulus almost invariably produces a transient stimulation of TRH, and hence TSH and thyroid-hormone secretions.

Interoceptive sensory signals can likewise be transmitted from internal sensory receptors to hypothalamic neurons. Baroreceptors, thermoreceptors, and proprioceptors are capable of delivering important signals from the peripheral nervous system to relevant hypothalamic centers, thereby effecting changes in the activity of a particular axis. A sudden drop in blood pressure, for example, is registered and transduced by baroreceptors located in the atria, carotid bodies, and elsewhere in the arterial system. The information is transduced into neural signals, which are thereafter conveyed up the neuroaxis, and eventually delivered to hypothalamic centers, which mediate stress responses. The result is a marked activation of CRH neurons (along with vasopressinergic and other neuronal groups) and a stimulation of CRH release into the portal vasculature. Restoration of blood pressure is accompanied by a return of the HPA axis to a basal activity state.

Interoceptive signals can also be conveyed by changes in circulating concentrations of metabolic intermediates, osmolytes, or growth factors. As will be described in chapters to follow, it is clear that specific hypothalamic neuronal populations function as sensory receptors, endowed with the capacity to monitor blood levels of one or more of these key physiological regulators, and to relay appropriate commands to one or more homeostatic regulatory systems. Classic examples of such cues include stimulation of GHRH/GH secretion by elevated amino acid concentrations in blood, and induction of CRH/ACTH/glucocorticoid secretion by hypoglycemia. The physiological importance of these regulatory responses are discussed in subsequent chapters.

3.5. One Homeostatic Axis May Influence Another Through Crosstalk

Hypothalamic control systems do not operate independently of one another. Indeed, regulatory mechanisms have evolved to most efficiently coordinate the activities of several homeostatic systems to yield the most adaptive overall set of responses to a given physiological situation. In many cases, the benefit to the organism is derived from the enlistment of several different systems to achieve the same physiological goal. For example, stressful stimuli are known to evoke neurosecretion of both CRH and vasopressin from the same parvicellular terminals in the median eminence. This pool of vasopressin clearly functions to amplify and supplement ACTH secretion that would have occurred in response to CRH alone.

In other cases, activation of one system leads to suppression of another. In lactating women, the stimulation of prolactin secretion is often accompanied by a temporary cessation of ovulatory cyclicity. This lactational amenorrhea is most likely mediated by an inhibitory action of prolactin on GnRH and gonado-

tropin secretion. This mechanism serves to ensure that sufficient maternal physiological resources, and parental attention, is dedicated to the care of a given newborn prior to the arrival of the next offspring. Other examples of cross-signaling between axes, such as the inhibition of reproductive hormone secretions by hormones of stress, are described in corresponding chapters.

3.6. Homeostatic Settings are Changed During Stages of Development

The ontogeny of many neuroendocrine systems appears to involve major readjustments of homeostatic set points. Such resettings are known to occur perinatally, during sexual maturation, and in aging. In the HPG axis, for example, an increase in the frequency and/or amplitude of GnRH pulse generation is believed to mediate the pubertal activation of the reproductive axis. The process almost certainly represents a fundamental shift in the set-point of the activity of the neuroendocrine axis. This shift may be entirely because of an intrinsic neural maturation process that directs acceleration of the GnRH pulse generator, or a diminishment in hypothalamic responsiveness to negative feedback. Nevertheless, the pubertal activation of GnRH pulsatility is sustained throughout the adult reproductive lifespan, as is the new, adult-like equilibrium between feedforward and feedback regulation in the HPG axis. Other major readjustments of neuroendocrine homeostatic settings may occur in response to environmental cues, or they may accompany somatic disease states, changes in nutritional status, chronic stress, or psychiatric disorders.

3.7. Neuroendocrine Homeostatic Settings Can Be Altered Over Hours, Days, Months, or Years

The pubertal activation of GnRH release is a clear-cut example of set-point adjustment that is maintained over many years within a lifetime. In other situations, set-points may be altered over hours, days, or months. Within the circadian period, the set-point for neurohormone secretion can vary from hour to hour, likely as a function of regulatory signals from the biological clock residing in the suprachiasmatic nucleus (*see* Chapter 22); activity within the HPA axis varies with a rhythm that peaks in the morning hours and drops to a nadir in the evening.

Sustained changes in the set-point of an axis can also occur over the course of several days. Pheromonal cues, for example, can trigger sustained inhibition or activation of the reproductive axis in many species; social and psychological cues can alter food intake and energy homeostasis in humans.

Seasonal breeding represents a major example of set-point adjustments that extend over periods of months. The HPG axis receives photoperiodic cues through a signaling cascade, which includes the retinohypothalamic pathway, synaptic pathways leading to the pineal gland, melatonin secretions, and ultimately neural circuitries governing GnRH release (*see* Chapter 23). Through these pathways, information regarding day-length is registered, transduced, and conveyed to the reproductive axis as stimulatory or inhibitory signals for reproductive status. The resulting periods of reproductive activity and inactivity are thereby sustained throughout the months that are most adaptive for the reproductive success of the particular species.

3.8. Dysregulations in Neuroendocrine Homeostatic Systems Have Major Clinical Consequences

Virtually all major dysfunctions of neuroendocrine homeostatic axes have profound effects upon the health and well-being of an individual. The clinical consequences of neuroendocrine pathophysiologies are discussed in relevant chapters that follow, although in general they fall into the categories of infertility, growth and developmental impairments, oligo- or galactorhea, hypo- or hyper-thyroidism, hypo- or hyper-adrenocorticolism, and specific symptoms of diseases which present as a syndrome (e.g., polycystic ovarian syndrome or McCune-Albrights disease). As with any disease, the underlying causes of neuroendocrine dysregulation are varied, and can be heritable, acquired, associated with tumorogenesis and/or carcinogenesis, or otherwise associated with another medical condition. Proper diagnosis and treatment must always take into account the multidimensional nature of the controlled neuroendocrine system in which the hormone in question plays a functional role.

Neuroendocrine diseases are considered *primary, viz.* involving the major target organ of the axis, or *secondary, viz.* defects at antecedent levels (pituitary or hypothalamus). Moreover, the impairments may be manifest as hypofunction or hyperfunction in nature. Given the feedback relationships that prevail in neuroendocrine axes, it can prove difficult to accurately locate and diagnose the endocrinopathy, as it is not

Table 1
Expected Results of Diagnostic Tests for Basic Types of Neuroendocrine Disturbances

	End-organ (Hyper)	*End-organ (Hypo)*	*Pituitary (Hyper)*	*Pituitary (Hypo)*	*Hypothalamic (Hyper)*	*Hypothalamic (Hypo)*
Serum [pituitary hormone]	Low	High	High	Low	High	Low
Serum [end-organ hormone]	High	Low	High	Low	High	Low
End-organ responsiveness to tropic hormone	High	Low	N	N	N	N
Pituitary responsiveness to releasing factor	Low or N	High or N	High	Low	N	N
Pituitary responses to hypothalamic secretagogue	Low or N	High or N	High	Low	High	Low

always clear whether a diminished or excessive level of hormone results from primary alterations in the activity of glandular cells, or secondarily from changes in the level of feedback regulation by the succeeding organ. This is particularly true in cases where X-rays, MRIs, or other scanning procedures fail to reveal any obvious irregularity in the suspect tissue.

Neuroendocrine disturbances can often be confirmed by a combination of (1) measurement of the pituitary and end-organ hormones in serum, and (2) assessment of the responsiveness of the target organ to the stimulatory hormone. The information provided by these measurements and tests can thus be used to logically rule in or out a primary defect at the end-organ, anterior pituitary gland, or hypothalamus. Expected consequences of these tests are given in Table 1 for primary (end-organ), secondary (pituitary), or secondary (hypothalamus) neuroendocrine disturbances, along with examples of likely results in specific diagnoses.

4. NEUROENDOCRINE REFLEXES

4.1. How Does a Neuroendocrine Reflex Work?

Neuroendocrine reflexes mediate acute physiological responses to sensory and/or somatic signals. A sensory stimulus, such as the suckling by young at a mother's nipple, can evoke activity in an afferent pathway, which in turn prompts an appropriate activation of an effector neuronal population, such as the magnocellular oxytocinergic neurons. The net result is a physiological response, e.g., oxytocin-mediated milk ejection, that is qualitatively, quantitatively, and temporally appropriate for the original stimulus. Thus, neuroendocrine reflexes are like their neuromotor counterparts in that they have afferent and efferent loops, usually with one or more synapses intervening within the reflex circuitries (Fig. 5). The neuromotor reflexes, however, consist of strictly neuronal afferent and efferent pathways; the neuroendocrine reflexes can instead be comprised of a humoral input and neural output, humoral input and hormonal output, or neural input and hormonal output. Moreover, some neuroendocrine reflexes occur as stereotyped, *fixed-action responses* to a specific stimulus, similar to neuromotor reflex responses, while others may mediate *graded responses* to stimuli of varying magnitude. Major examples of each are given below.

4.2. Fixed-Action Neuroendocrine Reflexes

As detailed in Chapters 8, 9, and 14, oxytocin and vasopressin are produced in the magnocellular neurons of the supraoptic and paraventricular nuclei, transported intracellularly to neurovascular terminals in the posterior pituitary gland, and released upon electrophysiological stimulation into the peripheral circulation. As noted in the foregoing example, one major action of oxytocin is to stimulate contractions in myoepithelial cells of the mammary glands, prompting milk ejection. The reflexive secretion of oxytocin and its actions at the mammary gland represent a fixed-action response, *viz.* a relatively stereotyped output signal that is evoked by a specific input signal. The afferent limb of the milk-ejection reflex consists of sensory receptor endings in the nipple, primary sensory afferents, and multiorder synaptic relays leading up the neuroaxis to the supraoptic and paraventricular nuclei. The suckling stimulation leads to transient and robust increases in the frequency of action potentials in magnocellular oxytocinergic neurons, which is thereafter coupled to an acute increase in oxytocin neurosecretion. The oxytocinergic neurons and the circulation of oxytocin to the mammary gland thus constitute the efferent hormonal loop of this neuroendocrine reflex. The molecular and

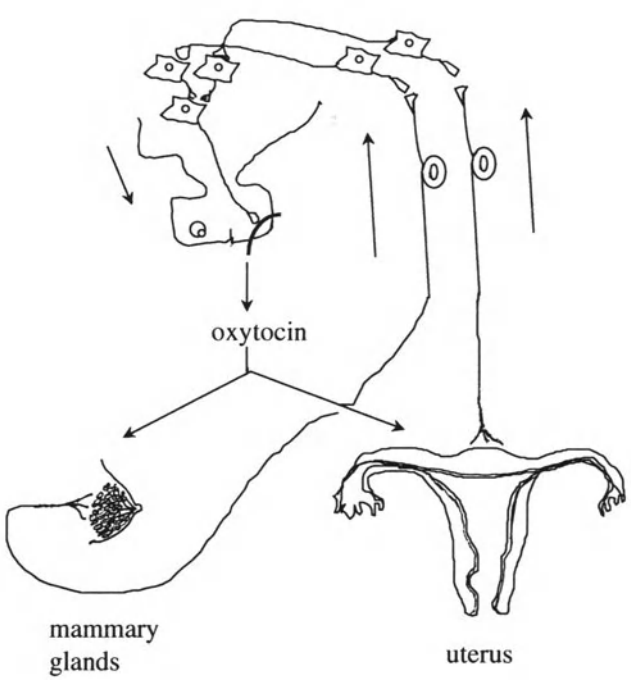

Fig. 5. Schematic representation of fixed-action neuroendocrine reflexes mediated by oxytocinergic neurons. Shown are two different reflex loops. In one, stimulation of sensory receptors in the nipple evokes neural impulses in afferent pathways that ascend the spinal cord and reach the hypothalamus. Stimulation of this afferent loop leads to reflexive activation of magnocellular oxytocinergic neurons in the supraoptic and paraventricular nuclei of the hypothalamus, and hence, neurosecretion of oxytocin into the peripheral circulation. The oxytocin stimulates smooth muscle contractions in the mammary glands, leading to increased intramammary pressure and milk-ejection. Thus, suckling by the young activates a neuroendocrine reflex that culminates in milk-ejection. In the second reflex, sensory receptors in the cervix are stimulated during labor, resulting in a transmission of neural signals up the neuroaxis to the hypothalamus. Activity in this afferent loop results in an activation of magnocellular oxytocinergic neurons, leading to an acute increase in the release of oxytocin into the peripheral circulation, and a stimulation of additional uterine contractility.

cellular events governing the secretion of oxytocin is examined in detail in Chapters 8 and 9. For the purposes of this discussion, the suckling-oxytocin response represents a simple reflex circuit consisting of a neural input and hormonal output, that exhibits a fixed-action response to a specific stimulus.

The release and actions of oxytocin during parturition can similarly be described in terms of a neuroendocrine reflex. In the latter stages of parturition, oxytocin appears to be released in response to dilation of the cervix. A neural afferent loop conveys this mechanoreceptive information to the hypothalamus and neural signals activate oxytocin neuronal activity and neurohormone secretion; the increase in circulating oxytocin stimulates an increase in myometrial contractility and hence, the intensity of labor. Again, this reflexive release of oxytocin is mediated by a neural input and hormonal output and represents a fixed-action response to a specific stimulus.

4.3. Graded Neuroendocrine Reflexes

The second major magnocellular neurohormone, vasopressin, is also released as an efferent component of neuroendocrine reflexes. One major action of vasopressin is exerted at receptors on the distal tubules of the mammalian kidney, where it facilitates the resorption of H_2O from the collecting ducts. The antidiuretic actions of vasopressin thus lead to increases (usually restoration) of blood volume. Vasopressin also induces the contraction of vascular smooth muscle cells, thereby contributing to elevations in blood pressure. Thus, the two major stimuli for release of vasopressin are (1) decreases in blood pressure and volume, and (2) increases in body fluid osmolality. Two functionally distinct feedback mechanisms govern the release of vasopressin by these two stimuli, the net effect of the secretory induction being the restoration of prestimulus osmolality, blood volume, and/or blood pressure.

Both neuroendocrine reflex mechanisms occur as responses that are proportional to the initiating stimulus. The first reflex response proceeds as follows. A significant increase in blood osmolality is registered and transduced by osmoreceptive cells in the hypothalamus and these signals are thereafter conveyed through afferent projections to magnocellular vasopressinergic cells. The basal electrophysiological activity of these cells is thereby altered, likely as an alteration in the phasic firing patterns of many neurons (more specifically, an increase in intraburst spike frequency and burst duration, see Chapter 3-4). The altered firing activity is coupled to an increase in vasopressin secretion into the peripheral circulation, which acts at the level of the renal tubules to promote water resorption and hence, dilution of body fluid osmolytes. The distinguishing feature of this neuroendocrine reflex is that it exhibits graded vasopressin responses that are proportional to the magnitude of the initial increase in blood osmolality. Moreover, the reflex loop consists of a humoral input and hormonal output.

A second type of reflex is triggered by low blood volume and/or low blood pressure, such as that which occurs consequent to major hemorrhage. Barorecep-

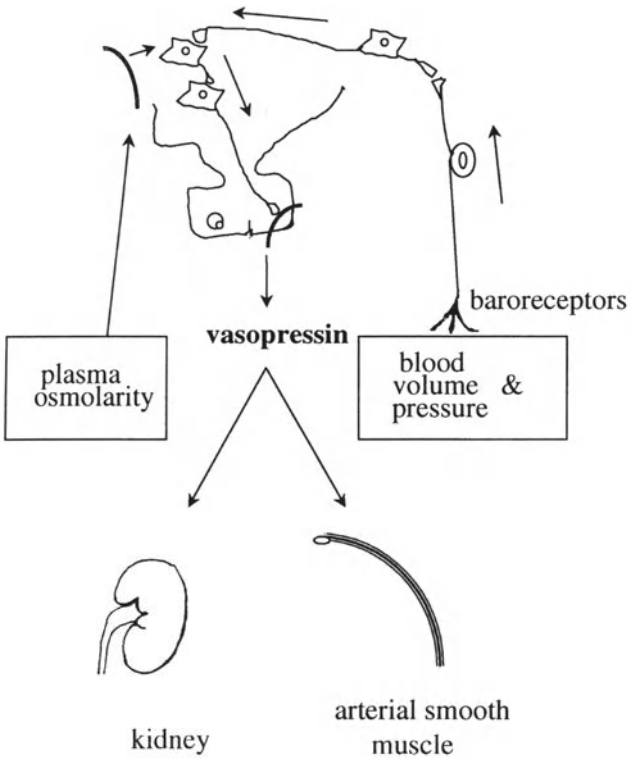

Fig. 6. Schematic representation of graded neuroendocrine reflexes mediated by vasopressinergic neurons. Shown are two different afferent reflex loops. In one reflex, a drop in plasma osmolarity is registered by specialized receptor cells in the hypothalamus and the amplitude of this change is encoded as neural impulses; these are then transmitted through synaptic connections to magnocellular vasopressinergic neurons in the supraoptic and paraventricuar nuclei. These afferent signals evoke a graded change in the activity of the vasopressinergic neurons, resulting in an alteration in vasopressin into the peripheral circulation. Vasopressin promotes reabsorption of water in the distal renal tubules, and stimulates vascular smooth muscle contraction. These actions result in dilution of blood osmolytes, defense of blood volume, and increased blood pressure. The second reflex is triggered by decreases in blood pressure, which are registered and transduced by baroreceptors in the carotid sinus, aorta, and elsewhere in the arterial system. Neural signals are conveyed up the neural axis to the hypothalamus, resulting in a proportional stimulation of activity in vasopressin neurons, and neurosecretion of vasopressin into the circulation.

tors located in the aortic arch, carotid sinus, and right atrium, register a suprathreshold drop in volume and pressure and transduce the drop into neural signals that are conveyed through multisynaptic pathways up the neuroaxis and ultimately, to magnocellular vasopressinergic neurons. The activity of these neurons is increased accordingly, and neurosecretion of vasopressin is stimulated. Increased plasma vasopressin levels thereafter mediate both antidiuretic and pressor responses to the original depressor or hypovolumetric stimulus. This response is likewise a graded one, which proceeds with an amplitude of vasopressin secretion that is proportional to the stimulus.

The distinct afferent loops distinguish these two major reflex responses from each other. The osmoreceptor response involves a humoral input and hormonal output, whereas the baroreceptor response is described as having a neural input and hormonal output. The two reflexes are similar, however, inasmuch as they both lead to graded vasopressin responses that are proportioned so as to evoke appropriate antidiuretic and pressor responses. In this regard, it is fair to consider how a graded neuroendocrine reflex differs from a neuroendocrine homeostatic mechanism. One difference is that the controlled variable and feedback regulator in a homeostatic system is usually a hormone that provides a continuous "readout" of the prevailing feedforward within the system; in the case of the foregoing reflexes, a systemic physiological variable (body fluid osmolality, blood volume, and blood pressure), which is monitored and may direct changes in neurosecretion. More importantly, physiological variables must be altered significantly above or below the limits of a normal range of values before any response is evoked within the system. Thus, under normal circumstances, very little tuning of the vasopressin secretion rate occurs in response to fluctuations within the acceptable (*viz.* nonharmful) physiological range. In essence, they function less as homeostatic regulatory systems, and more as a acute responses to potentially life-threatening systemic stresses.

5. MOLECULAR AND CELLULAR MECHANISMS OF NEUROENDOCRINE INTEGRATION

Homeostatic regulatory systems and neuroendocrine reflexes are critically dependent upon the proper functioning of the cellular signal transduction mechanisms of their constituent parts. Perhaps the most critical processing of information in this regard occurs within specific hypothalamic neurons. A given hypothalamic cell is endowed with a certain complement of receptors, second messenger systems, transcriptional regulators, and ion channels, all of which may determine the hypothalamic cell's role in receiving, integrating, and transmitting neural signals that are vital to the operation of a given homeostatic or reflex sys-

tem. A major challenge that faces neuroendocrinologists today is to acquire an understanding of how a cell registers neural and hormonal signals and integrates them in a process leading to the production of a physiologically appropriate output signal. Compounding the difficulty of this task is the incalculable complexity of cell–cell connections and interactions within the hypothalamus and the likelihood that individual neurons may be charged with integrating not just one type of signal, but many different types of signals conveyed simultaneously. Indeed, an integrative process may be one in which (1) synaptic activation via afferent neural pathways leads to the activation of a neuron, (2) a circulating hormone binds its cognate receptor in a cell and thereby alters the secretory activity of that cell, or (3) both of these types of signals impact the neuron simultaneously. Moreover, hormonal or neurotransmitter actions may be directly stimulatory or inhibitory, or they may be permissive, *viz.* they render a cell more or less responsive to another particular stimulus. In any event, the cellular and molecular pathways through which these important integrative processes occur are now subject to more direct study, using many of the modern tools of molecular biology.

5.1. Spontaneous Electrophysiological Activity in Neuroendocrine Cells

For simplicity, we define a neuroendocrine cell as one which either secretes neurohormone at neurovascular junctions, receives hormonal or other blood-borne signals, or functions as an interneuron between these two cell types. In most respects, all three varieties of neuroendocrine cells exhibit electrophysiological properties that are similar to neurons elsewhere in the central nervous system. They have dendrites, soma, and axons that resemble neurons elsewhere, and they exhibit relatively normal resting potentials, synaptic potentials, and action potentials. Moreover, they can display a range of intrinsic and synaptically driven activity patterns that are fairly common among other brain cell types. One fundamental property that is relatively specific to many neuroendocrine cell groups, however, is the propensity to release neurohormone or neurotransmitter in synchrony and at regular intervals. This coordinated, intermittent release pattern by a population of cells is referred to as *neuroendocrine pulsatility.* The functional importance of pulsatile neurohormone secretion has been clearly demonstrated; presentation of continuous neurohormonal stimuli is less effective, or even inhibitory, in releasing pituitary hormones. By contrast, administration of regular pulses of the same neurohormone can continue to evoke anterior pituitary hormone secretions for virtually unlimited periods. It is likely that the pulsatile pattern of release prevents down-regulation of signal transduction events within the target anterior pituitary cell. The pulsatile pattern of neurohormone secretion has also been found to be of profound clinical importance, as described in subsequent chapters.

The phenomenon of pulsatility has been noted in the majority of neuroendocrine axes, but it has been most extensively studied in the HPG axis. Here, the rhythmic release of GnRH (and hence, LH) is most robust, and as a result, it has been best characterized. The frequency of GnRH pulses in vivo can vary among species, age, sex, and physiological circumstances. Frequencies may range from as fast as one pulse/15 min in gonadectomized rodents, to as slow as one pulse/6 h or more in anestrous sheep. In all species individual pulses consist of a burst of neurosecretion lasting 0.5–5 min, after which the local concentration of GnRH in the median eminence extracellular spaces declines according to the rate of transport through the portal vasculature. Degradation in the extracellular fluid may also impact the waveform of an individual pulse. Fig. 7 depicts the patterns of GnRH and LH release as measured in two ovariectomized sheep. Electrophysiological correlates of pulsatile GnRH release have been characterized using hypothalamic electrode recordings in monkeys, sheep, goats, and rats. Such studies have demonstrated that pulsatile hormone secretion is governed by some set of neurons that periodically fire in unison a high-frequency volley of action potentials, which result in the neurosecretion of a pulse of GnRH into the portal vessels. These studies demonstrate that there must exist at least two important elements of the pulse generating process: a pacemaker or pseudo-pacemaking mechanism, and a mechanism for electrophysiological synchronization among neurosecretory cells. Underscoring the importance of these two elements is the demonstration that immortalized neuroendocrine neurons have the capacity for pulsatile release of neurohormone in vitro; through some unknown mechanism, pulses must be initiated and communicated among these cells in culture.

One simple model for pulsatility holds that the intrinsic rhythmic activity of one or more "pacemaker" cells can "drive" the activity of other cells in the population. To date, such rhythmic volleys generated in individual cells have not been demonstrated, at least with the temporal characteristics that

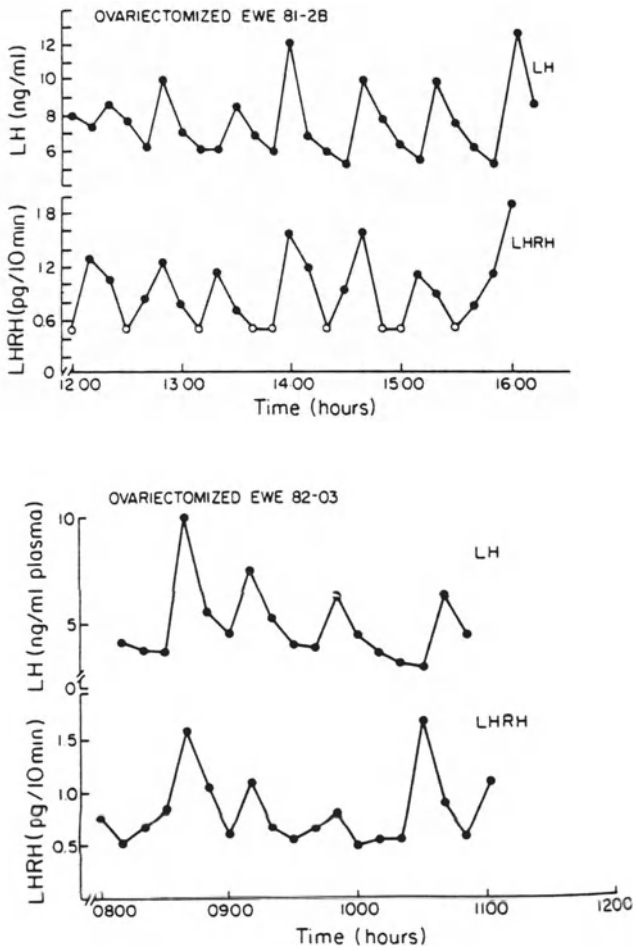

Fig. 7. Examples of neuroendocrine pulsatility. Pulsatile GnRH and LH release profiles were determined in two different ovariectomized ewes. The GnRH patterns were measured in push–pull perfusates of the hypothalamus, and LH patterns were determined in sequential blood samples obtained via jugular catheters. In these animals, all GnRH pulses were temporally associated with LH pulses, and they occurred at a frequency of approximately 1 pulse/30–40 min. (Reprinted with permission from Levine JE, Pau K-Y F, Ramirez VD, Jackson GL. Simultaneous measurement of luteinizing hormone-releasing hormone and luteinizing hormone release in unanesthetized, ovariectomized sheep. *Endocrinology* 1982; 111:1449–1455).

would be expected if the cell's activity underlies and stimulates the overall pattern of pulsatility. More likely, a "pseudo-pacemaking" mechanism may underlie pulsatile neurohormone secretion. In this scenario, any neuron within a pulsing network can initiate a pulse and the pulsatile rhythm follows from the continued repetition of (1) random autoactivation of a neuron within the interconnected cell network, (2) stimulation of other neurons in the network by the excited cell, (3) a refractory period, during which no neurons fire, and (4) autoactivation of another neuron in the network. This stochastic model does not depend upon the activity of a distinct pacemaker cell, but is instead driven by the emergent, random activity of an interconnected network of cells. In either model, some mechanism must operate to synchronize the pulsatile release activity among neurons. The simplest mechanism achieves synchronicity through intercellular signaling via synaptic contacts or electrical coupling among pulsing neurons. Other possibilities include the production of other intercellular synchronizing factors that mediate "volume" neurotransmission over a relatively wide multicellular area.

5.2. How are Signals Received, Transduced, and Integrated Within Neuroendocrine Cells?

Both neural and endocrine inputs can regulate the spontaneous electrophysiological and secretory activity of neuroendocrine cells. The molecular mechanisms through which these actions are manifest are not well understood and are currently subject to intensive study. The extracellular signals may be conveyed by neurotransmitters, circulating hormones, or nonhormonal metabolic stimuli (e.g., osmolytes, glucose). Signaling may be initiated either through *plasma membrane receptors,* as is the case for most neurotransmitters or by occupation and activation of *intracellular hormone receptors.* The latter receptors include virtually all members of the intracellular steroid receptor superfamily.

At the level of the plasma membrane, signaling can proceed through the activation of receptor, second messengers, and downstream intracellular effectors. In some cases, second messengers may be generated, which also act through effects on ion fluxes at the level of the plasma membrane. Additionally, neurotransmitters and hormones may bind and activate ligand-gated ion channels and rapidly evoke changes in cell excitability. Activation of intracellular receptors most commonly leads to transcriptional regulation of target genes, which in turn results in changes in the expression of proteins that function in the control of cell excitability and/or secretion.

Depicted in Fig. 8 is a hypothetical neuroendocrine neuron, indicating several possible cell processes that may ultimately be regulated by extracellular signals. The propagation of the extracellular signals within a cell involves *transduction* of the original signal into meaningful alterations in the activity of intracellular messengers; in many cases the initial signal can ram-

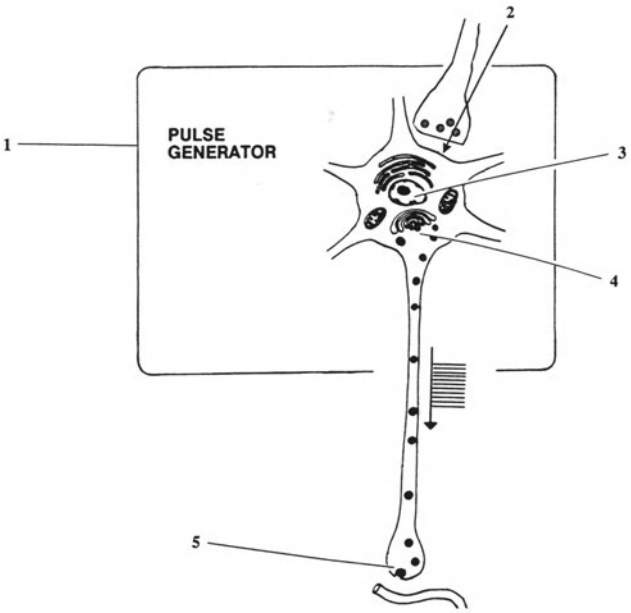

Fig. 8. Extracellular signals can evoke changes in neurosecretion through many signaling pathways. Shown are several general cellular processes that may be altered by synaptic and hormonal signals in hypothalamic cells, leading to regulated changes in the rate or pattern of secretion from that cell. Processes that may be regulated include (1) a neuroendocrine pulse generating mechanism, which may in turn depend upon cell–cell connectivity and local signaling, (2) membrane conductances and resultant alterations in membrane resting or action potentials, (3) transcription, RNA processing, or translation of neurotransmitter or neurohormone; the expression of some signaling molecules, which act within that cell may also be regulated, e.g., membrane receptors or protein kinases, resulting in altered responsiveness of the cell to other neuroendocrine signals, (4) posttranslational processing, and (5) exocytosis (stimulus-secretion coupling) or cellular reuptake of neurotransmitter or neurohormone. Cellular signal integration is likely a function of the net effect of many extracellular signals on one or more of these processes.

ify in many directions, simultaneously influencing a number of different cell activities. Conversely, multiple signals may be propagated along pathways that converge to regulate one or more of the same processes. As noted in Fig. 8, processes ultimately regulated as a result of extracellular signals include membrane conductances, active or facilitated transport, stimulus-secretion coupling, transcription, RNA processing, translation, post-translational processing, intracellular transport, exocytosis, and cellular reuptake.

Cellular integration occurs when a cell assesses the weight of each of these signals, computes their net effect, and directs alterations in output signals. The final output signal is encoded in changes in the rate or pattern of neurosecretion from that cell. Thus, any signal *to a cell* may alter one or more of the foregoing cellular processes; these alterations can thus lead to marked changes in the release of neurohormone or neurotransmitter *from that cell.* Examples of integrative mechanisms that may mediate the rate or temporal pattern of neuroendocrine secretions are given below.

5.2.1. Amplitude Modulation

The rate of neurotransmitter or neurohormone release can be stimulated or inhibited by a variety of mechanisms; these include (1) postsynaptic summation of signals, which leads to sufficient depolarization or hyperpolarization of membrane potential to impact the firing rate of the cell, (2) increased or decreased availability or activity of intracellular signaling molecules, (3) altered production, packaging, or transport of neurotransmitter or neurohormone, or (4) altered stimulus-secretion coupling dynamics, *viz.* amount of transmitter released from axon terminals in response to a given set of propagating impulses.

Activation and inhibition of CRH neurons can be considered as examples of amplitude modulation. Stressful stimuli lead to the neural stimulation of CRH neurosecretion into the hypophysial portal vessels. Circulating glucocorticoid hormones conversely inhibit the amplitude CRH neurosecretion as the major negative feedback component of the HPA axis. In both cases, the overall rate of neurohormone release/time is increased or decreased as a function of these signals. The neural activation of CRH release is rapid and appears to be mediated in part by synaptic signals conveyed through synaptic circuitries; the magnitude and quality of the stressful stimulus is likely encoded by the strength and origin of the synaptic signals impinging upon the CRH neurons, which in turn adjust their firing rates accordingly. The integration of signals, in this case, likely occurs at the level of the plasma membrane through computation of the net effect of the afferent set of synaptic signals; additional effects may be exerted through rapid effects of second messengers on ion channel conductances.

By contrast, the inhibitory effects of glucocorticoids are likely manifest through regulation of gene expression in CRH cells. The steroids bind intracellular receptors and the activated complex binds DNA response elements to regulate transcriptional activation of target genes. In this case, it has been demonstrated that it is the transcription of the CRH gene itself that is likely subject to inhibition by glucocorticoids (*see* Chapter 15). The net consequence for the

CRH cell is that the rate of production of CRH is diminished, leading to a likely reduction in the pool of neurohormone that is available for exocytotic release at the CRH axon terminal. Whereas other mechanisms may also contribute to glucocorticoid-mediated suppression of CRH release, this transcriptional regulation is considered to be a major mechanism mediating this physiological signal.

5.2.2. Pulse Frequency Modulation

The frequency of neurosecretory episodes can also be increased or decreased as a function of signal integration in neuroendocrine neurons. This phenomenon is not to be confused with regulation of impulse frequency in neuroendocrine neurons. All neurons act as FM to AM converters, in that the frequency of action potentials that invade an axon terminal determines in large part the amplitude of transmitter that is released per unit time; thus, alterations in the frequency of action potentials *per se* are eventually reflected as a change in the amplitude of signaling from the cell, as noted above. As stated earlier, however, a common pattern of secretion in neuroendocrine cells is one which is intermittent or pulsatile in nature. Changes in the frequency of the action potential volleys, which mediate release of pulses will, in turn, alter the frequency of pulsatile neurohormone discharges; the net output signal of the system can thus be encoded by pulse frequency modulation. Indeed, it has been clearly shown that pulse frequency modulation can occur in nature, and that it can have profound impact upon the quality and magnitude of the response of the pituitary gland.

Physiological regulation of neuroendocrine pulse frequency has been most extensively studied as it pertains to GnRH release in the HPG axis. In virtually all male and female mammals, GnRH release occurs as regular pulses that exhibit a periodicity that is largely characteristic of the physiological circumstance. The frequency of GnRH pulsatility is known to be altered as a function of developmental stage (e.g., at puberty), seasonal reproductive state, stage of estrous cycle, illness, nutritional state, and the prevailing degree of negative feedback regulation by gonadal hormones. For example, castration of male rats results in an acceleration of pulsatile GnRH release (Fig. 9). The GnRH pulse frequency, in turn, can dictate increases or decreases in gonadotropin secretion, and in some circumstances may direct differential release patterns of LH and FSH. Gonadal hormones appear to exert much of their negative feedback actions within the HPG axis through feedback

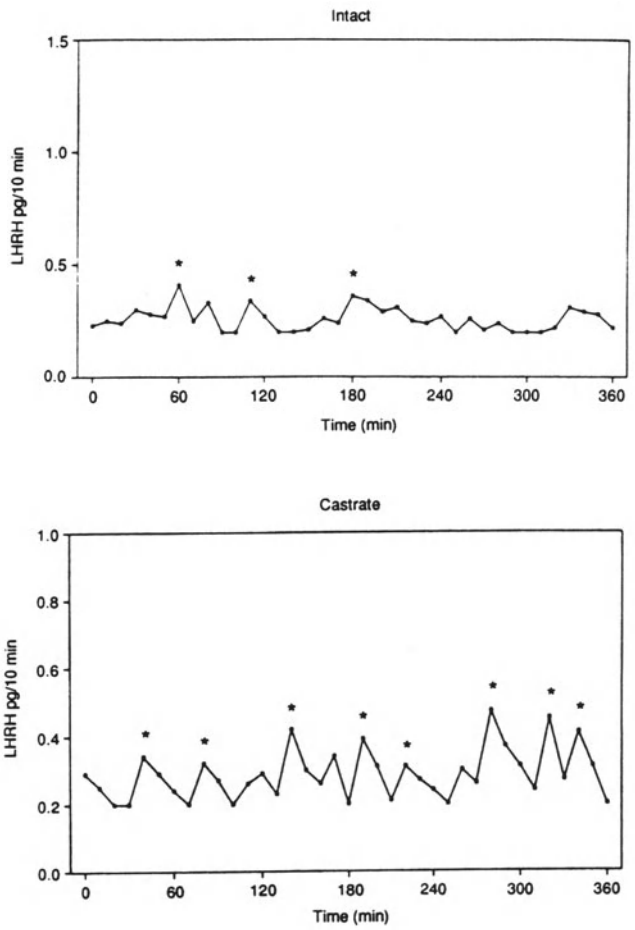

Fig. 9. Frequency modulation of neuroendocrine pulsatility. Pulsatile GnRH release patterns in an intact male rat (top panel) and a castrate male rat (bottom panel) are shown, with asterisks noting significant pulses. Frequency of GnRH pulsatility has been found to be significantly increased following castration. (Reprinted with permission from Levine et al., In vivo sampling and administration of hormone pulses in rodents. In: *Methods in Neuroscience,* "Pulsatility in Neuroendocrine Systems," 1994; pp. 129–161).

suppression of GnRH pulsatility. Intensive analysis of GnRH and gonadotropin patterns in rats, guinea pigs, sheep, rabbits, and monkeys have revealed that GnRH pulse frequency is accelerated following castration and suppressed following gonadal hormone replacement (Levine et al., 1991). What are the cellular mechanisms through which gonadal hormone actions may lead to suppression of GnRH pulse frequency? The mechanism may involve steroid hormone actions that result in altered levels of cell excitability; steroids may, for example, induce or reduce expression of some proteins that confer alterations in the duration of electrophysiological refractory peri-

ods. Alternatively, steroid hormones may slow the rate of a molecular processes underlying a clock-like mechanism in true pacemaker cells. Unfortunately, there is scant evidence supporting any hypothesis in this regard. It is likely that the fundamental, cellular basis of pulsatility must be characterized before progress can be made towards this end.

5.2.3. Permissive Effects

Some neural and endocrine signals may not in themselves alter the secretory activity of neuroendocrine cells, but they may render them more or less responsive to other signals. For example, an extracellular signal may induce expression of more receptor molecules for another extracellular signal. Similarly, hormones may evoke increased production or activity in second messenger cascades, thereby amplifying the intracellular responses to an extracellular regulator. These types of actions are referred to as *permissive,* as they essentially permit recurring signals to be transmitted in an up- or down-regulated state along a given signaling pathway, even though they do not directly initiate signaling in that pathway.

Many hormonal actions on neurons may be permissive, and indeed, evidence is accumulating that the integration of neural and endocrine signals may more often than not involve variations on this theme. Some of the actions of estrogen in hypothalamic neurons are representative of this type of action. For example, the induction of sexual receptivity in female rats by estrogen and progesterone treatments are mediated in part by permissive effects of these steroids on neurons in the ventromedial nucleus. Estrogen induces the expression of oxytocin receptors in this hypothalamic nucleus, and progesterone acts to stimulate distribution of these receptors. The net result of these actions is likely a larger response of these neurons to afferent oxytocin signals, and hence, an amplification of the lordotic behavior that is modulated by this neural circuitry. Other work has also shown that estrogen can regulate the coupling of receptor populations to G proteins, increase protein kinase activity, and alter the expression of ion channels, actions that may all ultimately modulate cellular responses to afferent transmitters.

5.2.4. Cellular Integration May Involve "Crosstalk" Between Signaling Pathways

In recent years, it has become increasingly evident that interactions may occur between molecules that have traditionally been considered components of different, and functionally distinct, signaling pathways. Steroid hormone receptors, for example, have been shown to be activated by intracellular second messengers such as cAMP, growth factors, and other mediators even in the absence of the steroid ligand for that receptor. On this basis, it has been proposed that such "molecular crosstalk" may be physiologically relevant in the integration of neural and endocrine signals. The progesterone receptor has been most studied in this regard. Recent studies have demonstrated that the progesterone receptor can be activated by stimulation of the cAMP/PKA signaling pathway, and convincing evidence has been presented that such a mechanism may be involved in the neural regulation of sexual behavior, secretion of gonadotropin surges (Levine, 1997), and in the GnRH self-priming mechanism in pituitary gonadotropes. One scenario for this integrative process holds that estrogen stimulates progesterone receptor expression in hypothalamic neurons and in gonadotropes; thereafter, neural signals for GnRH surges, and neurohormone signals (GnRH) for gonadotropin surges, can effectively stimulate appropriate cellular responses by ligand-independent activation of the progesterone receptors. Circulating progesterone subsequently amplifies the cellular response of hypothalamic neurons to GnRH. In this model, progesterone receptors are essentially serving the role of estrogen-inducible transcription factors that confer responsiveness of a cell to other afferent signals. This is a permissive hormone action, albeit one in which one type of molecule is induced (progesterone receptor) to permit convergence of two signaling pathways (cAMP/PKA and progesterone), resulting in an integration of both neural and endocrine signals and production of an appropriate and robust cellular response. Other examples of potential cross-talk mechanism include the ability of either circulating or locally produced steroid compounds to alter signaling through membrane-bound GABA receptors, or by stimulation of cAMP levels which, in turn, can regulate ion conductances at the level of the plasma membrane.

5.2.5. Trophic Signals

Neural and hormonal signals may have more long-lasting, if not permanent, effects on the function of a hypothalamic cell by virtue of their effects on cell growth, development, and morphology. Such trophic effects have been well documented in adrenal chromaffin cells, as their mature phenotype can be dramatically altered by treatment with either glucocorticoid or nerve growth factor. Similar actions of growth factors and hormones are likely exerted during

development of hypothalamic neurons. The well-known organizational effects of steroid hormones during fetal development are but one class of trophic signals that impact hypothalamic neuronal function. Other examples include the seasonal photic stimulation of changes in glial/neuronal juxtaposition and the apparent ability of ovarian steroid hormones to alter cell–cell contacts among neuronal endings, tanycytes, and glial elements in the median eminence. In puberty, moreover, considerable evidence implicates neurotrophins in glial-neuronal signaling, which contribute to the maturation of hypothalamic neurons governing GnRH release. Further research will hopefully shed light on the transduction and integrative mechanisms through which trophic signals may exert their effects on the structure, neurotransmitter phenotype, and function of hypothalamic neuroendocrine cells.

SUMMARY

The hypothalamus plays a central role in the regulation of visceral processes, motivated behavior, and responses of the autonomic nervous system. Hypothalamic cells are informed by signals received through sensory pathways, peripheral hormone secretions, and ill-defined afferent pathways originating in limbic and cortical structures. They register, transduce, and integrate these signals, and modulate accordingly their output "commands" to other cells. The outputs can consist of neurohormonal signals acting at distant peripheral organs (e.g., kidneys), neurohormonal signals to the anterior pituitary gland, or neural signals to circuitries that govern functions as diverse as feeding, thermoregulation, or sexual behavior. The integrative activities of hypothalamic neurons can often be considered as fundamental components of neuroendocrine homeostatic systems or neuroendocrine reflexes. The microanatomy of neuronal groups which may integrate signals in these systems has been studied extensively and is reviewed in relevant chapters in this volume.

How does signal integration occur in specific hypothalamic neurons? The molecular events that mediate these integrative processes have been extremely difficult to resolve, given the technical barriers that have limited experimentation on a specific cell, or population of cells, that subserve a specific integrative process. The advent of new transgenic animal models, gene "knock-out" paradigms, cell-activity markers, gene-transfer methodologies, immortalized cell lines, single-cell RNA analysis, and refined organotypic tissue-slice explants will hopefully permit the characterization of these critically important integrative processes.

SELECTED READINGS

Bluet-Pajot MT, Epelbaum J, Gourdji D, Hammond C, Kordon C. Hypothalamic and hypophyseal regulation of growth hormone secretion. Cell Mol Neurobiol 1998; 18:101–123.

Chrousos GP. Regulation and dysregulation of the hypothalamic-pituitary-adrenal axis. The corticotropin-releasing hormone perspective. Endocrinol Metab Clin N Amer 1992; 21:833–858.

Crowley WR, Parker SL, Armstrong WE, Spinolo LH, Grosvenor CE. Neurotransmitter and neurohormonal regulation of oxytocin secretion in lactation. Ann NY Acad Sci 1992; 652:286–302.

DeGennaro Colonna, Cell VSG, Locatelli V, Loche S, Ghigo E, Cocchi D, Muller EE. Neuroendocrine control of growth hormone secretion. Acta Paediatr Scand 1989; 349:87–92.

Frohman LA, Downs TR, Chomczynski P. Regulation of growth hormone secretion. Front Neuroendocrinol 1992; 13:344–405.

Joseph-Bravo P, Uribe RM, Vargus MA, Perez-Martinez L, Zoeller T, Charli JL. Multifactorial modulation of TRH metabolism. Cell Mol Neurobiol 1998; 18:231–247.

Kaltenbach JC. Endocrine aspects of homeostasis. Amer Zool 1988; 28:761–773.

Kaufman JM, Kesner JS, Wilson RC, Knobil E. Electrophysiological manifestation of luteinizing hormone-releasing hormone pulse generator activity in the rhesus monkey: influence of alpha-adrenergic and dopaminergic blocking agents. Endocrinology 1985; 116:1327–1333.

King JC, Rubin BS. Dynamic alterations in luteinizing hormone-releasing hormone (LHRH) neuronal cell bodies and terminals of adult rats. Cell Mol Neurobiol 1995; 15:89–106.

Knobil E. Patterns of hypophysiotrophic signals and gonadotropin secretion in the rhesus monkey. Biol Reprod 1981; 24:44–49.

Lechan RM. Neuroendocrinology of pituitary hormone regulation. Endocrinol Metab Clin N Amer 1987; 16:475–501.

Leong DA, Frawley LS, Neill JD. Neuroendocrine control of prolactin secretion. Ann Rev Physiol 1983; 45:109–127.

Levine J. New concepts of the neuroendocrine regulation of gonadotropin surges in rats. Biol Reprod 1997; 56:293–302.

Levine JE, Bauer-Dantoin AC, Besecke LM, Conaghan LA, Legan SJ, Meredith JM, Strobl FJ, Urban JH, Vogelsong KM, Wolfe AM. Neuroendocrine regulation of the luteinizing hormone-releasing hormone pulse generator in the rat. Rec Prog Horm Res 1991; 47:97–153.

Lincoln DW, Wakerley JB. Electrophysiological evidence for the activation of supraoptic neurons during the release of oxytocin. J Physiol 1974; 242:533–554.

Moore KE. Interactions between prolactin and dopaminergic neurons. Biol Reprod 1987; 36:47–58.

Plotsky PM, Thrivikraman KV, Meaney MJ. Central and feedback regulation of hypothalamic corticotropin-releasing factor secretion. Ciba Found Symp 1993; 172:59–75.

Power RF, Mani SK, Codina J, Conneely OM, O'Malley BW. Dopaminergic and ligand-independent activation of steroid hormone receptors. Science 1991; 254:1636–1639.

Robinson AG, Fitzsimmons MD. Vasopressin homeostasis: coordination of synthesis, storage, and release. Reg Pept 1993; 45:225–230.

Schumacher M, Coirini H, Pfaff DW, McEwen BS. Behavioral effects of progesterone associated with rapid modulation of oxytocin receptors. Science 1990; 250:691–694.

Tannenbaum GS, Painson JC, Lapoint M, Gurd W, McCarthy GF. Interplay of somatostatin and growth hormone-releasing hormone in genesis of episodic growth hormone secretion. Metabolism 1990; 39:35–39.

Wray S. Uterine contraction and physiological mechanisms of modulation. Amer J Physiol 1993; 264:C1–18.

6 Endocrine Targets in the Brain

Lothar Jennes, PHD and M. Chris Langub, PHD

CONTENTS

1. INTRODUCTION

1.1. Neuropeptide Receptors in the Brain

More than 50 neuropeptides have been identified to be synthesized in neurons of the hypothalamus. Most of these peptides are transported inside the axons to the median eminence where they are released into the perivascular space of fenestrated capillaries of the primary plexus. The peptides then enter the bloodstream and are carried to the secondary plexus of the anterior pituitary where they diffuse out of the modified capillaries into the perivascular space and bind to specific membrane receptors to regulate the synthesis and/or release of the anterior pituitary hormones.

In addition to these "neurohormone" actions, most neuropeptides are also released within the central nervous system to regulate the activity of various neurons and, in some cases, glial cells. In general, the neuropeptides are released either from a presynaptic axon terminal, which is followed by binding to and activation of postsynaptic receptors or they are released from extrasynaptic sites of "en passant" axons, which is followed by diffusion of the peptides to specific extrasynaptic membrane receptors. In addition, many neuropeptides are released into the cerebrospinal fluid and thus, circulated throughout the brain via the extracellular fluid. The peptide levels in the cerebrospinal fluid are of clinical importance because they parallel in most cases the amounts of peptide released in the median eminence. They can also be easily determined by radioimmunoassay of cerebrospinal fluid collected by spinal taps. Because the neuropeptides are not exclusively released at presynaptic terminals, but also at asynaptic sites, anatomical mismatches in the localization of the peptide-containing neurons and their relevant receptors are a common feature for most neuropeptide systems. In the following section, the localization of several neuropeptide receptors is described with focus on the hypothalamus and compared to the localization of the corresponding neuropeptides. The distribution patterns of peptide hormone receptors in the hypothalamus are summarized in Table 1.

From: *Neuroendocrinology in Physiology and Medicine*, (P. M. Conn and M. E. Freeman, eds.),

2. NEUROPEPTIDE RECEPTORS

2.1. Gonadotropin Releasing Hormone (GnRH)

GnRH is best known for its regulation of the anterior pituitary gonadotropes where it stimulates the release and synthesis of luteinizing hormone (LH) and follicle stimulating hormone (FSH). In addition to this "endocrine" function, GnRH is involved in the regulation of intracerebral processes, such as facilitation of reproductive behavior. These actions are mediated by binding of GnRH to and activation of specific membrane receptors. So far, only one GnRH receptor type has been cloned and sequenced. This receptor belongs to the family of seven transmembrane-spanning, G-protein coupled receptors. The GnRH receptor has the unique feature that a C-termi-

Table 1
Distribution of Neuropeptide Receptor mRNAs in the Hypothalamus

	GnRH-R	*CRH-R1*	*CRH-R2*	*TRH-R*	*Somato-statin R1*	*Somato-statin R2*	*Somato-statin R3*	*Somato-statin R4*	*Vasopressin*	*Oxytocin*
Preoptic area	–		+	+++	+++	+++	+++	+++	++	+++
Median preoptic n.	–				–	++			++	–
Anterior hypothalamic n.	–	+	+	+	+	++	++		++	++
Suprachiasmatic n.	–	–	+	+	++++	++	+++		++++	–
Retrochiasmatic area	–								++++	+++
Supraoptic n.	–	–	+++	–	+++	–	++++	++	–	++
Paraventricular n.										
Parvocellular	–	–	++	+	+++	+	+++		++	
Magnocellular	–	–	–							++
Arcuate n.	+++	–/+	–/+	+	++++	+++	+++	++	++	–
Dorsomedial n.		+++	–	+++	+	++	++		++	–
Ventromedial n.	++	+	++++		+	+++	+++		–	+++
Lateral hypothalamic area	–		+	+++					++	–
Mammillary body	–			+	+	+		+	++	++

nus intracellular loop is missing. After the cloning of the receptor, cRNA probes were generated and used for *in situ* hybridization to localize the cells that contain the mRNA. In general, GnRH receptor mRNA is restricted to a few nuclei in the brain, including the claustrum, amygdala, hippocampus, medial habenula as well as the hypothalamic arcuate and ventromedial nuclei. Most of these regions also contain the receptor protein as determined by in vitro autoradiography using the ^{125}I-labeled agonist [D-Ser (tbu)^{6}-Pro^{9}-NHEt)-GnRH (Buserelin, Hoechst) as ligand, however, several mismatches have been noted. For instance, the ventromedial nucleus contains moderate GnRH receptor mRNA levels and low levels of specific binding sites. The ventromedial nucleus sends extensive projections to the central gray, which contains abundant GnRH binding sites. The central gray is a site in the brain where local microinjections of GnRH facilitate reproductive behaviors. It is likely that the receptor protein is synthesized in the perikarya of neurons in the ventromedial nucleus and transported intraaxonally to the central gray. Similarly, GnRH binding sites have been detected in the interpeduncular nucleus where receptor mRNA is absent. Instead, the medial habenula contains GnRH receptor mRNA and it is likely that the receptor protein is transported through a major projection from the medial habenula through the fasciculus retroflexus to the interpeduncular nucleus. In general, there is a fairly good overlap between GnRH-neuropeptide containing regions in the brain and GnRH receptor expressing neurons. One notable exception is the hippocampus, which contains substantial amounts of receptor mRNA and protein, yet GnRH immunoreactive neurons or axons are absent from the hippocampus. Evidence suggests that GnRH from the cerebrospinal fluid can reach the hippocampus and bind to receptors in the strata oriens and radiatum.

The exact function of intracerebral GnRH receptors in the control of reproductive behaviors or other physiological events is not clear, however, it is known that the ventromedial nucleus is one of the critical sites in the brain involved in the generation of lordosis behaviors. It is possible that activation of GnRH receptors contributes to the expression of this behavior. The arcuate nucleus of the hypothalamus receives GnRH-containing axons and also contains GnRH receptor mRNA and protein, which suggests that GnRH innervates certain neurons in this nucleus. On the other hand, the neurons in the arcuate nucleus synthesize a variety of neuropeptides and catecholamines, including dopamine, neuropeptide Y, and proopiomelanocortin, which are transported to the median eminence where they can regulate GnRH release from axon terminals. It is likely that GnRH can influence the activity of these neurons and thereby participate indirectly in the control of the secretion of GnRH from axon terminals in the median eminence.

2.2. Corticotropin Releasing Hormone (CRH)

CRH is the principal neuropeptide that stimulates the release of ACTH from the anterior pituitary and it is therefore responsible for the control of the brain-pituitary-adrenal axis. The CRH neurons that regulate the anterior pituitary corticotropes are located in the paraventricular nucleus and, to a lesser extent, in the supraoptic nucleus. In addition, CRH is synthesized in many neurons in a variety of cortical and subcortical

regions, the hypothalamus and brain stem and it is thought that these neurons are involved in the control of autonomic functions. Thus, CRH participates in the regulation of memory, control of food and electrolyte balance, emotions, especially fear and aggression, as well as neurological disorders such as depression.

So far two different receptors for CRH have been cloned and the cDNA and amino acid sequences determined. Both proteins are G-protein coupled, they belong into the family of seven transmembrane spanning receptors and activation of both receptors stimulates cAMP production. The receptors differ in their structure/function relationships in that the peptides CRH, urotensin, and sauvagine are about equipotent in stimulating cAMP production through activation of the CRF-R1 ($EC_{50} \approx 4$ n*M*) whereas CRH, urotensin, and sauvagine are effective at the CRF-R2 receptor with EC_{50} of 20, 2, and 0.5 n*M*, respectively.

The localization of the mRNAs encoding the CRF-R1 and -R2 receptors is quite different, especially in the hypothalamus and pituitary. Thus, CRH-R1 mRNA is abundant in the anterior and intermediate lobe of the pituitary, whereas CRH-R2 mRNA is very low. In the hypothalamus, CRH-R1 mRNA is prominent in the dorsomedial nucleus and less in the supraoptic and ventromedial nuclei, as well as in the lateral hypothalamus. This receptor mRNA is absent or very low in all subdivisions of the paraventricular nucleus. Conversely, CRH-R2 mRNA is highly expressed in the ventromedial, supraoptic, and paraventricular nuclei, whereas less receptor mRNA is present in the preoptic and suprachiasmatic nuclei. CRH-R2 mRNA is absent in the dorsomedial nucleus. Both receptor mRNAs are absent in the hypothalamic arcuate nucleus. Both receptor mRNAs are present in many extrahypothalamic regions, again in an almost mutually exclusive pattern. CRH-R1 mRNA levels are high in the cortex, pons/medulla, and amygdala, especially in the basolateral and medial nuclei, whereas CRH-R2 mRNA is present in the posterior cortical nucleus of the amygdala and less in the medial nucleus. CRH-R2 mRNA is absent in the cortex and pons, except for certain neurons in the inferior collicle. There are only a few regions in the extrahypothalamic brain that contain both receptor mRNAs. These include the hippocampus, interpeduncular nucleus, accessory olfactory nucleus, and the ependyma.

The general localization of the CRH receptor mRNAs matches to a large extent the distribution of CRH-containing neurons and axons. Notable exceptions are the interpeduncular nucleus where immunoreactive fibers are absent or rare, yet both CRH-R1 and R2 mRNA is found in moderate amounts. Conversely, the locus ceruleus contains many CRH immunoreactive neurons and axons, yet neither CRH-R1 nor -R2 mRNAs have been detected.

Based upon the distribution of the two receptor mRNAs, it is clear that CRH action on ACTH release in the pituitary is mediated predominantly by CRH-R1. However, the neurons in the paraventricular nucleus that project to the median eminence, i.e., the neuroendocrine CRH neurons, express the CRH-R2 mRNA, which suggests that these neurons are regulated at least in part by CRH itself through CRH-R2-autoreceptors or a short-loop feedback system using the CRH-R2 receptor. A similar situation exists in the supraoptic nucleus where CRH is synthesized in a subpopulation of neurons and only CRH-R2, but not CRH-R1 mRNA is detected. These data suggest that CRH-R2 also participates in the control of the hormone release from the neural lobe, although it is not clear at present if either oxytocin- or vasopressin-containing neurons or both express the receptor. One can only speculate about the role of CRH receptors in other hypothalamic regions: for instance, the ventromedial nucleus is an important site of integration of autonomic-gastrointestinal functions and CRH has been implicated in the development of eating disorders. It is possible that these effects are mediated by activation of CRH-R2.

2.3. Thyrotropin-Releasing Hormone (TRH)

TRH is synthesized in the form of a large precursor molecule that contains five copies of the active tripeptide. TRH stimulates the release of thyroid-stimulating hormone (TSH) from the anterior pituitary thyrotropes and, to a lesser extent, of prolactin, but it has important additional functions inside the brain. Here, TRH is involved in the control of diverse processes including blood pressure, respiration, body temperature, nociception, and water and food intake. All actions of TRH are initiated by binding to a plasma membrane receptor that belongs to the seven transmembrane spanning, G-protein coupled receptors. Recently, the cDNA encoding this receptor has been sequenced and the amino acid sequence deduced. It appears that only one form of this receptor exists, although a splice variant has been described that contains an extended cytoplasmic C-terminal tail. However, no specific functional differences have been determined for this 19-20 amino acid extension.

"*In situ*" hybridization studies using cRNA or oligonucleotide probes have shown that the receptor mRNA is widely distributed throughout the hypothal-

amus. Thus, the medial and lateral preoptic areas and the periventricular anterior hypothalamus contain strongly labeled neurons, whereas fewer TRH-receptor mRNA containing cells are present in the parvocellular portion of the paraventricular nucleus and in an area surrounding the magnocellular subdivision. TRH-receptor mRNA has been found in the suprachiasmatic nucleus, the perifornical region of the lateral hypothalamus, and in the dorsomedial nucleus. The arcuate nucleus and the mammillary body contain only a few labeled neurons, whereas the posterior hypothalamus and the supramammillary region contain many labeled cells. Important extrahypothalamic regions with high TRH-receptor mRNA content include the accessory olfactory bulb, perirhinal cortex, various subdivisions of the amygdala, as well as the diagonal band of Broca.

The distribution of the receptor mRNA matches in most instances the location of the receptor protein, as determined by "in vitro" autoradiography. Thus, binding is strongest in the preoptic area followed by the posterior mammillary nucleus, the dorsomedial, and the ventromedial nuclei. The amount of binding in the paraventricular nucleus is still controversial since very little specific binding was measured with [^{3}H](3-Me-His^2)-TRH as ligand whereas high levels were measured with [^{3}H]-TRH. The finding that the receptor protein is located in the same regions as the corresponding receptor mRNA suggests that the receptor is not extensively transported to distant axon terminals, but it remains at or near the perikaryon probably functioning as a postsynaptic dendritic or somatic receptor.

2.4. Somatostatin

Somatostatin is the major inhibitor of growth hormone release from the anterior pituitary somatotropes. This inhibition is exerted by binding to and activation of specific membrane receptors on the somatotropes as well as certain neurons inside the brain. In the central nervous system, somatostatin is thought to inhibit the activity of growth-hormone releasing hormone producing neurons. So far, five different receptor subtypes for somatostatin have been cloned, sequenced, and the amino acid sequences have been deduced. All of these receptors are G-protein coupled and activation of the receptors leads to an inhibition of adenylate cyclase activity and a decrease of Ca^{++} influx through voltage-sensitive Ca^{++} channels. The expression of the five receptor subtypes is region-specific in the brain and under differential regulation during the pre- and postnatal development. In the adult, the mRNAs for the somatostatin receptors 1-4 are widely expressed throughout most regions of the cortex, olfactory system, hippocampus, amygdala, and septum, whereas somatostatin receptor-5 mRNA is found only in the cerebellum, dorsal motor nucleus of the vagus, and interpeduncular nucleus. The extensive extrahypothalamic distribution of the somatostatin receptor mRNAs suggests that somatostatin exerts additional functions, beside the regulation of growth hormone release.

In the hypothalamus, somatostatin receptor-1 mRNA levels are highest in the supraoptic, suprachiasmatic, arcuate, paraventricular nuclei, and medial preoptic area. Lower levels of the receptor mRNA are present in the periventricular and ventral premammillary nuclei and lowest in the anterior hypothalamic, ventromedial, dorsomedial, lateral mammillary, and medial tuberal nuclei as well as the lateral preoptic area. In contrast, somatostatin receptor-2 mRNA levels are highest in the medial tuberal, arcuate, ventromedial, and medial preoptic nuclei and lower levels of receptor mRNA are present in the anterior hypothalamic, suprachiasmatic, arcuate, and dorsomedial nuclei. The mRNA content is lowest in the lateral preoptic area, paraventricular, periventricular, and mammillary nuclei. Somatostatin receptor-3 mRNA levels are highest in the supraoptic, suprachiasmatic, ventromedial, arcuate, and magnocellular paraventricular nuclei and less abundant in the medial preoptic and anterior hypothalamic areas, the dorsomedial, and the mammillary nuclei. It is low or absent in the periventricular nucleus. The distribution pattern of the somatostatin-4 receptor mRNA is quite different in that its expression is highest in the medial preoptic area and less abundant in the supraoptic, arcuate, and mammillary nuclei. The mRNA of the somatostatin-5 receptor is not detectable in the adult hypothalamus.

The distribution of the somatostatin receptor 1-4 mRNAs overlaps to a large extent with the localization of the receptor proteins as shown by in vitro autoradiography and with the location of somatostatin immunoreactive perikarya or axons. Thus, the somatostatin receptor-2 selective ligand MK-678 binds to cells in the ventromedial and dorsomedial nuclei, conversely, somatostatin-28, which is more selective for the type-1 receptor, binds preferentially to cells in the paraventricular nucleus. In many instances, such as in the periventricular and paraventricular nuclei, extensive overlap exists in the distribution of somatostatin immunoreactive neurons and receptor mRNAs. This overlap suggests that a portion of these receptors may function as autoreceptors to

inhibit the activity of somatostatin neurons. Moreover, the localization of several somatostatin receptor subtype mRNAs in the same nucleus, for example, the arcuate nucleus, indicates that certain neurons express several different somatostatin receptor subtypes. Because growth-hormone releasing hormone neurons are prominent in the arcuate nucleus, it is likely that their activity is regulated, at least in part by somatostatin, thus establishing intrahypothalamic feedback loop systems that control either somatostatin or growth-hormone releasing hormone secretion from the median eminence.

2.5. Vasopressin

Vasopressin is a nonapeptide that is produced in the hypothalamic magnocellular neurons of the supraoptic and paraventricular nuclei, as well as in parvocellular neurons of the suprachiasmatic nucleus. The hormonal function of vasopressin is typically associated with the regulation of water-salt balance, ACTH release, blood pressure, and metabolic balance. In addition, vasopressin functions as a neurotransmitter in the central nervous system where it affects brain development, learning, memory, and body temperature.

Several membrane receptors for vasopressin have been characterized based upon differential binding properties of analogs and more recently, 3 cDNAs have been cloned that encode for the VP1a, VP1b, and VP2 receptors. All of these receptors are G-protein coupled and have seven transmembrane spanning hydrophobic regions. They differ, however, in their second messengers pathways in that activation of the receptors of the V1 class stimulates phosphatidylinositol turnover and Ca^{++} influx whereas the V2 receptor is coupled to adenylate cyclase. Binding of an agonist to the V1a receptor activates phospholipase-A_2, -C, and -D, but not cAMP production. These effects are not sensitive to pertussis toxin, which indicates that the receptor is coupled to $G_{q/11}$ proteins. The three different receptor subtypes are expressed differentially and so far only the V1a-receptor mRNA and protein have been identified in the central nervous system, whereas the V1b receptor is the dominant receptor in the anterior pituitary corticotropes and the V2 receptor is, in the adult, only expressed in the kidney.

V1a-receptor mRNA is present in many regions of the brain including the frontal and piriform cortex, islands of Calleja, olfactory system, hippocampus, most septal nuclei, and throughout the midbrain, pons, and medulla. In the hypothalamus, highest levels of V1a-receptor mRNA are present in the suprachiasmatic, arcuate, stigmoid, dorsomedial, and periventricular nuclei and in the lateral hypothalamic area. Intermediate levels of the V1a-receptor mRNA are present in the medial preoptic nucleus, anterior and posterior hypothalamic areas, parvocellular portion of the paraventricular nucleus, and ventral premammillary and supramammillary nuclei. Interestingly, V1a receptor mRNA is absent or very low in the supraoptic and ventromedial nuclei.

The location of V1a-receptor mRNA parallels well the areas in the hypothalamus that exhibit specific binding of [^{3}H]-arginine-vasopressin, such as the suprachiasmatic, arcuate, and stigmoid nuclei. These findings suggest that vasopressin receptors are localized to the perikaryal and/or proximal dendritic plasma membrane and are, in most cases, not transported to distant sites on axons or dendrites. Moreover, the presence of V1a receptor mRNA in the paraventricular and suprachiasmatic nuclei supports the view that vasopressin-synthesizing neurons in these regions express the V1a receptor and may therefore be regulated in part by vasopressin peptide in an autocrine or paracrine fashion. In fact, dual *in situ* hybridization for a simultaneous detection of vasopressin and V1a-receptor mRNAs showed that both mRNAs are colocalized in approx 50% of the vasopressin neurons in the suprachiasmatic nucleus. In addition, about one-half of the neurons that contain the receptor mRNA are negative for vasopressin and they may contain other neurotransmitters.

One region in the hypothalamus where extensive vasopressin binding, but only very little receptor mRNA has been detected, is the ventromedial nucleus. It is possible that the few neurons that contain the receptor mRNA are sufficient to translate the mRNA into large amounts of vasopressin-receptor protein or that the receptors that were thought to be specific for vasopressin are indeed receptors for oxytocin, which shares extensive amino acid sequence homology with vasopressin.

The role of the V1a receptor in the hypothalamus is best understood in relation to the suprachiasmatic nucleus where the mRNA content follows a distinct circadian rhythm with highest levels during the dark period. This is the exact opposite of the vasopressin mRNA, which increases about threefold during the early light period. The circadian expression of the V1a receptor is in good agreement with previous electrophysiological results that have shown that vasopressin is more effective in stimulating single unit activity of neurons during the night when compared to the day. Interestingly, the circadian rhythm of V1a-receptor mRNA is not dependent upon the presence

of the vasopressin peptide because the neurons of the suprachiasmatic nucleus of the Brattleboro rat, which does not synthesize vasopressin, exhibits the same circadian rhythm of V1a-receptor expression.

2.6. Oxytocin

Oxytocin is best known for its endocrine actions on the mammary gland where it causes contraction of the myoepithelial cells resulting in milk ejection and on the smooth muscle cells of the uterus to induce contractions during parturition. In the brain, oxytocin affects many autonomic and somatic functions, reproductive and maternal behaviors, learning and memory, as well as certain neuroendocrine circuits. Oxytocin is structurally closely related to vasopressin and differs only in two amino acids in position 3 and 8. The similarity of the two peptides made it difficult to characterize separate receptor proteins and although in vitro autoradiography identified distinct anatomical binding patterns for the two peptides, controversies remain for several hypothalamic nuclei.

Recently, one oxytocin receptor has been cloned and sequenced. This protein shows the appropriate rank order of binding affinities: oxytocin > 1-deamino(7-sarosine)-oxytocin > arginine vasopressin after transfection of COS cells. The receptor is G-protein coupled and exhibits 35–50% amino acid homology with the different vasopressin receptors. Very little is known about the signal transduction pathways that are activated by oxytocin although some preliminary evidence suggests that Ca^{++} mobilization is involved in agonist action.

Oxytocin receptor mRNA is widely distributed throughout the central nervous system with high concentrations in the olfactory system, caudate-putamen, bed nucleus of the stria terminalis, amygdala, and hippocampus. Lower levels of oxytocin-receptor mRNA are found throughout the mesencephalon and brainstem, except for the dorsal motor nucleus vagus, which contains larger amounts of the receptor mRNA. No mRNA has been detected in the pituitary. In the hypothalamus, high levels of oxytocin-receptor mRNA are present in the anterior medial and magnocellular preoptic nuclei, the retrochiasmatic area, and the ventromedial nucleus, especially the ventrolateral subnucleus. Intermediate levels of receptor mRNA are detected in the dorsomedial portions of the paraventricular and supraoptic nuclei and the lateral hypothalamic and supramammillary area. No oxytocin-receptor mRNA is found in the suprachiasmatic and arcuate nuclei.

In general, extensive overlap exists in the location of oxytocin binding sites as determined by in vitro autoradiography and oxytocin-receptor mRNA suggesting that most receptor protein is present in the plasma membrane of the perikarya and/or proximal dendrites. Small discrepancies exist in the anterior medial preoptic nucleus, which does not exhibit specific binding of oxytocin yet has high amounts of receptor mRNA. One explanation is that the receptor protein is transported along the axons and/or into the distal dendrites. Conversely, the posterior pituitary exhibits large amounts of oxytocin binding sites yet no receptor mRNA. Since certain neurons in the paraventricular and supraoptic nuclei contain the receptor mRNA and they project to the neural lobe, it is possible that the receptor protein is transported from the hypothalamic perikarya to the axon terminals to regulate the release activity in an autocrine or paracrine fashion.

Several hypotheses exist about the role of oxytocin in the hypothalamus. One is related to the control of osmotic balance because oxytocin is released from the neural lobe in response to hypertonic saline administration. The anterior medial preoptic nucleus is thought to contain osmosensitive neurons and, because oxytocin receptor mRNA levels are high in this nucleus, the existence of an oxytocin-mediated feedback system has been proposed that includes communication between the anterior medial preoptic nucleus and the magnocellular neurons in the paraventricular and supraoptic nuclei.

In the ventromedial nucleus, oxytocin is involved in the facilitation of lordosis behavior and, because large amounts of oxytocin-receptor mRNA and binding sites are present in this structure, it appears that oxytocin exerts these effects by activating perikaryal receptors. Interestingly, estradiol causes a significant increase in oxytocin-receptor protein in the ventrolateral portion of the ventromedial nucleus a few hours after administration of the steroid suggesting that classical nuclear steroid receptors are involved. This is surprising because a typical estrogen-response element has not been found in the oxytocin-receptor gene, however, it is possible that widely spaced half-palindromic motifs in the promoter region can be activated by estrogen receptors.

3. STEROID HORMONE RECEPTORS

The brain is an important target for circulating steroid hormones that originate from peripheral tissues or local production. Steroids exert their effects

Table 2
Distribution of Steroid Hormone Receptors in the Hypothalamus

Hypothalamus	*ER-α*	*ER-β*	*AR*	*PR*	*GR*	*MR*	*VDR*
Preoptic area	++++	++++	+++	+++	+	+	+
Periventricular n.	+++	+++	+++	+++	+	+	+
Median preoptic n.	++	–	++	++	+++	++	+
Anterior hypothalamic n.	+	+	+++	++	++	++	–
Suprachiasmatic n.	–	+	++	++	+	–	+
Supraoptic n.	–	+++	+	–	–	–	+
Paraventricular n.	–	++	+	–	+++	+	+
Parvocellular region							
Magnocellular region	–	++++	+	–	–	–	+
Arcuate n.	+++	+	++	++	+++	+	+
Dorsomedial n.	+	+	+	–	+++	+	–
Ventromedial n.	++	–	+++	+	++	+++	+
Lateral hypothalamic area	+	–	+	+	+	–	+
Mammillary body	–	–	+++	+	++	+	–

on brain function by binding to classical nuclear receptors and, in addition they can also interact with certain subunits of glutamate and GABA receptors. Nuclear steroid receptors are hormone-activated transcription factors that influence gene expression in a wide variety of central nervous system neurons. They exert profound influences on the brain throughout the lifespan of an individual, beginning with early development and extending into adulthood. Steroid hormones affect the morphology of neurons, cell survival, neurochemical phenotype, and connectivity, which has important consequences for the functioning of the entire body.

This section summarizes the present state of the localization of the nuclear receptors for estrogen, androgen, progesterone, glucocorticoid, mineralcorticoid, and vitamin D in the central nervous system from a perspective of their potential influence on a wide variety of hormone-dependent functions. The patterns of distribution of the steroid hormone receptors in the hypothalamus are summarized in Table 2 and examples of autoradiograms after *in situ* hybridization localization of steroid hormone-receptor mRNAs or of steroid-receptor proteins after immunohistochemical stainings are provided in Fig. 1.

3.1. Gonadal Steroids

3.1.1. Estradiol

Early studies have shown that intravenously administered radioactive estradiol binds to nuclear receptors in the target neurons and these can be identified with high resolution autoradiography. Based on these studies, it is clear that estradiol target neurons are widely distributed throughout the central nervous system and that the brain regions associated with neuroendocrine functions, such as the preoptic area, are particularly rich in estradiol-receptive neurons. More recently, a variety of molecular, biological, and immunohistochemical techniques have detected estrogen receptors in anatomically distinct regions of the brain throughout its rostrocaudal extent confirming the results of in vivo autoradiography. To date, two types of estrogen receptor, the ER-α and ER-β forms, have been identified and the distribution of the receptor mRNAs has been determined with *in situ* hybridization histochemistry. Brain cells that exclusively express ER-β mRNA are located in the olfactory bulb, supraoptic, paraventricular, suprachiasmatic, tuberal hypothalamic nuclei, zona incerta, substantia nigra/ventral tegmental area, cerebellum, retina, and the pineal. In contrast, cells in the ventromedial nucleus and subfornical organ expressed only ER-α transcripts. Both types of receptors are expressed in the perikarya of other brain regions including the bed nucleus of the stria terminalis, medial and cortical amygdaloid nuclei, preoptic area, arcuate nucleus, median eminence, lateral habenula, periaquaductal gray, parabrachial nucleus, locus ceruleus, nucleus of the solitary tract, and spinal trigemical nucleus. In the cerebral cortex, ER-β mRNA containing neurons appear to be more abundant than the ER-α positive neurons.

In the hypothalamus, many aminergic and peptidergic neurons are targets for estradiol. Thus, it has been shown that oxytocin and vasopressin containing

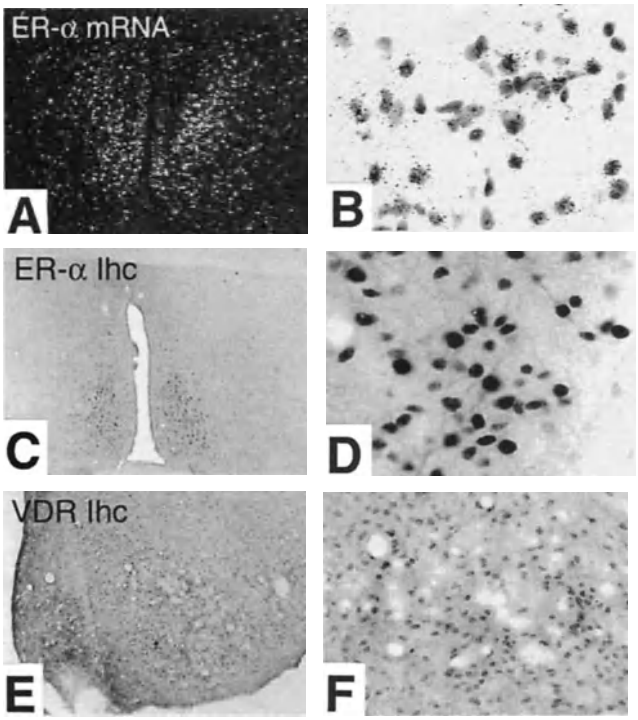

Fig. 1. Examples of localizations of estradiol receptor-α mRNA with *in situ* hybridization **(A,B),** estradiol receptor-α protein **(C,D)** and vitamin D receptor protein **(E,F)** with immunohistochemistry in the rat hypothalamus. **(A)** is a low-magnification dark-field micrograph of the preoptic nucleus showing accumulations of silver grains over neurons of the anteroventral preoptic subdivision. **(B)** is a high-magnification bright-field micrograph showing the location of estrogen-receptor-α individual neurons of the same region. **(C)** and **(D)** show the localization of the estrogen-α-receptor protein in the cell nuclei of neurons in the anteroventral preoptic subdivision with low **(C)** and high power **(D). (E)** and **(F)** show the location of vitamin D receptor protein with low **(E)** and high **(F)** magnification in the cell nuclei of certain neurons of the rat arcuate nucleus.

neurons also express estradiol receptors as do endorphin, somatostatin, or neuropeptide Y-synthesizing neurons, among others. The majority of estrogen-responsive neurons are found in areas known to be involved in some aspect of neuroendocrine functions. One important aspect of the function of estradiol in the brain is to provide the gonadal signal for positive and negative feedback regulation of GnRH neuronal activity, which controls the reproductive cycle in the female. Interestingly, the GnRH neurons do not express estradiol receptors, which implies that estradiol acts on afferent neurons that convey the hormone signal to the GnRH neurons. In addition, many target neurons are found in areas that are not typically considered to have a primary role in reproductive behavior or neuroendocrine function, such as the cortex or hippocampus. The function of estrogen in these areas are not clear at present, however, estradiol has been implicated to be important for diverse processes including learning, memory and neuroprotection, olfaction, vision, the central regulation of coordination and balance, muscle movement, and seasonal rhythms.

3.1.2. Androgens (Testosterone and Dihydrotestosterone)

The pattern of distribution of cells that accumulate radioactive androgens, contain the receptor protein or the receptor mRNA appears similar to that described of estrogen receptors in the brain. Androgen-responsive cells are located in the habenula, pineal, interpeduncular nucleus, area acusticolateralis, cerebellum, and motor nuclei of the medulla oblongata. The lateral septal nucleus, the medial and cortical nuclei of the amygdala, the amygdalo-hippocampal area, and the bed nucleus of the stria terminalis, Ammon's horn, also contain androgen responsive cells, as do both parts of the subiculum. Additional neurons that express androgen receptors are present in the vestibular nuclei, the cochlear nuclei, the medial geniculate nucleus, and the nucleus of the lateral leminiscus. The olfactory regions of the cortex and in both, the main and accessory olfactory bulbs, the nucleus of the solitary tract, the area postrema, and the subfornical organ also contain androgen-responsive cells.

In the hypothalamus, androgen-receptor-containing cells are concentrated in the medial aspects close to the third ventricle and they are less numerous in the lateral hypothalamic area. Specifically, androgen-responsive cells are present in the suprachiasmatic, supraoptic, and periventricular nuclei, as well as in the parvocellular and magnocellular regions of the paraventricular nucleus. Further caudally, androgen-receptor-containing neurons are found in the dorsomedial, ventromedial, and arcuate nuclei, retrochiasmatic area, median eminence, and the mammillary body. The highest concentration of cells that are responsive to androgen are in the preoptic-hypothalamus.

Androgens play a critical role in the regulation of neuroendocrine functions and reproductive behaviors and, based on their distribution in the hypothalamus, they are in a position to coordinate the neuropeptide and neurotransmitter systems that govern such functions and behaviors. Androgen may also alter the

central processing of vestibular and auditory information, as well as visceral sensory information.

3.1.3. Progesterone

Progesterone receptor-containing neurons are scattered throughout many brain regions including the central, medial, and cortical nuclei of the amygdala, the septum, subfornical organ, the lateral geniculate nucleus, the parietal and entorhinal cortex, and the central gray of the midbrain. In the hypothalamus, progesterone-sensitive neurons are concentrated in various preoptic nuclei including the medial preoptic, lateral preoptic, and periventricular preoptic nuclei. Further caudally, progesterone-receptive neurons are found in the suprachiasmatic, periventricular, arcuate, ventromedial, dorsomedial nuclei, and in the ventral and lateral mammillary region and lateral hypothalamus. The results of in vivo uptake of tritiated progestin correspond well with the results obtained with *in situ* hybridization for the progesterone receptor-transcript mRNA and with immunohistochemistry using specific antibodies to the receptor protein.

The brain regions that contain receptors for progesterone are known to regulate sexual behavior, the ovarian cycle, and gonadotropin release. Depending upon the timing and dose of progesterone administration, this steroid hormone can either inhibit or facilitate the estrogen-induced LH surge. Progesterone is also associated with inhibition of sexual receptivity and/or facilitation lordosis behavior. Little is known about the role of PR in other brain regions.

3.2. Adrenal Steroids

3.2.1. Glucocorticoid

In vivo autoradiography has shown that glucocorticoid-concentrating neurons are widely distributed throughout the brain with highest levels of receptors in the lateral septum, hippocampus, cortical, and thalamic regions. In the hippocampus, receptors are seen in the pyramidal neurons of areas CA1 and CA2 and in the granular cells of the dentate gyrus. Moderate labeling of a large number of neurons is observed in layers II, III, and VI of the neocortex and in many thalamic nuclei, especially the anterior and ventral nuclear groups, as well as several midline nuclei. Within the cerebellar cortex, strong labeling is seen in the granular layer. In the lower brain stem, strong labeling is found throughout the locus ceruleus and in the mesencephalic raphe nuclei rich in noradrenergic and serotoninergic neurons, respectively.

In the hypothalamus, moderate numbers of glucocorticoid receptor-containing neurons are present in the periventricular preoptic and suprachiasmatic nuclei, whereas moderate to strong labeling is seen in the median preoptic nucleus, anterior hypothalamus, and the mammilary body. The most intense labeling is found in the parvocellular neurons of the paraventricular hypothalamic nucleus and in the neurons of the arcuate and dorsomedial nuclei. No labeled cells are present in the supraoptic nucleus. These results are in good agreement with recent *in situ* hybridization or immunohistochemical studies.

3.2.2. Mineralocorticoid

In vivo uptake studies of [^{3}H]-mineralocorticoids have shown that the highest density of positive neurons exists in the hippocampus and lower density in the hypothalamus. Other brain regions that express the mineralocorticoid receptor include the septum, allocortical regions, and brain stem reticular formation and motor nuclei of cranial nerves and the meninges. The distribution of labeled cells in the hypothalamus region mirrors that of the glucocorticoid receptor distribution with the difference being that fewer labeled cells are present in a specific nuclei. Thus, mineralocorticoid receptors are present in certain neurons of the preoptic area, including the median preoptic nucleus, the periventricular, arcuate, and dorsomedial nuclei, as well as the anterior hypothalamic nucleus and the mammillary body. The highest number of labeled neurons is seen in the ventromedial nucleus. One main difference in the localization of mineralocorticoid- and glucocorticoid-receptor-containing neurons is seen in the paraventricular nucleus of the hypothalamus where only a few neurons contain the mineralocorticoid receptor. These data suggest that modulation of the hypothalamic-pituitary-adrenal stress axis in the paraventricular nucleus involves only to a minor extent the mineralocorticoid receptors.

Adrenocorticosteroid receptors are intimately linked to stress and adaptation, a system of homeostasis. Response to stress occurs following a wide variety of physiological and psychological stimuli that influence the activity of the neurons with receptors for the adrenal steroids. Glucocorticoid receptors are viewed as modulators of the neuroendocrine stress response in that they translate the stress stimuli into specific gene transcription products targeted to enhance the capacity of an individual to adapt to the changing environment. The existence of dual receptor systems for the adrenal steroids suggests a tight coordination of responses to stress stimuli. The glucocorticoid receptor system mainly functions as a negative-

feedback regulator of the hypothalamic-pituitary-adrenal axis whereas, the mineralocorticoid receptors are thought to play an additional important role in the central regulation of body fluid and electrolyte balance by working together with the angiotensin neural network. Elevation of blood pressure appears to be mediated by actions of mineralocorticoids on responsive cells in the brain, as well as in the kidney and vascular smooth muscles.

3.3. Vitamin D

Although 1,25-dihydroxy vitamin D_3 has been associated for a long time with the maintenance of calcium homeostasis and bone development, relatively recent data based on in vivo autoradiography have shown that this steroid hormone acts on many different target tissues in the body, including the brain. In the central nervous system, major target sites for 1,25-dihydroxyvitamin D_3 include the central nucleus of the amygdala, the bed nucleus of the stria terminalis, the reticular nucleus of the thalamus, ventral hippocampus, dorsal raphe, parabrachial, ambiguous nuclei, and the nucleus tractus solitarius, motor nucleus of the cranial nerves, cerebellum, and substantia geletinosa. In the hypothalamus, vitamin D receptors are present in the periventricular nucleus, the preoptic area, the parvocellular region of the paraventricular nucleus, arcuate, supraoptic, and suprachiasmatic nuclei. These data were recently confirmed with *in situ* hybridization to identify the receptor mRNA and with immunohistochemistry to identify the receptor protein.

Based upon the distribution of vitamin D receptors throughout the brain several functions can be suggested. Many target cells are positioned in sensory pathways, the motor system, and the neuroendocrine-autonomic system. Thus, a regulation of the autonomic system can be achieved through target neurons in the dorsal raphe nuclei, the parabrachial, and ambiguous nuclei, as well as the nucleus tractus solitarius. Modulation of sensory pathways can be processed in target neurons in substantia geletinosa, the parabrachial nucleus, and the reticular nucleus of the thalamus. The presence of vitamin D receptors in the choroid plexus, preoptic, and hypothalamic regions suggests a major role of vitamin D receptors in regulation of neuroendocrine functions including sexual and reproductive functions, hypothalamic-pituitary-adrenal stress axis, hypothalamic-pituitary-thyroid axis, and central body fluid regulation.

SUMMARY

Peptide and steroid hormones exert profound effects in the central nervous system ranging from regulation of adaptive, behavioral, developmental, aging, neuroplastic and neurodegenerative to neuroendocrine processes, among others. Defining the roles and regulation of peptide and steroid hormone action in the brain is important for our understanding of many clinical conditions that are caused by a malfunction of the neuroendocrine brain.

SELECTED READINGS

Aronsson M, Fuxe K, Dong Y, Agnati LF, Okret S, Gustafsson JA. Localization of glucocorticoid receptor mRNA in the male rat brain by in situ hybridization. Proc Natl Acad Sci USA 1988; 85:9331–9335.

Beaudet A, Greenspun D, Raelson J, Tannenbaum GS. Patterns of expression of SSTR1 and SSTR2 somatostatin receptor subtypes in the hypothalamus of the adult rat: relationship to neuroendocrine function. Neuroscience 1995; 65:551–561.

Calza L, Giaardino L, Ceccatelli S, Zanni M, Elde R, Hokfelt T. Distribution of thyroptropin-releasing hormone receptor messenger RNA in the rat brain: an in situ hybridization study. Neuroscience 1992; 51:891–909.

Chalmers DT, Lovenberg TW, De Souza EB. Localization of novel corticotropin-releasing factor receptor (CRF_2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF_1 receptor mRNA expression. J Neurosci 1995; 15:6340–6350.

Jennes L, Conn PM. Gonadotropin releasing hormone and its receptors in rat brain. Front Neuroendocrinol 1994; 15:51–77.

Ostrowski NL, Lolait SJ, Young WS. Cellular localization of vasopressin V1a receptor messenger ribonucleic acid in adult male rat brain, pineal, and brain vasculature. Endocrinology 1994; 135:1511–1528.

Shughrue PJ, Lane MV, Merchenthaler I. Comparative distribution of estrogen receptor-alpha and -beta mRNA in the rat central nervous system. J Comp Neurol 1997; 388:507–525.

Simmerly RB, Chang C, Muramatsu M, Swanson LW. Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. J Comp Neurol 1990; 294:76–95.

Stumpf WE, Grant LD, eds. Anatomical Neuroendocrinology. Basel: Karger, Basel, 1975.

Stumpf WE, O'Brien LP. $1,25(OH)_2$ Vitamin D_3 sites of action in the brain: an autoradiographic study. Histochemistry 1987; 87:393–406.

Thoss VS, Perez J, Duc D, Hoyer D. Embryonic and postnatal mRNA distribution of five somatostatin receptor subtypes in the rat brain. Neuropharmacology 1995; 34:1673–1688.

Yoshimura R, Kiyama H, Kimura T, Araki T, Maeno H, Tanizawa O, Tohyama M. Localization of oxytocin receptor messenger ribonucleic acid in the rat brain. Endocrinology 1993; 133:1239–1246.

PART II

Neuroendocrine Regulation of Reproduction, Parturition, Lactation, Growth, Development, Metabolism, and Fluid Balance

7 Neuroendocrine Regulation of Pituitary Function

General Principles

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CONTENTS

1. INTRODUCTION

The human pituitary gland weighs no more than 1 g, but nonetheless controls all the major endocrine systems and is indispensable for life. Located at the base of the brain, and closely surrounded by protective dense bone and fibrous membranes, the gland is comprised of the neurohypophysis or neural lobe and the adenohypophysis. Embryologically derived from a neural downgrowth, the neural lobe is a bag of axons which project from nerve cells in the *hypothalamus* and terminate on capillaries of the inferior hypophysial artery. This is the site at which the nonapeptides, vasopressin, and oxytocin are released into the systemic circulation. Synthesized in the supraoptic and paraventricular nuclei, vasopressin, also termed the antidiuretic hormone, controls the volume of body water whereas oxytocin is concerned mainly with stimulating milk ejection during lactation, and contraction of the uterus (womb) during parturition.

The adenohypophysis is derived from an outgrowth of the roof of the mouth and, in subhuman species, is divided into the pars distalis and the pars intermedia. The pars distalis is more commonly called the *anterior lobe* and the pars intermedia and the neural lobe or pars nervosa together form the *posterior lobe.* There is no distinct pars intermedia in the human. The anterior pituitary gland controls the adrenal and thyroid glands, the gonads, body growth, and development of the breast and lactation by way of secreting adrenocorticotropin (ACTH), thyrotropin (TSH), the gonadotropins, growth hormone (GH) and prolactin, respectively. In addition to being growth promoting ("trophic") and stimulating immediate hormonal or cellular events ("tropic"), these hormones all affect metabolism. This is especially the case for ACTH, GH, and TSH. The loss of ACTH with the consequent loss of adrenocortical hormone secretion, and perhaps

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to a lesser degree GH and TSH, makes removal of the gland ("hypophysectomy") so lethal.

Anterior pituitary hormone secretion is under the control of the brain modulated by the feedback of hormones secreted by the pituitary target organs, i.e., the thyroid and adrenal glands and the gonads, and as shown recently, fat tissue by way of leptin. Neural control of the anterior pituitary hormones is mediated by the hypothalamic-pituitary regulatory neurohormones (formerly termed "factors") which are released into the exquisite *hypophysial portal vessels* and transported by them to the anterior lobe, where they either stimulate or inhibit the release of the anterior pituitary hormones. The hypothalamic-pituitary regulatory factors (*see* Chapter 3) are termed neurohormones because instead of being released at synapses between nerve cells, they are released and transported to their target cells in the blood stream. Neural control of the pars intermedia, which secretes the melanocortin, melanocyte stimulating hormone (MSH), is mediated mainly by nerve projections from neurons in the hypothalamus, however, portal vessel blood does reach this part of the gland and so neurohormones that control MSH release may exist.

The hypothalamic-pituitary system is the interface between the central nervous and the endocrine systems by which external factors, such as day-length and stress, and internal factors, such as emotion, trigger endocrine responses. It was therefore termed the *neuroendocrine system,* and the pituitary gland is said to be under *neuroendocrine control.* In addition to the hypothalamic-pituitary system, the circumventricular organs (*see* Section 2), of which the pineal gland and subfornical organ are perhaps the most important, also satisfy the criteria of neuroendocrine systems. Furthermore, since the term "neuroendocrine" was coined, it has also been applied to interactions between nerve and endocrine cells in the periphery, and especially the viscera, of which the gastrointestinal system and its appendages are the most prominent.

This chapter focuses on the principles of neuroendocrine control of the pituitary gland and illustrates how the hypothalamic-pituitary system was used to demonstrate that peptides satisfy the criteria of neurotransmitters or neurohormones, played a significant role in the development of our understanding of gene transcription, translation and posttranslational processing, and could be used as a "window" to study brain function in the conscious human ("neuroendocrine window of the brain"). Also outlined are the effects of pituitary target hormones on the brain-pituitary stem: in particular, the way that hormones secreted by the anterior pituitary target glands play an important role in brain differentiation and plasticity, affect central neurotransmission and thereby mood, mental state and memory, and feedback on the brain-pituitary system as the afferent limb of a *homeostatic* regulatory system that ensures that the output of pituitary hormones is maintained at a preset and functionally optimal level.

2. HYPOTHALAMIC-PITUITARY AXIS

2.1. Brief History

The central importance of the anterior pituitary gland as "conductor of the endocrine orchestra" was not understood until the early 1930s when P.E. Smith published his parapharyngeal method for removing the gland ("hypophysectomy"). The effects of hypophysectomy proved to be so dramatic that for a short period most scientists in the field, including the distinguished neurosurgeon, H. Cushing, thought that the pituitary gland was autonomous. However, around the same time, W. Rowan, working in Alberta, Canada, on the annual migration of birds showed that day-length had a potent effect on the growth of the gonads. Rowan's experiments together with those on seasonal breeding in animals, H. Selye's observations on the effect of stressful stimuli on endocrine organs and especially the adrenal gland and the effects of brain lesions on pituitary hormone secretion led to the concept that the anterior pituitary gland must be under central nervous control, a view that Cushing soon adopted. The observational and experimental evidence which supported this concept was summarized by F.H.A. Marshall in his 1936 Croonian lecture.

It had long been known that the pituitary gland and brain were connected by the pituitary stalk, but several lines of evidence suggested that neural control of the anterior pituitary gland was mediated, not by nerve fibers in the pituitary stalk, but by chemical substances released into the hypophysial portal vessels. These vessels, first described by G.T. Popa and U. Fielding in 1930, surround the pituitary stalk linking a primary plexus of capillaries at the base of the hypothalamus with a second capillary plexus in the anterior pituitary gland. Throughout the 1930s and 1940s, a debate raged about the direction of blood flow in the portal vessels. Based on histological evidence, this debate could have been avoided had someone read the 1935 report by B. Houssay et al. that in the living toad, blood flowed from the hypothalamus down to the pituitary gland. But Houssay's paper was published in French.

The *neurohumoral hypothesis* of the control of the anterior pituitary gland was first formally advanced by H.B. Friedgood in 1936 and J.C. Hinsey in 1937. However, it was the elegant pituitary graft experiments of G.W. Harris and D. Jacobsohn in 1952 that showed beyond doubt that the anterior pituitary gland was controlled by substances released at nerve terminals in the median eminence at the base of the hypothalamus and transported to the pituitary gland by the hypophysial portal vessels. The characterization of the first three of these substances, thyrotropin-releasing factor, luteinizing hormone-releasing factor, and somatostatin, was to take a further 18 to 21 years of hard work in the laboratories of R. Guillemin and A. Schally for which they were awarded the 1977 Nobel Prize for Physiology and Medicine. Soon after its characterization as a decapeptide in 1971, Fink and Jamieson measured luteinizing hormone-releasing factor (LRF: later abbreviated to LHRH or GnRH) by radioimmunoassay in hypophysial portal blood of the anesthetized rat and showed that its release could be increased fivefold by a small electrical stimulus applied to the medial preoptic area of the hypothalamus. The neurohumoral hypothesis of neural control of the anterior pituitary control had been proved.

2.2. General, Anatomy, and Development

The pituitary gland is linked to the hypothalamus at the base of the brain (Fig. 1). The hypothalamus is comprised of a medial part adjacent to the third cerebral ventricle and in which are located the major hypothalamic nuclei (*see* Chapter 2) and a lateral part comprised mainly of a large cable of nerve fibers that carries reciprocal fiber tracts between midbrain and forebrain, the medial forebrain bundle (Fig. 2), in which are embedded a few aggregations of nerve cell bodies. Axons from nerve cell bodies located in the hypothalamic nuclei project to the median eminence where they either terminate on the loops of primary capillaries of the hypophysial portal vessels in the external layer of the median eminence or form a cable that passes through the internal layer of the median eminence to form the bulk of the pituitary stalk and then the neural lobe (Fig. 3). The *median eminence,* so called because it protrudes as a small dome in the midline from the base of the hypothalamus, forms the floor of the third ventricle and is delineated by the optic chiasm in front, the mammillary bodies behind and a depression (hypothalamic sulcus) on either side (*see* Chapter 2). Arising from the median eminence is the neural stalk which links the pituitary gland to the brain.

The pituitary gland is located in a fossa in the basisphenoid bone at the base of the skull, the "sella turcica," so called because its shape resembles a Turkish saddle. The close proximity of the hypothalamus and pituitary gland to the optic chiasm means that tumors either in the hypothalamus or the pituitary gland may press on, or more rarely invade, the optic chiasm or tracts, and thereby lead to visual defects. It is for this reason that a thorough endocrine examination of the patient must include ophthalmoscopic examination of the retina, and perimetry of the visual fields to ascertain whether there are any defects in the visual fields, a sign of pressure on or damage to the optic chiasm or tracts.

The hypothalamic-pituitary axis is divided functionally into two systems. The hypothalamus, hypophysial portal vessels, and adenohypophysis constitute the *hypothalamo-adenohypophysial* axis. The hypothalamus, neural stalk, and neural lobe constitute the *hypothalamo-neurohypophysial* axis. The neural stalk is made up of numerous nerve fibers that project mainly from the paraventricular and supraoptic nuclei (PVN and SON) of the hypothalamus to terminate on a capillary bed derived from the inferior hypophysial artery and located in the neural lobe of the pituitary gland. Nerve fibers in the neural lobe (or pars nervosa) are surrounded by pituicytes which are equivalent to glial cells. Also present in the neural stalk are nerve fibers of other types of chemical neurotransmitter, such as the endogenous opioids and dopamine, which are present in nerve fibers that project from the arcuate nucleus and innervate the pars intermedia. Because the stalk and median eminence are continuous, these dopaminergic neurons are a continuation of a dense palisade of dopaminergic fibers that also terminate on the primary plexus of the hypophysial portal vessels enmeshed with nerve fibers that contain other neurohormones. The pituitary stalk and median eminence are covered by a single layer of cells termed the pars tuberalis which is continuous with the pars distalis.

The adenohypophysis develops from an outgrowth of the ectodermal placode which forms the roof of the embryonic mouth (or "stomadeum"). This ectodermal outgrowth forms Rathke's pouch and meets the neurohypophysis that grows down from the floor of the embryonic third ventricle. Rathke's pouch closes and separates from the roof of the mouth. The caudal (rear) part of the pouch remains thin to form the pars intermedia that becomes tightly juxtaposed to the rostral surface of the neurohypophysis (Fig. 4). The rostral part of the pouch develops into the pars distalis (Fig. 5). Vascularization of the median emi-

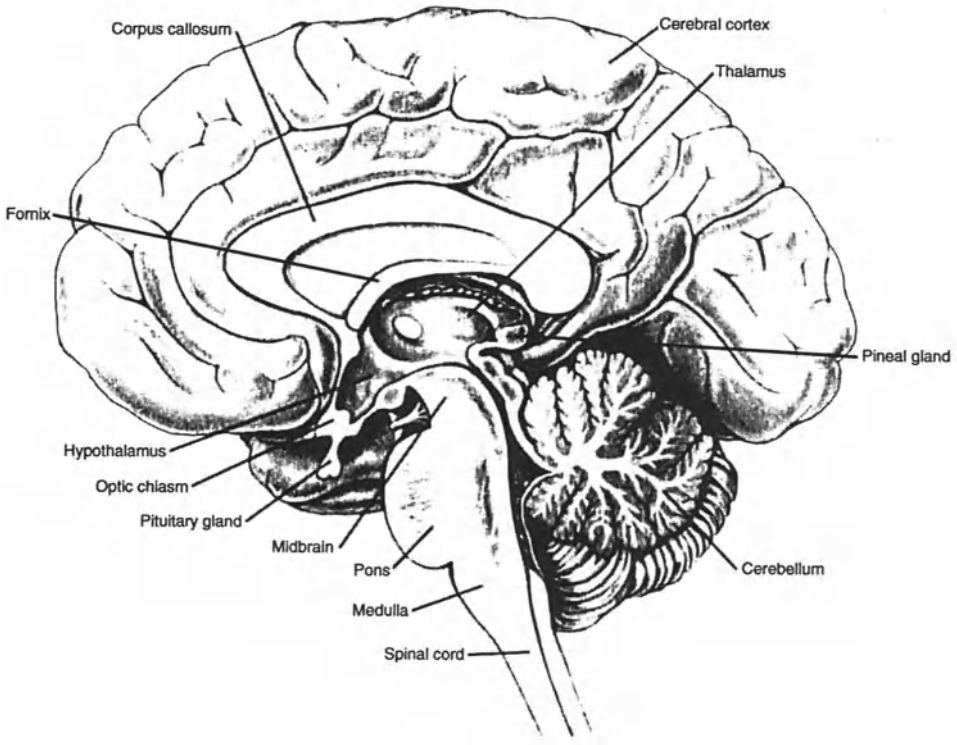

Fig. 1. A midsagittal section of the human brain showing the inside surface. Note the pituitary gland is attached by way of the pituitary stalk to the floor of the hypothalamus. The hypothalamus and the thalamus, which lies above it, form the wall of the third cerebral ventricle at the posterior end of which is the pineal gland.

nence and the pituitary gland begins at about day 15 of embryonic (E 15) life in the rat, and the hypophysial portal vessels (*see* Section 3) become defined by E 18. Nerve terminals in the median eminence with granular vesicles (presumably neurohormones) are first evident on E 16 and the first secretory granules appear in pars distalis cells on E 17. This sequence of embryonic development suggests that the appearance of secretory cells in the pars distalis may depend in part upon the differentiation of nerve terminals in the median eminence and the anlage of the hypophysial portal vessels.

3. NEUROHEMAL JUNCTIONS AND CIRCUMVENTRICULAR ORGANS

Neurohemal junctions are the fundamental functional modules of the major central neuroendocrine system, the median eminence. They are comprised of nerve terminals and capillaries that are closely juxtaposed and thereby facilitate the release of chemical messengers from nerve terminals into the blood stream and vice versa (Fig. 6). Neurohemal junctions are also the fundamental units of the neurohypophysis, and of the circumventricular organs such as the organum vasculosum of the lamina terminalis, subfornical organ, and the pineal gland that are located at various sites around the third cerebral ventricle and the area postrema located in the fourth cerebral ventricle. All the circumventricular organs are characterized by the fact that their vessels are fenestrated (Fig. 6) and that the blood brain barrier is inoperative at these sites. The neurohemal junctions in the median eminence, neurohypophysis, and pineal gland facilitate the transport of neurohormones from the nerve terminals or nerve cell derivatives (pineal) into the blood stream, whereas at the other circumventricular organs, the neurohemal junctions facilitate the transport of neurohormomes from the blood to nerve cells. The latter mechanism has been implicated in the "cross-talk" between peripheral organs and the brain so that, for example, the peptide angiotensin increases blood pressure by activating neurons of the subfornical organ that have a high density of angiotensin receptors. In addition to their importance as sites for the transfer of chemical messengers from nerve terminals into blood vessels or vice versa, the neurohemal junctions of circumventricular organs are also sites at

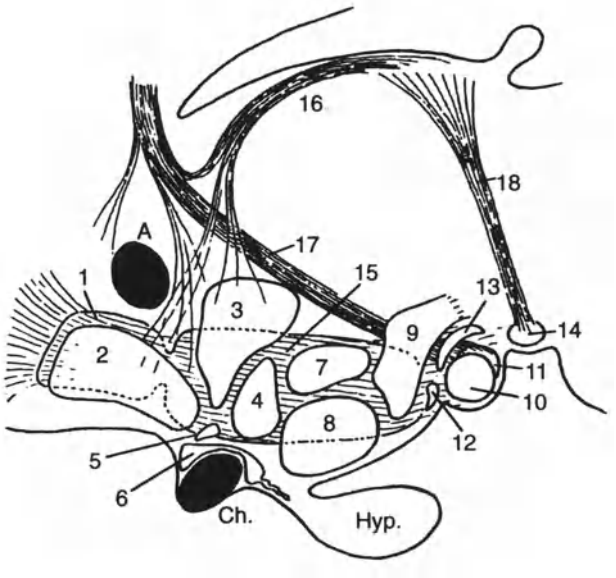

Fig. 2. Diagram showing the relative positions in a sagittal plane of the hypothalamic nuclei in a typical mammalian brain, and their relation to the fornix, stria habenularis, and fasciculus retroflexus. *A.* Anterior commissure. *Ch.* Optic chiasma. *Hyp.* Hypophysis (pituitary gland). 1. Lateral preoptic nucleus (permeated by the medial forebrain bundle). 2. Medial preoptic nucleus. 3. Paraventricular nucleus. 4. Anterior hypothalamic area. 5. Suprachiasmatic nucleus. 6. Supraoptic nucleus. 7. Dorso-medial hypothalamic nucleus. 8. Ventro-medial hypothalamic nucleus. 9. Posterior hypothalamic nucleus. 10. Medial mamillary nucleus. 11. Lateral mamillary nucleus. 12. Premamillary nucleus. 13. Supramamillary nucleus. 14. Interpeduncular nucleus (a mesencephalic element in which the fasciculus retroflexus terminates). 15. Lateral hypothalamic nucleus (permeated by the medial forebrain bundle). 16. Stria habenularis. 17. Fornix. 18. Fasciculus retroflexus of Meynert (habenulo-peduncular tract) (after Le Gros Clark 1938 In: *The Hypothalamus,* Le Gros Clark et al., eds. Edinburgh, Oliver and Boyd).

which drugs or toxins that are normally excluded by the blood brain barrier can penetrate the brain and affect brain function.

4. THE HYPOTHALAMO-ADENOHYPOPHYSIAL SYSTEM

4.1. Neurohormonal Control of Anterior Pituitary Hormone Secretion

The transmission of signals between the brain and anterior pituitary gland is mediated by chemical messengers (neurohormones; *see* Chapter 3) that are transported by the hypophysial portal vessels from the hypothalamus to the anterior pituitary gland (Figs. 3–5) where they either stimulate or inhibit the release

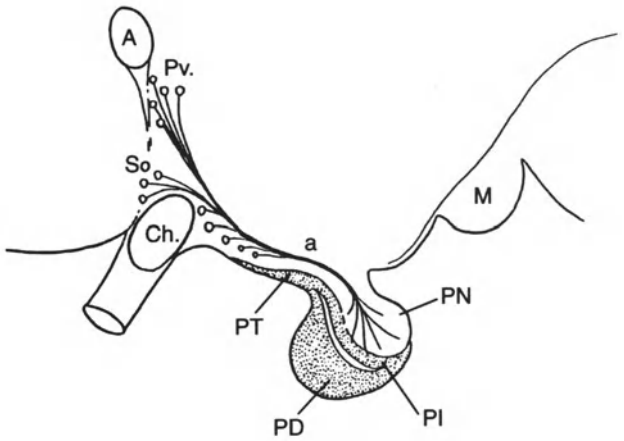

Fig. 3. Schematic section of the mammalian hypothalamus and pituitary gland showing the neurohypophysial tract (labeled a) comprised mainly of fibers derived from the paraventricular (Pv) and supraoptic (So) nuclei. *A.* Anterior commissure. *Ch.* Optic chiasma. *M.* Mamillary bodies. *PT.* Pars tuberalus. *PD.* Pars distalis. *PI.* Pars intermedia. *PN.* Pars nervosa. (Modified after Le Gros Clark 1938.)

of anterior pituitary hormones. Synthesized in nerve cells of the hypothalamic nuclei, the neurohormones are released from nerve terminals into the plexus of primary capillaries of the hypophysial portal vessel system. These capillaries are derived from the superior hypophysial arteries and coalesce to form the hypophysial portal veins that run on the surface or through the pituitary stalk to the anterior pituitary gland where they form a secondary plexus of vessels called the pituitary sinusoids. The vessels on the surface of the stalk are the long portal vessels, whereas those within the substance of the stalk are the short portal vessels. The latter have been implicated in so-called short-loop feedback, a controversial issue discussed under "Feedback."

4.2. Hypophysial Portal Vessels

The portal vessels are so-called because they transport the chemical messengers from one capillary bed (primary capillaries) to a second capillary bed before entering the general circulation. In principle, this is identical to the hepatic portal system which transports substances from the primary bed of capillaries in the intestine and its appendages (e.g., pancreas) to a second bed of capillaries or sinusoids in the liver. Both the primary and secondary (sinusoids) plexus of capillaries are fenestrated (Fig. 6) that presumably facilitates the transport of substances across the capillary wall. The hormones released from anterior pituitary

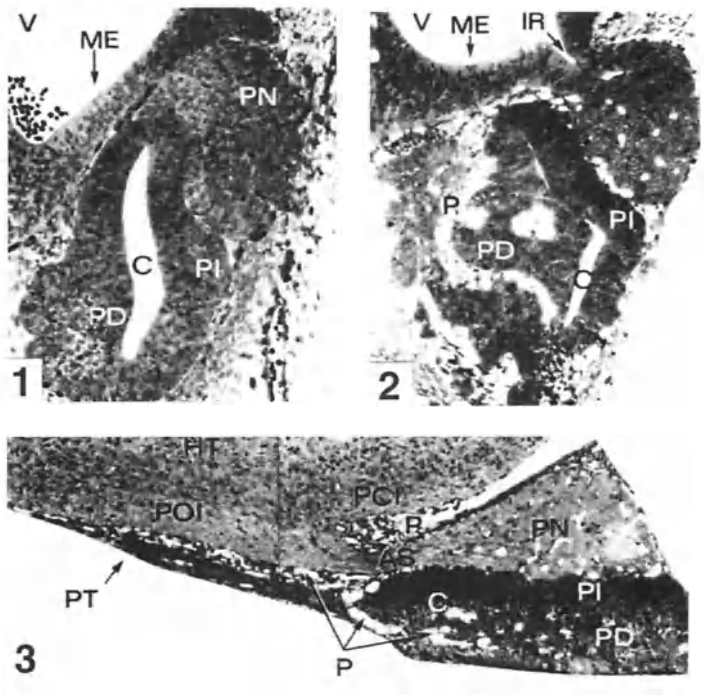

Fig. 4. Development of the pituitary gland in the rat. Photomicrographs of midline sagittal sections through the hypothalamic-pituitary complex of rats at embryonic days 15 (Fig. 1), 17 (Fig. 2) and 20 (Fig. 3). Figure 1 shows the pituitary anlage shortly after closure of Rathke's pouch which migrates dorsally to meet the neurohypophysial downgrowth from the floor of the hypothalamus. Rotation of the pituitary gland caudally through 135° with respect to the base of the diencephalon (hypothalamus) is seen, as is the invasion of the pars distalis (*PD*) by the leash of portal vessels (*P*) at E17. *AS* anatomical stem; *C* hypophysial cleft; *HT* hypothalamus; *IR* infundibular recess; *ME* median eminence; *PCI* pars caudalis infundibuli; *PI* pars intermedia; *PN* pars nervosa; *POI* pars oralis infundibuli; *V* third ventricle. Iμ Araldite sections, toluidine blue stain. Figs. 1, 2 × 120. Fig. 3 × 80 (Reproduced with permission from Fink and Smith, Z. Zellforsch 1971; 119:208–226.)

cells are transported by pituitary veins into the systemic circulation by which they are transported to their major target organs, the gonads and the adrenal and thyroid glands.

4.3. Hypothalamic Neurohormones

4.3.1. Outline

Most of the neurohormones that mediate neural control of anterior pituitary hormone secretion are peptides (*see* Chapter 3) that are synthesized in discrete hypothalamic nuclei. The neurohormone-secreting neurons are "the final common pathway" neurons for neural control of the anterior pituitary gland, a term borrowed by G.W. Harris from Sherrington's description of the alpha motor neurons of the spinal cord which innervate and control the contraction of skeletal muscles. Like the alpha motor neurons, the hypothalamic neurons are controlled by inputs to the hypothalamus from the brain stem and midbrain as well as from higher brain centers. The hypothalamic neuroendocrine neurons are, therefore, connected with many other regions of the nervous system, and in particular, the components of the limbic system that is involved in several important higher brain functions including emotion, olfaction, and memory.

The neural control of all established anterior pituitary hormones is mediated by at least one or more neurohormones. In some cases, two neurohormones may act synergistically, as is the case for adrenocorticotropin (ACTH), the release of which is stimulated by both the 41 amino acid residue peptide, corticotropin-releasing factor-41 (CRF-41), and the nonapeptide, arginine vasopressin (AVP). The control of ACTH secretion is further complicated by the fact that urocortin, a new member of the CRF-41 family of peptides, acts on CRF type 2 receptors to inhibit AVP release, and studies on hypophysial portal blood and immunoneutralization suggest that ACTH secretion may be inhibited by atrial natriuretic peptide (ANP). Urocortin, through its action on the CRF type 2 receptor may also be involved in the regulation of blood pressure.

In other cases the neurohormones act antagonistically, as is the case for growth hormone (GH), the release of which is stimulated by the 44 amino acid residue peptide, GH-releasing hormone (GHRH-44), and inhibited by the 14 or 28 residue peptide, somatostatin-14 or -28 (*see* Chapter 3). The neural regulation of pituitary GH secretion is even more complex than originally thought in that Bowers et al. discovered in 1981 that a hexapeptide, referred to as GH-releasing peptide-6 (GHRP-6) is a potent GH-releasing factor. Subsequent studies suggested that GHRP-6 or a related compound may be endogenous to the brain and controls GH release by way of actions mediated by GHRH-44.

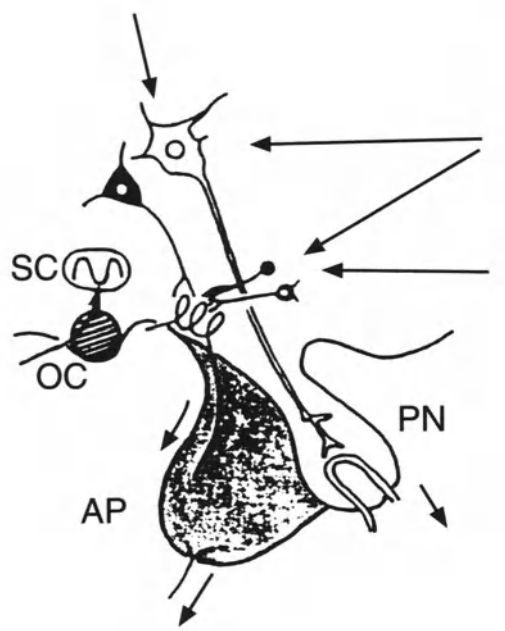

Fig. 5. A schematic diagram of the hypothalamic-pituitary system showing the magnocellular (white) projections directly to the systemic capillaries of the pars nervosa (PN) and the parvocellular (black) projections to the primary plexus of the hypophysial portal vessels which convey neurohormones to the pars distalis of the anterior pituitary gland (AP). Dorsal to the optic chiasm (OC) are the suprachiasmatic nuclei (SC) that receive direct projections from the retina and play a key role in the control of circadian rhythms (indicated by the sinusoidal curve). The activity of the intrinsic neurons of the hypothalamus is greatly influenced by projections (arrows) from numerous areas of the forebrain, midbrain and hindbrain, particularly the limbic system, as well as by hormones, mainly estrogen, and progesterone in the case of the hypothalamic-pituitary-gonadal system.

Prolactin seems to be the only anterior pituitary hormone that is predominantly under inhibitory control of the brain. The evidence for this came first from the studies of Everett and Nikitowitch-Winer who showed that prolactotropes were the only cell type that did not undergo atrophy in pituitary grafts under the kidney capsule where they are far removed from central neural control. This histological observation was confirmed by the finding that prolactin concentrations in plasma are increased in animals bearing pituitary grafts under the kidney capsule. Dopamine has long been thought to be the prolactin inhibitory factor (PIF). Although direct proof for this is still not available, dopamine agonists such as bromocriptine are highly effective in treating hyperprolactinemia, a relatively common cause of infertility in women. Hyperprolactinemia is frequently due to benign tumors of the anterior pituitary gland—and these too can often be controlled or eradicated by treatment with dopamine agonists.

Because prolactin secretion is stimulated by stress, it is also conceivable that hyperprolactinemia may, in some women, be due to stress. In the human, the prolactin response to stress is as sensitive as that of ACTH and adrenal glucocorticoids. The significance of this is illustrated by studies in groups of monkeys (e.g., rhesus macaques) where the dominant male or female have high testosterone or estrogen levels and low to absent prolactin levels—the converse is the case for the subordinate animals—high prolactin and low sex steroid levels. This can easily be manipulated by switching the animals around into different groups—if a subordinate is placed in a social setting in which he/she becomes dominant, prolactin concentrations in plasma fall and sex steroid concentrations increase. This suggests that the hyperprolactinemia in the subordinate animal is stress-induced. The functional consequence of this relationship between prolactin, sex steroids and social environment is that high prolactin levels are associated with low to absent fertility—the neurochemical details are not understood, but high prolactin levels in primates (except for the marmoset) are nearly always associated with low gonadotropin [LH and follicle stimulating hormone (FSH)] levels.

In addition to a PIF, a search for a prolactin releasing factor (PRF) has continued for many years. Potential candidates have included thyrotropin-releasing hormone, vasoactive intestinal peptide, GnRH-associated peptide (GAP) and, recently, two novel, closely related hypothalamic peptides (termed PrRP31 and PrRP20, respectively) discovered in 1998 by Hinuma et al. when searching for a ligand for an orphan receptor (hGR3). However, although all of these compounds are capable of stimulating prolactin release from pituitary cells in vitro, and in some cases under pharmacological conditions in vivo, robust proof that any of these or other compounds are the physiological PRF remains to be established.

The neural control of the gonadotropins, LH and FSH, is mediated by one and the same decapeptide, GnRH, and is discussed in greater detail below.

4.3.2. Criteria for Neurohormones and Neurotransmitters

Werman, in 1972, summarized the criteria for a candidate compound to be classified as a neurotransmitter (or neurohormone) thus:

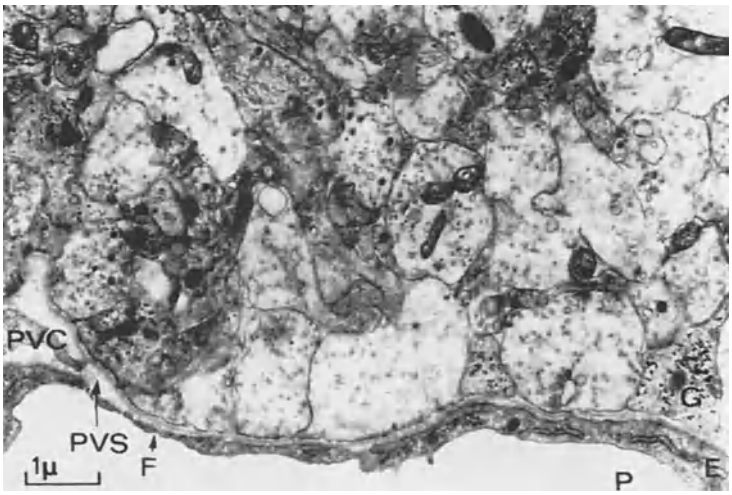

Fig. 6. Electromicrograph of the external layer of the median eminence of a rat at the first postnatal day. Note the high density of nerve terminals around part of a primary portal capillary vessel (P) which is fenestrated (F). Note also the large number of agranular and granular vesicles in the nerve terminals. These vesicles contain the packets (quanta) of neurohormone or neurotransmitter that are released on nerve depolarization as a consequence of nerve action potentials. The neurohormones are released into the perivascular space and from there move rapidly into portal vessel blood for transport to the pituitary gland. This arrangement is typical of neurohemal junctions found in the several circumventricular organs of the brain (see text). *E* endothelial cell; *F* fenestration; *G* glial process; *P* portal vessel; *PVC* perivascular cell; *PVS* perivascular space. ×13200. (Reproduced with permission from Fink and Smith, Z Zellforsch 1971; 119:208–226.)

"If it can be shown that a substance is released into the extracellular space from presynaptic nerves in quantities consistent with the amount and rate of stimulation and the physiology of transmitter release at that junction, and if it can be shown that the material released acts on postsynaptic membranes by using molecular mechanisms identical with those used by the physiologically evoked transmitter, then that substance is a transmitter."

Werman's two principal criteria—endogenous release (collectibility) and exogenous mimicry (identity of action)—should be supplemented by a third criterion invoking the topochemistry of the mechanisms for the synthesis and inactivation of the transmitter.

The surgical accessibility of the hypophysial portal vessels in the anesthetized rat has made it possible to satisfy these criteria for all the hypothalamic-pituitary neurohormones outlined above, with the exception of dopamine. Thus, although dopamine is released into portal blood, the rate of its release is not consistent with a role as a PIF, whether the portal blood was collected by the transpharyngeal or the parapharyngeal route. In the sheep, where Clarke and Cummins devised a method for collecting hypophysial portal blood from conscious animals, no detectable dopamine is released into portal blood even under conditions in which prolactin levels are low. These negative findings on dopamine suggest that the role of this monoamine as a physiological PIF needs to be reexamined.

The technique of collecting hypophysial portal blood has also made it possible to clarify the physiological significance of posttranslational processing. This is exemplified by the fact that somatostatin 14 as well as 28, derived from the same precursor, are released into hypophysial portal blood. The measurement of neurohormone release into hypophysial portal blood has also made it possible to ascertain whether newly discovered hypothalamic compounds could serve as hypothalamic-pituitary regulatory factors. This is exemplified by work in our laboratory which showed that the concentrations of the cardiac peptide ANP were about four times greater in portal than in systemic blood—a finding which lead to immunoneutralization studies which suggest that ANP is an ACTH inhibiting factor. Studies of hypophysial portal blood can also exclude the neurohormonal role of a candidate neurotransmitter. Thus, although angiotensin and cholecystokinin are present in the median eminence, their concentrations in portal blood are not greater than in peripheral blood, and this makes it unlikely that either plays a role as a neurohormone.

All of the hypothalamic-pituitary regulatory neurohormones are present in regions of the nervous system outside the hypothalamus—here Werman's criteria for a neurotransmitter, mentioned above, need to proved by determining whether the neurohormones are released (by push-pull cannulae or dialysis), activate cells (e.g., electrophysiologically) and are responsible for a behavioral effect. With respect to the criterion of evidence for the presence of mechanisms for neurotransmitter inactivation, the inactivation of peptides differs from the monoamines in that the latter are inactivated by specific uptake systems (the serotonin, norepinephrine, and dopamine trans-

porters) while peptides are inactivated by degradative enzymes, which are present at a high concentration in the central nervous system, as well as by diffusion into the extracellular space.

4.3.3. Neurohormone Receptors

The hypothalamic neurohormones and their receptors, all G-protein-coupled, underscore the adage that the evolution of hormones is not dependent on structural changes in the hormones, but rather to the uses to which they are put. The latter depends entirely on the receptors and the signal transduction systems that mediate receptor activation, a point illustrated by the receptors that mediate the neurohormone control of growth hormone and adrenocorticotropin secretion.

The human GHRH receptor cDNA was first cloned and sequenced in Michael Thorner's laboratory in 1993 and found to be a member of a family of receptors that includes secretin, calcitonin, vasoactive intestinal peptide, and parathyroid hormone. The activity of the GHRH receptor promoter is enhanced by the pituitary-specific transcription factor Pit-1. The importance of Pit-1, the prototypic POU domain protein, for the development and activity of the GHRH receptor is demonstrated by the fact that the GHRH is not present in the pituitary gland of dwarf mice (dw/dw) that lack Pit-1. Glucocorticoids stimulate whereas estrogen inhibits GHRH-receptor gene expression, actions that are likely to be mediated by the glucocorticoid and estrogen response elements present in the promoter and explain in part the physiological effects of glucocorticoids and estrogen on GH secretion. The pituitary expression of GHRH receptor mRNA in the rat increases with embryonic development to reach a peak at embryonic day 19.5 (two days before parturition) followed by a decline through to postnatal day 12 and then an increase to day 30 followed by a decline with age. If a similar pattern occurs at the corresponding times in the human then that would be a major factor in the decline in plasma GH concentrations with age in the elderly.

There are five receptors for somatostatin (SSTR) encoded by five distinct genes. All five receptors are present in the anterior pituitary gland of which SSTR-5 is the most prominent on the somatotrophs, followed by SSTR-2. Hypothalamic arcuate neurons that secrete GHRH express the SSTR-2 and SSTR-1, supporting the proposition that the action of somatostatin may involve an effect on the release of GHRH. Relatively large amounts of somatostatin are also present in the cerebral cortex and hippocampus, regions which express the SSTR-2(a), a splice variant of the SSTR-2.

The accumulation of cyclic AMP possibly leads to the activation of Pit-1 by protein kinase A-mediated phosphorylation of the transcription factor CREB. Whether there is a direct action of cyclic AMP via CREB on the GHRH receptor gene remains to be determined as does the possibility that this is the common pathway for the action of both GHRH and somatostatin which inhibits GH secretion by suppressing cyclic AMP.

The actions of CRF-41 in brain and periphery are mediated by three receptors: CRF1, CRF2 alpha, and CRF2 beta. The CRF1 receptors are expressed predominantly in brain and pituitary whereas the CRF2 receptors are present mainly in heart and skeletal muscle. A CRF-binding protein is also present in the circulation. In addition to subserving the action of CRF-41, the cyclic AMP-linked CRF receptors also mediate the action of urocortin, the mammalian homologue of urotensin in fish, and of sauvagine, a homologue of CRF-41 derived from the South American tree frog. In addition to its role in mediating the neural control of ACTH release, CRF-41 and urocortin exert actions on the brain and the cardiovascular system. The ACTH releasing action of CRF-41 is potentiated by AVP that binds to the $V_{1\beta}$ receptor. The $V_{1\beta}$ receptor activates the inositol phospholipid cycle. Synergism between CRF-41 and AVP involves calcium activated cyclic AMP production. ACTH secretion is inhibited by ANP, an action mediated by two guanylyl cyclase-coupled receptor subtypes—A and B.

The complexity of the receptors involved in the control of ACTH and GH secretion contrasts markedly with the apparent biological efficiency of the gonadotropin control system where GnRH mediates the neural control of both LH and FSH, and the apparent simplicity of the thyrotropin and prolactin control systems in which there is evidence for only one neurohormone, TRH and the PIF, dopamine, respectively. Dopamine inhibition of prolactin release is mediated by the dopamine 2 (D2) receptor. Alternative splicing results in a long and short form of the D2 receptor, and this allows coupling to different types of G protein. The D2 receptor when activated inhibits adenylyl cyclase.

Whether the complexity of the neurohormones and receptors involved in the neural control of GH and ACTH release is a consequence of evolutionary convergence of different regulatory systems that are not deleterious and therefore "retained" or whether complexity has developed as a consequence of selective pressure to provide flexibility or increase the number

of variables that can influence the two systems remain to be determined.

4.3.4. Clinical Use

All of the hypothalamic neurohormones have been used extensively as tests of anterior pituitary function, sometimes inappropriately termed "pituitary reserve" rather than "pituitary responsiveness." In addition, neurohormone agonists and antagonists have been deployed for a variety of diagnostic tests and therapeutic regimes. The use of dopamine agonists, such as bromocriptine, for the treatment of hyperprolactinemia and prolactin secreting pituitary adenomata has been mentioned above, and the use of GnRH superactive agonists and antagonists will be outlined in subheading 4.7. Somatostatin, or its synthetic mimetics such as Octeotride, has been used in the treatment of GH-releasing tumors in the pituitary gland which lead to acromegaly and also ectopic (i.e., tumors outside the pituitary gland) hormone secreting tumors—most commonly small cell carcinomas of the lung or tumors of the gastrointestinal system. Somatostatin and its agonists are also effective in the treatment of pancreatitis. Orally active, nonpeptide mimetics of GHRP, such as MK-677, have recently been developed and these may have therapeutic value in the treatment of children of short stature and adult GH deficiency. The use of GHRH, GHRP, and its mimetics are also being explored as useful agents for the treatment of metabolic deficiencies that occur in aging.

Analogs of CRF-41 and urocortin have been developed with a view to their possible use in the treatment of depression, anxiety, anorexia nervosa, and stroke.

4.4. Teleological Advantages of Neurohormonal Control

The hypothalamic-pituitary axis illustrates the remarkable economy of physiological systems. First, and perhaps most impressive are the hypophysial portal vessels which, by transporting the neurohormones from the hypothalamus to the pituitary gland, undiluted by mixture in the systemic circulation, ensure that hypothalamic neurohormones released in very small amounts from the hypothalamus will reach the pituitary gland at concentrations that are several orders of magnitude greater than in the systemic circulation and therefore sufficient to exert their effects. The corollary of this is that the relatively little neurohormone needs to be released to exert its effect and that therefore only a small amount of new neurohormone needs to be synthesized. This is exemplified by the fact that while electrical stimulation of the medial preoptic area of the hypothalamus produces a significant increase in the output of GnRH into portal blood and massive LH release into the systemic circulation, there is no detectable change in the amount of GnRH stored in the hypothalamus. The "metabolic economy" of the hypophysial portal system is brought into sharp relief by the fact that hypothalamic concentration and total content of the hypothalamic-anterior pituitary regulatory neurohormones are three or more orders of magnitude lower than those of the neurohypophysial nonapeptides, vasopressin, and oxytocin, which reach their peripheral targets by the systemic circulation.

Second, the transport of neurohormones at effective concentrations by the hypophysial portal vessels also protects the body from potential adverse effects of the high concentrations of the neurohormones necessary to stimulate or inhibit pituitary hormone secretion. Thus, for example, the high portal blood concentrations of somatostatin may, in the systemic circulation, have adverse effects on the gut and on insulin secretion by the β-cells of the pancreas. Similarly, as shown by Fink et al., the portal plasma concentrations of atrial natriuretic peptide that inhibit ACTH secretion would, in the systemic circulation, cause a lethal drop in blood pressure.

Third, the neurohormones in the hypothalamo-adenohypophysial system are also chemical messengers in other systems; that is, as is also the case for the monoamines, acetylcholine, GABA and excitatory amino acids, the same substances serve as chemical messengers in different systems depending on the upon the presence of appropriate receptors and intracellular signaling mechanisms. Most of the neuropeptides of the hypothalamic-pituitary system have been implicated as neurotransmitters, neuromodulators, or neurotropins elsewhere in the nervous system, although robust evidence for their precise function needs to be established. Thus, for example, somatostatin is secreted by cells of the pancreatic islets and inhibits insulin secretion. CRF-41 is present in higher brain centers and has been implicated in stress-related behaviors, and in the periphery with cardiovascular control. GnRH is present in the placenta, too, at high concentrations affects the gonads and has also been implicated in mating behavior.

The gene regulation, expression and processing of each hypothalamic-pituitary regulatory neurohormone is discussed in Chapter 3. Here the "efficiency"

of hypothalamic-pituitary regulatory neurohormones and their neuroendocrine control will be illustrated by reference to gonadotropin-releasing hormone (GnRH).

4.5. Gonadotropin-Releasing Hormone

4.5.1. A Pluripotent Peptide

Gonadotropin-releasing hormone exemplifies the pluripotent nature of neuropeptides. Thus, in addition to stimulating the synthesis and release of the gonadotropins, FSH, and LH, GnRH also maintains the structural and functional integrity of the gonadotropes and has the capacity to increase by several fold the responsiveness of the pituitary gland to itself—the *self-priming effect of GnRH.*

As discussed in greater detail elsewhere (*see* Chapter 10), the primary function of FSH in the female is to stimulate the development of ovarian follicles and estrogen secretion. LH triggers ovulation, corpus luteum formation, and progesterone secretion. In the male, FSH stimulates spermatogenesis, whereas LH stimulates testosterone secretion. The fact that LH and FSH release is stimulated by the same decapeptide, GnRH, came as a surprise and remains a conundrum. In spite of intensive investigations carried out over more than 30 yr, no specific FSH-releasing hormone has been discovered. Nonetheless, because it is difficult to prove the non-existence of a factor, some journals and authors prefer the term LH-releasing hormone (LHRH), which leaves open the possibility of the existence of a separate FSH-RH. Several lines of evidence suggest that LH release is under precise minute by minute control of GnRH, whereas once the release of FSH has been triggered by GnRH, its continued release may no longer be dependent on GnRH.

For most of reproductive life, the secretion of LH and FSH is moderated by a *negative feedback* action of gonadal steroids, estrogen in the female and testosterone in the male. The physiological power of steroid hormone negative feedback is shown by the marked increase in the plasma concentrations of LH and FSH which occurs after menopause when the ovary stops secreting estrogen. The secretion of FSH is further modulated by the peptides, inhibin, activin, and follistatin, which are members of the transforming growth factor-beta superfamily. Inhibin, which inhibits FSH release, is secreted primarily by the gonads, but activin is also present in brain and is known to play a key role in embryogenesis. Subunits of inhibin and activin interact with Pit-1, a transcription factor important for the embryogenesis of the pituitary gland (as already discussed).

The menstrual cycle in the human female and estrous cycle in the female rat are punctuated by a massive surge of LH that triggers ovulation (Figs. 7 and 8). The LH surge, in turn, is triggered by the spontaneous surge of estradiol-17β that occurs during the late follicular phase in the human and reaches a peak at noon of proestrus in the rat. This *positive feedback* action of estrogen involves: 1) an action of estradiol-17β on the brain to stimulate the surge release of GnRH and/or to increase the pulse frequency of GnRH release, and 2) an increase in pituitary responsiveness to GnRH. In the human and rat, the increase of pituitary responsiveness to GnRH just before occurrence of the ovulatory LH surge is of the order from 20- to 50-fold (Fig. 9). This increase in pituitary responsiveness, generated by estrogen and the self-priming effect of GnRH, is pivotal for the occurrence of the ovulatory surge since the amount of GnRH released during the surge or pulses is far too small by itself to release an ovulatory surge of LH (Fig. 10). GnRH self-priming serves also to coordinate the release of GnRH with the increase in pituitary responsiveness to GnRH so that both events reach a peak at the same time and thereby ensure the occurrence of the ovulatory LH surge (Fig. 11).

4.5.2. Site of GnRH Synthesis—Kallmann's Syndrome

The sites and mechanisms of synthesis of the hypothalamic-pituitary neurohormones are described in detail in Chapters 2 and 3. Here, brief mention will only be made of the GnRH neurons that are located mainly in the medial preoptic area from where they project to the median eminence as well as to the organum vasculosum of the lamina terminalis. The function of the latter remains unresolved.

There are several remarkable features of the GnRH neurons. First, and most striking, is the fact that as shown by Schwanzel-Fukuda and Pfaff, these neurons arise in the epithelium of the medial olfactory pit and migrate from the nose into the forebrain along nerve fibers rich in neural cell adhesion molecules. Independent studies by Schwanzel-Fukuda and Pfaff and C. Petit et al. showed that a genetic defect responsible for failure of the normal migration of the GnRH neurons as well underdevelopment of the olfactory bulbs and tracts are the cause of the X-linked form of *Kallmann's Syndrome,* which is characterized by *hypogo-*

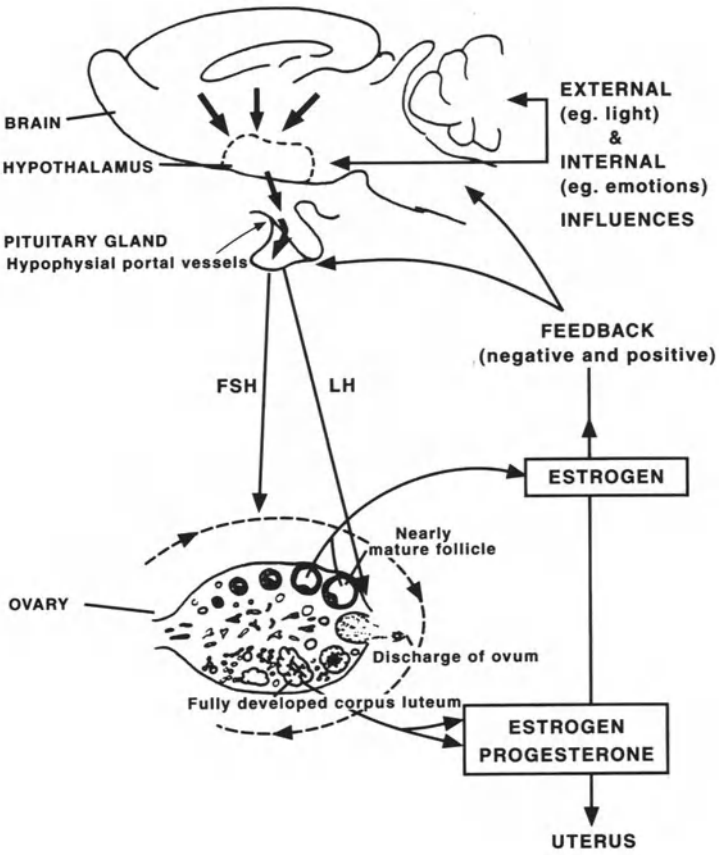

Fig. 7. Schematic diagram of the control of the ovarian cycle by follicle-stimulating hormone (FSH) and luteinizing hormone (LH) released from the anterior pituitary gland. The secretion of LH and FSH is controlled by the brain by way of gonadotropin releasing hormone (GnRH), a decapeptide that is released from hypothalamic neurons into the hypophysial portal vessels. The release of GnRH from hypothalamic neurons is influenced by external and internal factors acting by way of central nervous pathways, and the system is regulated by positive- and negative-feedback control involving estrogen and progesterone secreted by the ovary. Estrogen and progesterone act on the uterus to prepare the endometrium for implantation of the zygote should fertilization occur. Not shown for the sake of clarity is the peptide inhibin that is secreted by ovarian follicles and inhibits FSH release. FSH secretion is also influenced by activin and follistatin. (Reproduced with permission from Fink 1988b.)

nadism and *ansomia.* The *Kal-1* gene responsible for X-linked Kallman's Syndrome encodes a putative secreted protein of 680 amino acid residues which contains four fibronectin type III repeats and a four disulfide core motif. The majority of cases of Kallmann's syndrome are sporadic and few of these (less than 8%) have deficiencies in the Kal-1 gene—and so further research for the cause of this syndrome is required. Nonetheless, it seems that the anosmia and hypogonadism of Kallman's Syndrome are likely to be because of a defect in the extracellular matrix, which plays a key role in ensuring the correct migration of neurons to their programmed location in the brain.

Second, very few GnRH neurons reside in the medial basal hypothalamus, the putative site of the GnRH pulse generator, and so the generation of GnRH pulses, which in the rodent do not depend upon the integrity of connections with the preoptic area, remains to be determined.

Third, ultrastructural studies show that there are dendrodendritic connections between the GnRH neurons that could play a role in the synchronization of GnRH neuronal activity and/or in autoinhibition of GnRH neuronal firing.

4.5.3. GnRH Surge and Pulse Generators

Numerous studies in the rat have shown that the integrity of the medial preoptic area and the nearby suprachiasmatic nuclei is essential for the occurrence of regular estrous cycles and the spontaneous surge of GnRH and LH. This has led to the concept that this most rostral area of the hypothalamus (Fig. 2) is the site for the GnRH "*surge generator.*" Pulsatile GnRH release is thought to be generated by the medial basal hypothalamus, the "*pulse generator.*" The rhesus monkey differs from the rat in that, as shown by Knobil, Krey et al., the LH surge and ovulation can occur even after total surgical isolation of the medial basal hypothalamus. Experimental studies suggest that in the rheseus monkey, the LH surge is triggered by pulses of GnRH with a frequency of about one per hour (Knobil 1980), the optimal frequency for GnRH self-priming in an estrogen-primed rat and similar to the frequency of LH pulses in the human female towards midcycle. This pulse frequency is also

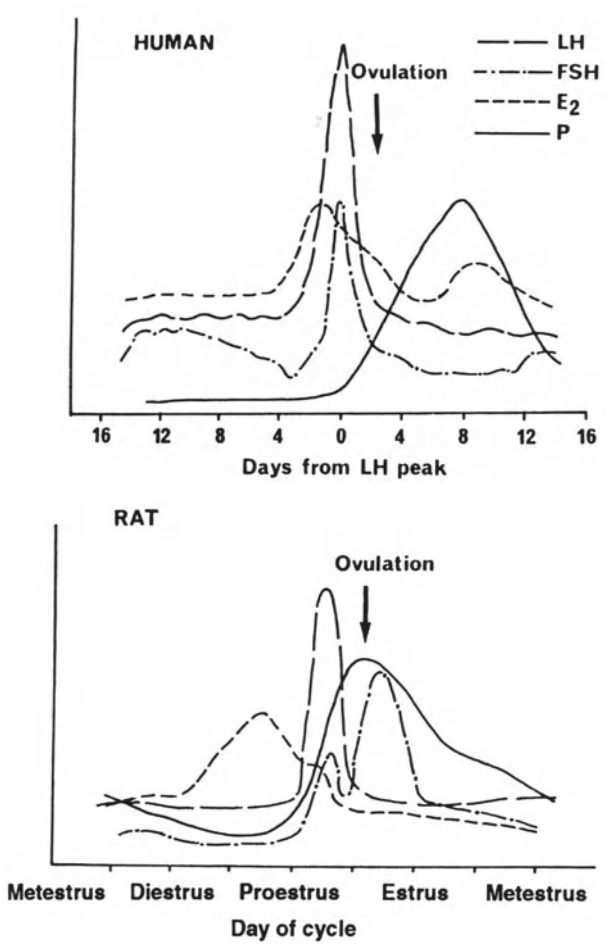

Fig. 8. Schematic diagram of the key hormonal changes during the human menstrual cycle and rat estrous cycle. Ovulation is preceded by a surge of luteinizing hormone (LH), which is triggered by a surge of estradiol-17β (E_2) and accompanied and followed by a surge of progesterone (P). In the human, most of the progesterone is secreted by the ovary in response to LH, but in the rat, a small amount of progesterone is secreted by the adrenal gland just before the onset of the LH surge. In the human, the timing and magnitude of the FSH surge are less consistent than that of the LH surge, and in the rat the major peak of the FSH surge occurs on the morning of estrus (after or about the time of ovulation). (Reproduced with permission from Fink, 1988b.)

optimal for generation of an LH surge by administering exogenous GnRH in the estrogen-primed human female. Thus, while the occurrence and importance of the spontaneous GnRH surge cannot be discounted in the human, the GnRH pulse generator may play a pivotal role in the control of ovulation in the monkey and the human. Because the GnRH pulse generator also plays a crucial role in the onset of puberty and in the control of seasonal reproduction, pulse frequency proves to be as powerful a signal as pulse amplitude.

The precise mechanism of the neural control of both the pulse and the surge generators has yet to be established. Work in Pfaff's laboratory showed that GnRH neurons do not possess estradiol receptors, and so the stimulation of GnRH release by the positive feedback effect of estradiol must be mediated by neurons that project to the GnRH neurons. Present evidence suggests that several stimulatory (e.g., serotonergic, noradrenergic, glutamatergic and peptidergic—neuropeptide Y, and galanin) and inhibitory (e.g., GABA, CRF-41, and endogenous opioids) mechanisms control the GnRH surge. The same neurotransmitter systems also appear to be involved in the control of GnRH pulses, but do not necessarily act in the same direction as in the control of the surge. Thus, for example, central noradrenergic neurons stimulate the GnRH surge, but inhibit GnRH pulses. The existence of an array of neurotransmitter systems for GnRH control might be expected because the appropriate release of GnRH is crucial for reproduction of mammalian species.

4.6. Self-Priming Effect of GnRH

The self-priming effect of GnRH is indispensable for the ovulatory surge of LH, and therefore reproduction of spontaneously ovulating mammals. The effect is a prime example of a neuroendocrine *servomechanism.* The self-priming effect of GnRH has two actions: first, it increases pituitary responsiveness to GnRH, and, second, it coordinates the exponential increase in pituitary responsiveness with the increased release of GnRH, either as a surge or increased frequency of GnRH pulses. As a consequence, both events reach a peak simultaneously, thereby ensuring a massive release of LH that triggers ovulation. Thus, a very small amount of GnRH, either in the form of a surge or a series of pulses, can induce the ovulatory surge of LH (Fink 1988). Figure 12 shows that 1) the self-priming effect is greatest when the pulses of GnRH are separated by 60 min, which is significant in terms of the optimal pulse frequency for LH release (as already described), and 2) the effect has a "memory" in that it is almost extinguished 240 min after the first pulse of GnRH. At the time of the midcycle in women, when the pituitary gland has already been primed by the endogenous surge of estradiol-17β, one pulse of GnRH can increase the LH response to a second pulse administered 2 h later by more than two-fold. In the proestrus rat, the GnRH-induced

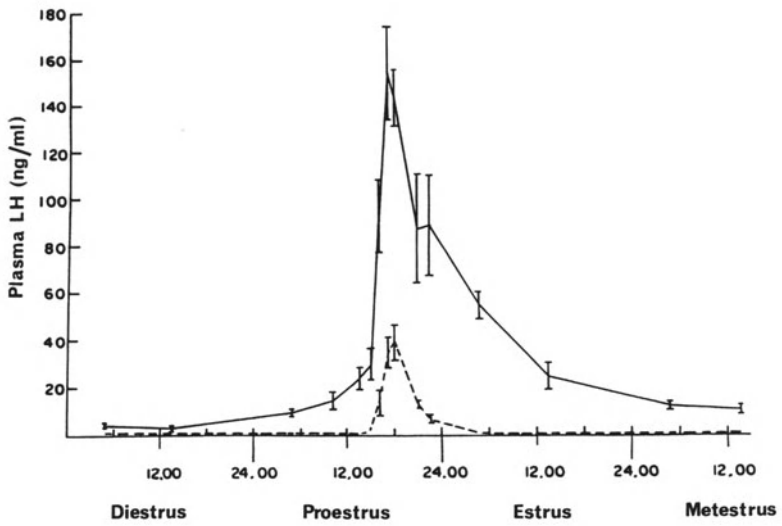

Fig. 9. Changes in pituitary responsiveness to GnRH during the estrous cycle of the rat. Note the exponential increase in the LH response to GnRH injections just before the spontaneous ovulatory LH surge. The figure shows the mean (±SEM) preinjection concentrations (----) and mean maximal increments (——) in plasma LH concentrations (ng NIH-LH-S13/mL) in animals anesthetized with sodium pentobarbitone 30–60 min before the injection of 50 ng GnRH/100 g body weight at different stages of the estrous cycle. (Reproduced with permission from Fink 1988b.)

increase in pituitary responsiveness to a second pulse of GnRH administered 1 h after the first is about seven-fold.

GnRH-self priming involves postreceptor changes that result in potentiation of the GnRH intracellular signaling cascades and the movement of secretory granules toward the cell membrane ("margination"). The latter is brought about by changes in the cytoskeleton possibly induced by protein kinase C (PKC) acting on myristoylated alanine-rich C kinase substrate (MARCKS) and MAP kinase. GnRH self-priming is also associated with the synthesis of a new protein—hormone induced protein 70—recently shown to be a protein disulfide isomerase that is likely to be involved in posttranslational processing of glycoproteins and intracellular redox actions.

4.7. GnRH Agonists and Antagonists

Superactive *GnRH agonists* have proven to be powerful clinical tools in that, by virtue of tachyphylaxis (downregulation or desentization of GnRH receptors), prolonged exposure to GnRH superactive agonists turns off gonadotropin secretion. These agonists are thus effective for "chemical ovariectomy or castration" that is required as adjunct therapy of tumors of the breast and prostate gland. For the same reason, superactive GnRH agonists are effective in the treatment of precocious puberty, in which inappropriately high gonadal steroid secretion results in premature development of secondary sex characteristics and epiphyseal bone growth and closure. If left untreated, the premature epyphyseal bone growth and closure results in dwarfism. Superactive GnRH agonists are also used for the treatment of uterine leiomyomas (fibroids) and can also be used as contraceptives.

In vitro evidence suggests that superactive GnRH agonists may exert direct inhibitory effects on breast cancer cells, which do in fact, express GnRH receptors. Administered as pulses (which do not cause tachyphylaxis), superactive GnRH agonists can, by virtue of the self-priming effect of GnRH, result in an ovulatory LH surge, and this fact is utilized in the treatment of infertility.

Potent *antagonists* of GnRH have been developed mainly as a consequence of the energetic work carried out in A.V. Schally's laboratory. Early antagonists triggered anaphylactic reactions because they released histamine. This problem has now been overcome and trials of GnRH antagonists are now ongoing to determine the therapeutic efficacy of these antagonists in the treatment of cancer of the breast, ovary and prostate, which all express GnRH receptors and which are also dependent on gonadal steroids. The advantage of using antagonists is that unlike the GnRH agonists, there is no flare effect; that is, superactive GnRH agonists, but not the antagonists, at first, stimulate gonadotropin and thereby gonadal steroid release that could stimulate tumor growth. The GnRH antagonists

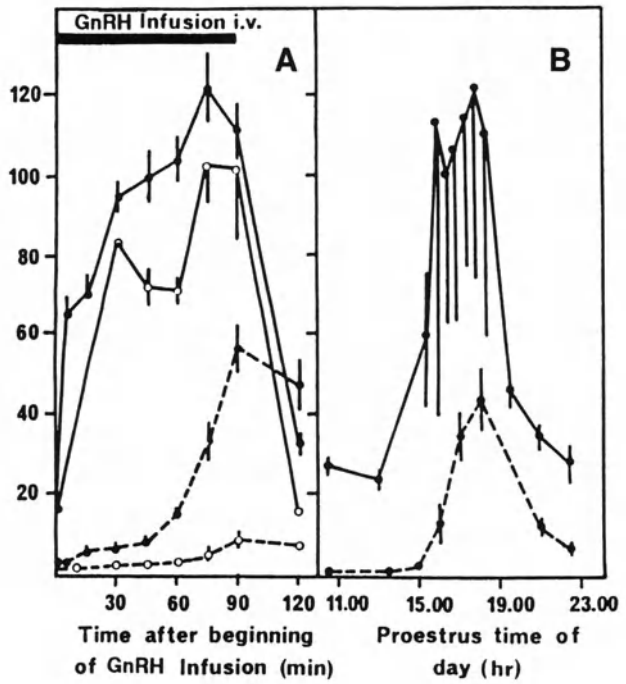

Fig. 10. Shows that the spontaneous surge of GnRH is too small to produce a spontaneous surge of LH except on proestrus, when the responsiveness of the anterior pituitary gland is 20 to 50 times greater than on diestrus. Units on the ordinate are nanograms for LH and picograms for GnRH per ml plasma. A: the mean (±SEM) peripheral plasma concentrations of LH (ng NIH-LH-S18) (----) and GnRH (——) in either proestrous (○; n = 11) or diestrous (●; n = 5) rats that were infused intravenously with synthetic GnRH at a rate of 0.167 ng/100/g/min for a period of 90 min after anesthesia with sodium pentobarbitone at 13.30 h. B: The mean (±SEM) concentrations of GnRH in hypophysial portal plasma (——); n = 9–52) and LH (NIH-LH-S13) in peripheral plasma (– – –; n = 6–9) during the day of proestrus. (Reproduced with permission from Fink 1988b.)

are also being tested, either alone or in combination with testosterone, as a possible male contraceptive. The antagonists are also useful for the treatment of uterine leiomyomas and, in combination with GnRH agonists, for in vitro fertilization.

5. THE HYPOTHALAMO-NEUROHYPOPHYSIAL SYSTEMS

5.1. Overview

The neural lobe is the site of release of the nonapeptides, oxytocin, and vasopressin, into the systemic circulation. Presumably because a considerable distance removes the target cells of these peptides from their site of release in the neural lobe, and because there is massive dilution in the systemic circulation, the rate and amount of synthesis of oxytocin and vasopressin and their content in the hypothalamus is about three orders of magnitude greater than that of the neurohormones concerned in anterior pituitary control (above). This fact, together with their synthesis in discrete hypothalamic nuclei, their ease of assay, and that both nonapeptides contain disulphide bridges which allow easy incorporation of the radioactive tracer, ^{35}S-cystine, contributed to four important landmarks of neuroendocrinology. First, oxytocin and vasopressin were the first of the hypothalamic neurohormones to be sequenced (by Du Vigneaud et al.). Second, the glycoprotein components of their precursor proteins led to their conspicuous staining and thereby to the concept of *neurosecretion* (the Scharrers), a term applied to neurons whose main purpose is to secrete neurohormones rather than transmit signals by propagated action potentials. Third, the fact that both peptides have disulfide bridges and their synthesis involves the incorporation of cystine, which can be labeled with the relatively high energy radionuclide, ^{35}S, made them excellent models for the first studies of neuropeptide synthesis and transport (first shown by Sachs et al.). Fourth, oxytocin and vasopressin-containing neurons were the first neuroendocrine neurons from which electrophysiological recordings were made (by J. Green and B. Cross in 1959).

The concept of neurosecretion (above) was soon discarded because it was shown that the magnocellular neurons are no different from others—i.e., they propagate action potentials and secrete neurohormones or neurotransmitters. In fact, oxytocin and vasopressin-containing neurons facilitate electrophysiological recording because they are large and because their axons terminate in the surgically accessible neural lobe. This makes it relatively easy to locate and verify by antidromic stimulation the identity of the neurons and record from them. Furthermore, the electrophysiological activity of the hypothalamic magnocellular neurons can be correlated with secretion of vasopressin and oxytocin. Finally, the neural lobe is a "bag" of nerve terminals and, therefore, proved to be a useful model for the study of *exocytosis* and *stimulus-secretion coupling*—that is, the coupling between the cascade of ion fluxes through membrane channels (membrane depolarization) triggered by action potentials and the calcium-dependent release of neurohoromones or neurotransmitters. The neurohormones like all other known neurotransmitters and peptide and protein hormones are packaged in secretory vesicles (Fig. 6) which are released in packets or "quanta."

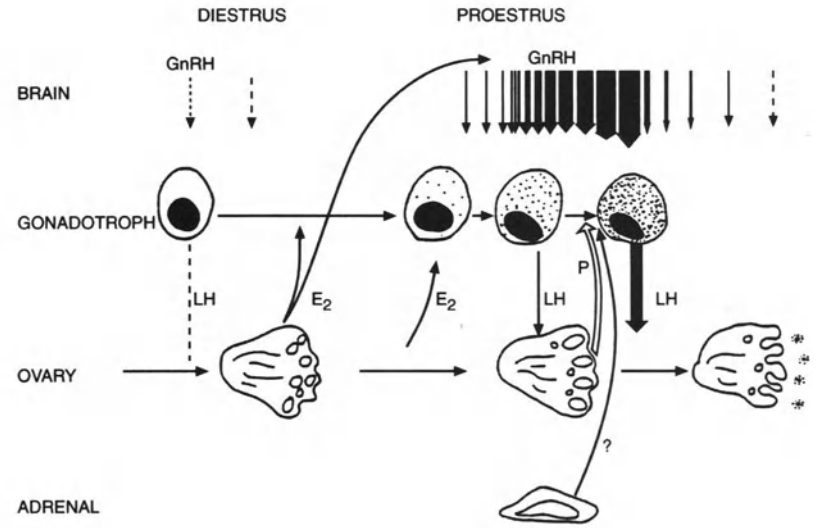

Fig. 11. Schematic diagram which shows the cascade of events which generates the spontaneous ovulatory LH surge in the rat. The increase in plasma concentrations of estradiol-17β (E_2) increases the responsiveness of the pituitary gonadotropes (increased stippling) to GnRH and also triggers the surge of GnRH. Pituitary responsiveness to GnRH is further augmented by the priming effect of GnRH, the unique capacity of the decapeptide to increase pituitary responsiveness to itself. Progesterone (P) secreted by the ovary in response to the LH released during the early part of the LH surge may also enhance pituitary responsiveness to GnRH. The priming effect of GnRH coordinates the surge of GnRH with increasing pituitary responsiveness so that the two events reach a peak at the same time. The conditions are thereby made optimal for a massive surge of LH. This cascade, which represents a form of positive feedback, is terminated by destruction of a major component of the system in the form of the rupture of the ovarian follicles (ovulation). The human female shows a similar increase in pituitary responsiveness and GnRH self-priming just before the midcycle LH surge.

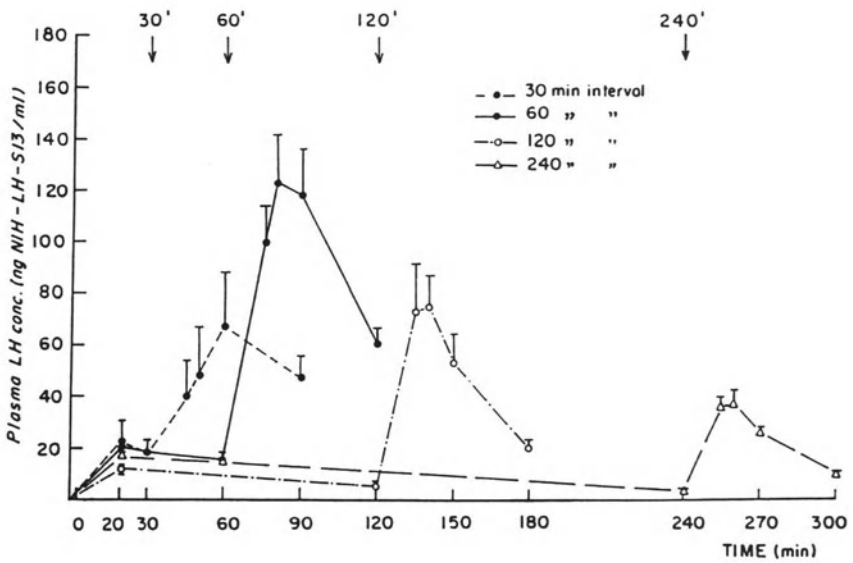

Fig. 12. The self-priming effect of GnRH in the rat. Mean (±SEM) plasma LH concentrations (ng NIH-LH-S13/mL) after two successive intravenous injections of 50 ng GnRH/100 g body weight. The first dose of GnRH was injected 30–60 min after the administration of sodium pentobarbitone at 13.30 h of proestrus; the second dose of GnRH was injected either 30, 60, 120, or 240 min after the first. Note that the response to the second injection of GnRH was greatest when the two injections were separated by 60 min and that by 240 min the priming effect had almost been extinguished. (Reproduced with permission from Fink 1988b.)

5.2. Oxytocin: Lactation and Parturition

Oxytocin is concerned mainly with milk ejection during lactation and parturition (the birth process), which are two perfect examples of a neuroendocrine reflex. Oxytocin is the neurohormonal component of the milk ejection reflex whereby suckling at the nipple of lactating mothers triggers volleys of impulses that travel through the mammary nerves to the spinal cord and by way of a multisynaptic pathway to reach the hypothalamus where they trigger the release of oxytocin. Oxytocin transported by the systemic circulation, stimulates the contraction of the myoepithelial cells of the breast acinar resulting in milk ejection. During parturition, oxytocin coordinates and reinforces uterine contractions. Here, too, a reflex is involved in that as uterine contractions force the head of the fetus against the cervix, volleys of impulses are triggered which ascend through multisynaptic pathways involving the pelvic nerves and the spinal cord to the hypothalamus to trigger the release of oxytocin that acts on the smooth muscle cells of the uterus. This is a classical positive feedback system (see subheading 8.1.2.).

Vasopressin (or the anti-diuretic hormone) is concerned mainly with the control of body water, although, as its name implies, it also induces vasoconstriction and thereby can increase blood pressure, although this usually occurs only after a substantial loss of blood volume. The vasopressin cells of the supraoptic and paraventricular nucleus respond to osmotic stimuli—an increase in plasma osmolality triggers the release of vasopressin which increases water reuptake in the nephron as a consequence of which there is an overall increase in body water with a fall in plasma osmolality. This is a perfect example of a homeostatic regulatory mechanism. In addition to its role in osmoregulation, vasopressin synthesized in the smaller (parvicellular) neurons of the PVN, acts synergistically with CRF-41 to release ACTH.

6. THE INTERMEDIATE LOBE OF THE PITUITARY GLAND

6.1. Model for Posttranslational Processing

The major secretion of the intermediate lobe of the pituitary gland is αmelanocyte stimulating hormone (αMSH), a 13 amino acid residue peptide that, together with ACTH and β endorphin, is derived from the precursor *proopiomelanocortin* (POMC). In addition to αMSH, the pars intermedia also contains other derivatives of POMC, βMSH, γMSH, CLIP, and β-endorphin that, together with the ACTH, comprise the *melanocortins*. The fact that in the pars distalis, ACTH is the major hormone derived from posttranslational processing of POMC, whereas in the pars intermedia, αMSH is the major active hormonal product of POMC processing, reflects the presence of different enzymatic processing pathways in the two parts of the gland. The release of αMSH is inhibited by dopaminergic neurons that originate in the arcuate nucleus and reach the intermediate lobe by way of the neural stalk. Man is conspicuous among mammals in that the pars intermedia is not defined as a separate lobe of the human pituitary gland.

6.2. Melanocortins

Although they are all derived from POMC, the melanocortins have diverse physiological functions. Alpha-MSH, first thought to stimulate the growth of melanocytes and pigment formation (melanogenesis), is also involved in fever, inflammation, and the immune response. Indeed, several of the melanocortins, including ACTH, affect cytokine-induced effects on thymocytes, T- and B-lymphocytes and neutrophils. The actions of the melanocortins are mediated by five melanocortin receptor subtypes (MC1–MC5). Although all of the melanocortin receptors are seven transmembrane G-protein-coupled, the tissue distribution of the five receptors is quite distinct. Thus, the MC1 is located on melanocytes and mediates MSH-stimulated melanocyte proliferation and melanogenesis, whereas the MC2 receptor is located in the adrenal cortex where it mediates the ACTH-induced secretion of glucocorticoids and mineralocorticoids. The MC3 and MC4 receptors are both located in the brain—the function of MC3 is unknown, but MC4 is involved together with leptin (a protein produced by adipocytes) and neuropeptide Y in the regulation of body weight. Studies on mutant ob/ob mice show that the action of leptin in regulating body weight is mediated, at least in part, by hypothalamic melanocortin activation that results in decreased food intake. The importance of hypothalamic melanocortin activity for moderating food intake is illustrated by the agouti peptide, which is a high-affinity antagonist for the MC1 receptor and induces obesity in the mouse. The MC4 receptor is also involved in grooming behavior. The MC5 receptor, whose function remains unknown, is widely distributed in body organs including the spleen, thymus, skin, and bone marrow. Cutaneous pigmentation is a function of the MC1 receptor and MC1 gene variants may predispose to red hair and light skin color that may make the individual susceptible to melanoma.

7. PINEAL GLAND AND PHOTOPERIODIC CONTROL OF REPRODUCTION

The pineal gland is a circumventricular organ (Fig. 1) that secretes melatonin into the circulation and plays an important role in the photoperiodic control of reproduction. The gland deserves special mention here because it reinforces the principles of neuroendocrine control. Philosophers (Descartes' "seat of the soul") and scientists have long been intrigued by the pineal. The secretion of melatonin is exquisitely sensitive to light. Pinealocytes in submammalian species are photoreceptors and the gland offers an excellent experimental model for studies of the transduction of light into nerve impulses and neurohormone secretion.

The outer segment (sensory pole) of the pinealocyte in fish, ampibia, and reptiles has all the ultrastructural characteristics of a true photoreceptor, but these features are only vestigial in mammals and intermediate forms exist in birds. In fish, amphibia, and reptiles, the effector pole of the pinealocyte "synapse" with secondary pineal neurons that give rise to the pineal tract which propagates signals to the central nervous system. In birds and mammals, however, the pinealocytes secrete melatonin directly into the circulation (or cerebrospinal fluid) in a neuroendocrine manner.

Melatonin, a derivative of serotonin, is synthesized within the pinealocytes in two steps. First, serotonin is converted by the rate limiting enzyme, *N*-acetyltransferase (NAT), to *N*-acetyl serotinin which is then converted to melatonin by hydroxyindole-*O*-methyltransferase. Because little, if any, melatonin is stored, the rate of melatonin secretion is tightly linked to its synthesis that depends upon NAT action, which in turn, depends upon noradrenaline release from the dense sympathetic innervation of the gland. In mammals, the control of melatonin secretion by light is mediated by a multisynaptic pathway that starts at the retina of the eye and successively involves synapses in the suprachiasmatic nuclei, the PVN, the intermediolateral column of the spinal cord, and the neurons of the superior cervical ganglion of the sympathetic nervous system. Sympathetic terminals in the pineal gland release noradrenaline that stimulates melatonin secretion by an action on adrenoreceptors on pinealocytes. Cyclic AMP seems to be the main intracellular second messenger that mediates the action of noradrenaline in this system, and recent studies suggest that the main point of regulation is NAT which, depending on the species, can be affected at the level of NAT gene expression as well as by posttranslational modifications which alter the activity of the enzyme.

The secretion of melatonin starts with the onset of the dark period (night) and stops with the onset of the light period (day). The secretion of melatonin during the dark period is stopped abruptly by exposure to light. In blind persons, the secretion of melatonin takes on the typical 25 h free running period. Taken together, these and other data suggest that the secretion of melatonin is predominantly controlled by light exposure superimposed upon the intrinsic rhythm of the major neural clock, the suprachiasmatic nuclei.

The action of melatonin is predominantly inhibitory with respect to reproduction, and the effects of pinealectomy and manipulation of melatonin levels is most pronounced in seasonal breeding animals such as the wallaby, hamster, vole, and sheep. However, the precise role of melatonin in reproduction has yet to be established.

What is well established is that the suprachiasmatic nuclei constitute the central generator of circadian rhythms of the body and that the functional integrity of these nuclei are indispensable for normal reproductive rhythms. The relative importance of the pineal gland and its precise role in reproductive control in relation to the suprachiasmatic nuclei await to be determined. With the discovery of genes that regulate circadian rhythms and melatonin synthesis the prospects of defining what is likely to be a remarkably elegant and robust molecular control system seems excellent.

8. HORMONAL EFFECTS ON THE NERVOUS SYSTEM

While neuropeptides such as angiotensin affect central neural mechanisms, steroid and thyroid hormones, the secretions of the three major pituitary target organs, have by far the most prominent effects on brain function. The effects of the steroid and thyroid hormones may be classified in terms of (1) feedback actions, (2) brain differentiation and neural plasticity, (3) neurotransmission, and (4) membranes and ion channels.

8.1. Feedback Actions

The feedback actions of steroid and thyroid hormones have been known since the 1930s. There are two types of feedback—negative and positive.

8.1.1. Negative Feedback

Negative feedback is deployed in most systems of the body, and its "purpose" is homeostasis (a term

introduced by Walter Cannon); that is, to maintain the functioning of a system at a constant predetermined level. Man, in the design of machines and control systems, has borrowed the concept of negative feedback from biology. The analogy most often used to explain negative feedback is central heating of a house. Central heating systems are comprised of a heater, which is controlled by a thermosensitive device and can be set to maintain the house at a certain temperature. The thermosensor is comprised of a detector, comparator, and a drive. If the temperature of the house drops below the preset temperature, the thermosensor switches on the heater. Once the preset temperature has been reached or exceeded (say because of the high ambient temperature) the thermosensor switches the heater off. Similarly, the range of the blood concentrations of gonadal steroid hormones (especially estrogen, testosterone, and progesterone) adrenal corticosteroids (especially cortisol in man) and thyroid hormones (thyroxine and tri-iodothyronine) have been preset at levels consistent with requirements for normal body function. The sensor-comparator that "measures" and compares the hormonal level with the preset level and the drive or regulator are probably located within the hypothalamic-pituitary unit, and the setting is probably determined genetically. Decrease of the gonadal, adrenal or thyroid hormones below the preset level results in the increased secretion of the pituitary gonadotropins, ACTH or thyrotropin (TSH) which increase the synthesis and release of gonadal steroids, adrenal steroids or thyroid hormone, respectively. Increased levels of the target hormones above the preset level reduces or inhibits the secretion of the corresponding pituitary "tropic" hormone—hence "negative feedback." As illustrated in Fig. 13, negative feedback control is more complex than outlined above, because the strength of the feedback signal depends on the amount of free plasma target-organ hormone. The latter is determined, not only by the rate of secretion, but by the concentrations of hormone binding protein in plasma and the rate at which the target-organ hormone is metabolized and cleared from plasma.

Interruption of negative feedback results in oversecretion of the pituitary hormones as occurs, for example, at the menopause, when gonadal steroid concentrations in blood are low or absent and pituitary gonadotropin concentrations reach very high levels.

The negative feedback actions of the pituitary target hormones have been used extensively in the clinic. The negative feedback of estrogen and progesterone, for example, is the basis of the "contraceptive pill," different formulations of which are used widely to block the secretion of FSH and LH and thereby prevent the development of ovarian follicles and ovulation.

8.1.2. Positive Feedback

Positive feedback, where increased output of the system increases the drive of the regulator, is far less common than negative feedback. The likely reason for this is that positive feedback can only be terminated by destruction of a component of the feedback loop. A moment's reflection suggests that the replacement of negative by positive feedback in the hormonal systems mentioned above or in the blood glucose or blood pressure control systems would be deleterious and possibly lethal. Nevertheless, positive feedback is essential for two crucial events in reproduction—ovulation and parturition, which have been outlined above. In both systems, termination of the positive feedback cascade is in fact associated with the "destruction" of the output component of the system—rupture of the ovarian follicle and ovulation in the case of estrogen positive feedback and expulsion of the fetus in the case of the parturition-oxytocin reflex. However, whereas in the latter the cause of feedback termination is clear (i.e., expulsion of the fetus prevents further ascending nerve volleys from the uterine cervix which are necessary for triggering the reflex release of oxytocin), the factors which determine the cessation of the ovulatory LH surge have yet to be determined.

The positive feedback effect of estradiol in the rat (Fig. 14) illustrates again the economy of neuroendocrine systems. Thus, the same hormone, estrogen, is able to trigger a cascade of events which ensure that ovulation and mating can occur in a precisely timed manner so as to ensure fertilization of the ova and, therefore, reproduction of the species.

8.1.3. Short-Loop and Ultrashort-Loop Feedback

Short-loop and ultrashort-loop feedback refer to the concept that the release of hypothalamic-pituitary regulatory neurohormones is moderated, respectively, by the appropriate pituitary hormones and the neurohormones themselves. The mechanistic basis for short-loop feedback is that some workers have observed reverse (pituitary to hypothalamus) blood flow in the hypophysial portal vessels. However, careful scrutiny of these reports, notably that of Török, shows that the animals were either in extremus or that the stalk was twisted in order to expose the vessels,

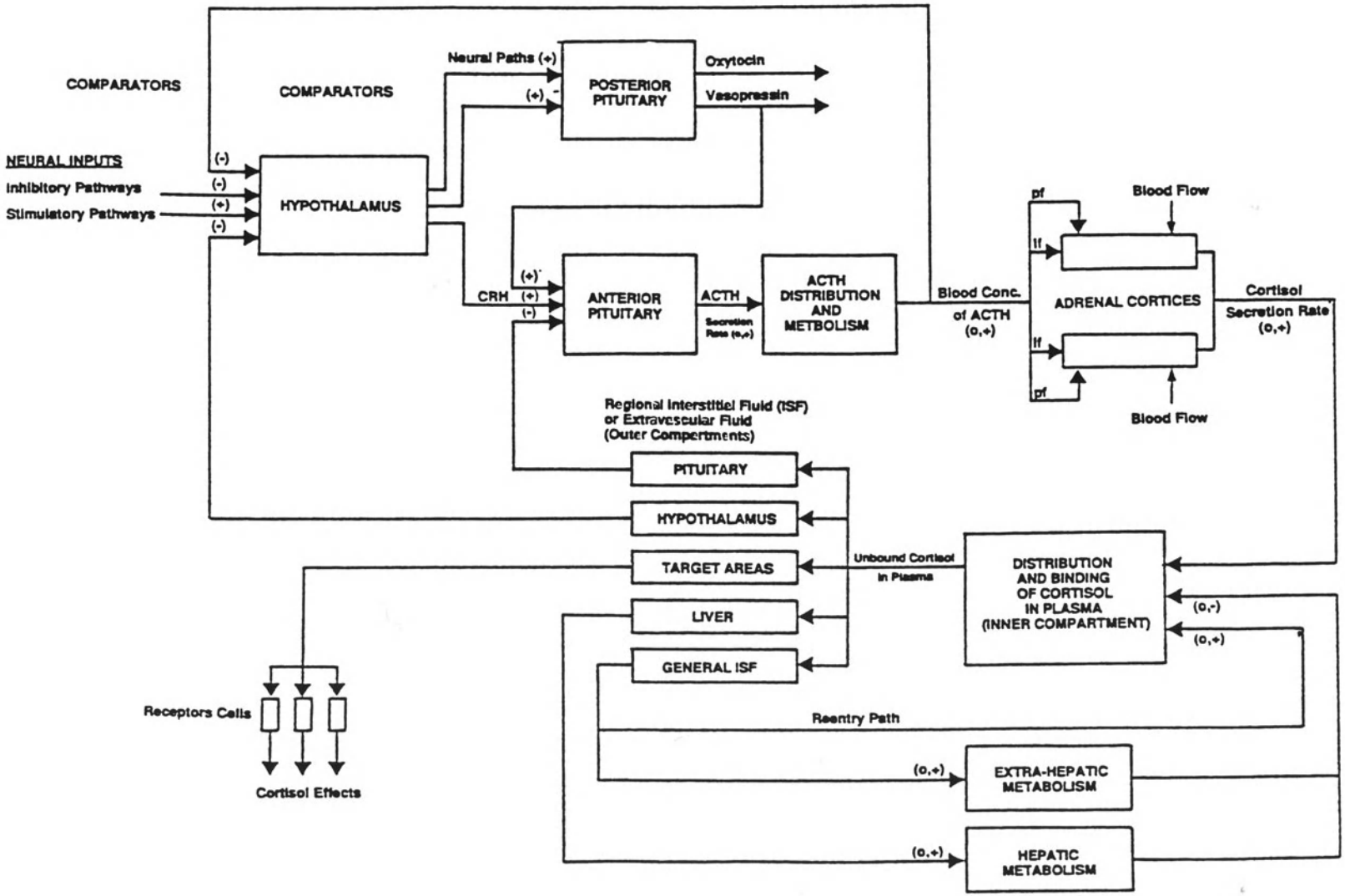

Fig. 13. Block diagram of the hypothalamic-pituitary-adrenal glucocorticoid control system that illustrates the complexity of a physiological negative feedback system. The "comparator" is a theoretical construct that encompasses the brain-pituitary system and "compares" the free plasma concentrations of cortisol with a preset value, which is probably genetically imprinted. The power of the feedback signal depends upon the plasma concentration of free cortisol, a function of the amount secreted, bound to cortisol binding protein in plasma and metabolized by the liver. *If,* input forcing of adrenal by ACTH; *pf,* parametric forcing of adrenal (hypertrophic effect) caused by ACTH over a longer time period. Parametric effect of changes in adrenal blood flow is also indicated. The designators 0,+ and 0,– indicate that signals in pathways are restricted in values (e.g., there are no negative masses or frequencies and removal processes or inhibitors are negative in effects). (Reproduced with permission from Yates and Maran 1975.)

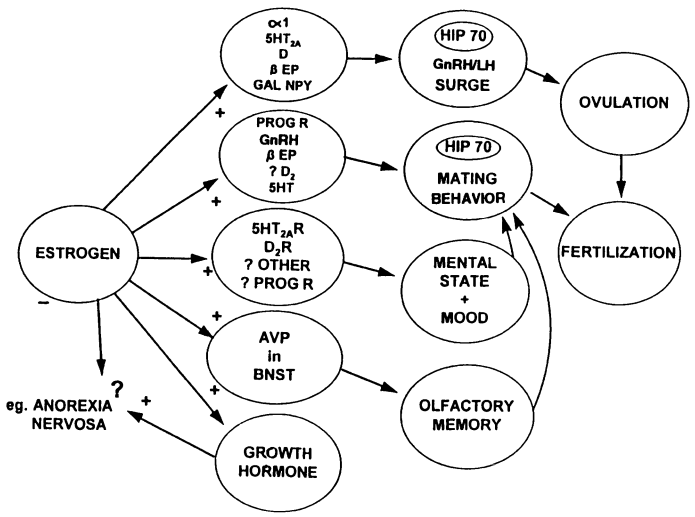

Fig. 14. Demonstration that fertilization and, therefore, procreation and the continuation of the mammalian species depends upon an orderly cascade of events orchestrated by a surge of estradiol-17β. This surge triggers the ovulatory surge of GnRH and LH and mating behavior. Mating depends upon the right mental state or mood and olfactory memory. Estrogen controls and coordinates all four modalities by way of different central mechanisms listed in the ellipsoids on the left. These mechanisms are only examples of key mechanisms and are by no means complete. There is a major interaction between total body mass, fat weight, and estrogen secretion, so that below a certain level of total body and fat weight, estrogen secretion is switched off. The mechanism is unknown, but could conceivably involve growth hormone, the secretion of which is stimulated by estrogen. The mechanism by which low total body and fat weight switches estrogen secretion off in anorexia nervosa could be a safety mechanism for protecting against fertility when the mental state is not appropriate for maternal behavior. *Abbreviations:* α_1, α_2 adrenoreceptors; 5-HT2A receptors; D, dopamine mechanisms; β EP, β endorphin; GAL, galanin; NPY, neuropeptide Y; Prog R, progesterone receptor; GnRH, gonadotropin releasing hormone; D2, dopamine 2 receptor; 5-HT, 5-HT mechanisms; AVP, arginine vasopressin; BNST, bed nucleus of the stria terminalis; HIP70, hormone-induced protein which is stimulated by GnRH in the pituitary and estrogen in the brain and is thought to play a role in estrogen induction of mating behavior and the GnRH induction of the self-priming effect. (Reproduced with permission from Fink 1996.)

thereby blocking normal hypothalamic to pituitary flow of blood. Robust direct evidence that a pituitary hormone inhibits the release of its own neurohormone remains to be obtained.

However, there is ultrastructural evidence for dendro-dendritic connections in the case of GnRH neurons, allowing the possibility (as mentioned above) that GnRH neurons may affect the activity of one another. This, however, is not a feedback system, but rather a neural network which subserves synchronization or autocrine-inhibition of neuronal activity.

Engineering models suggest that no control benefit would be derived from either a short- or ultrashort-loop feedback system.

8.2. Brain Differentiation and Plasticity

Thyroid and gonadal steroid hormones exert both reversible and irreversible effects on brain structure, connectivity, and synapses. Thus, if left untreated, congenital lack of thyroid hormone results in irreversible cretinism owing to serious defects in brain development. Reduced secretion of thyroid hormones in adult life (myxedema) also results in cognitive and other neurological deficits which can be reversed by thyroid hormone administration. Here, attention will be focused on gonadal steroids and their irreversible effects on sexual differentiation of the brain as well as reversible effects on certain neuronal systems.

8.2.1. Sexual Differential of the Brain

The early studies of Steinach (in 1913) and Pfeiffer (in 1936) showed that in the rodent the differentiation of neural control of reproductive function (cyclical in female and acyclial in the male) is determined by exposure to sex steroids rather than the genetic sex of the individual. Thus, transplantation of testes to genetic female rodents before a critical period of brain development permanently abolishes estrous cycles and ovulation and induces male behavior. The classical studies with pituitary grafts carried out by Harris and Jacobsohn (in 1952) and confirmed by Adams Smith and Peng (in 1966), showed that this action of the gonads was on the brain. Androgens were found to be as effective as the testis in producing masculini-

zation of the brain (Barraclough 1961). By the early 1960s, it was clear that, irrespective of the genetic sex, in the rodent as well as in several other mammals, the brain is at first neuter or feminine, and in the male is converted to the masculine form by exposure to androgens either *in utero* or during the early neonatal period.

The effect of testosterone is mediated by its enzymatic conversion by aromatase to estradiol-17β. The female brain is thought to be protected from the masculinizing effects of circulating estradiol by the presence of α-fetoprotein, which has a very high affinity for estrogen. In male animals, the brain is protected from the masculinizing actions of androgens by castration shortly after birth. They have the capacity to show estrous cyclicity—as assessed by the formation of corpora lutea in grafted ovaries—as well as female mating behavior.

The mechanism of action of androgens and estrogens in masculinizing the brain has not been established. Estrogen has been shown to stimulate neurite outgrowth from hypothalamic explants in culture, and ultrastructural studies demonstrated sex differences in synapse formation in hypothalamus. The most striking morphological sex difference in the mammalian brain is the sexually dimorphic nucleus of the preoptic area first shown by R. Gorski et al. in 1984. In the male rat, this nucleus is about three to five times the size of that in the female, and the size of the nucleus in the female can be converted to that in the male by the administration of androgens before the critical period of brain development (postnatal day 5 in the rat). However, in spite of its anatomical prominence, the function of the sexually dimorphic remains unknown. Functionally meaningful effects of sex steroids on sexual differentiation of the brain come from studies, pioneered by Nottebohm et al., on the sex differences in the telencephalic nuclei that control song in some songbirds because the functional anatomy of these nuclei is reasonably well understood. Androgens switch the song system on, but it seems that the central nuclei, which control song, are only responsive to androgen in the adult if the bird has been exposed to a surge of androgen or estrogen at hatching. The female brain in birds may be protected from the masculinizing effects of androgens by enzymatic inactivation.

What significance do these findings have for humans? Well, human sexual outlook and behavior is governed more by gender assignment and social factors and, therefore, the precise role and importance of sexual differentiation of the brain by sex steroids is more difficult to identify. However, there are three genetic deficiencies that suggest that androgen exposure *in utero* and possibly during the early neonatal period does influence sexual differentiation of the brain in the human.

First, there is the adrenogenital syndrome in which, because of a mutation in one of several enzymes, the adrenal gland secretes large quantities of androgen during fetal and postnatal life. A cohort of women with the adrenogenital syndrome, followed at Johns Hopkins hospital for about 20 yr by Money, Ehrhardt et al., showed that although most have normal menstrual cycles they also have a greater degree of male characteristics on psychosocial tests compared to women with androgen insensitivity or Mullerian duct aplasia.

Second, there is the testicular feminization or androgen insensitivity syndrome that occurs also in cattle, rats, and mice. Individuals with the disorder are genotypically male, but phenotypically female as a consequence of the fact that androgen receptors are absent or defective owing to a variety of mutations of the androgen receptor gene. Thus, although testes are present and secrete androgens, the tissues cannot respond to testosterone or 5α-dihydrotestosterone. As a consequence of normal plasma concentrations of testicular estrogens, the individual undergoes a "feminizing" puberty that results in the development of breasts and female appearance, but no pubic or axillary hair. Individuals with testicular feminizing syndrome show feminine behavior as would be predicted from androgen-deprived rodents. However, given the fact that in the rodent, sexual differentiation of the brain is effected by estradiol rather than testosterone itself, the feminine self-perception of subjects with androgen insensitivity syndrome may at first sight seem puzzling. A possible explanation is that because the androgen receptor is known to be important for normal activity of the aromatase enzyme, defective androgen receptors may lead to a deficiency in aromatase and, therefore, an inability to convert testosterone to estradiol.

Third, testosterone is normally converted in the periphery to 5α-dihydrotestosterone, a powerful androgen. This steroid cannot masculinize the brain, but is important for masculinizing the genitalia. Imperato-McGinley and associates carried out an important study in the Dominican Republic on pseudohermaphrodites with a deficiency of 5α-reductase that converts testosterone to 5α-dihydrotestosterone. At birth, the external genitalia of these individuals are female in appearance and consequently the affected individuals

are raised as girls. Under the influence of testosterone secreted in large amounts at puberty, the external genitalia become male in appearance, and in spite of the fact that they have been brought up as girls, the individuals now take on a male gender role. This led Imperato-McGinley et al. to conclude that exposure of the brain to testosterone *in utero,* neonatally, and at puberty, appears to contribute substantially to the formation of male gender identity. The syndrome of 5α-reductase deficiency is in keeping with the rodent model of sexual differentiation of the brain which predicts that 5α-dihydrotestosterone plays no significant role in masculinizing the brain; the brain in this syndrome will have been masculinized by testosterone or estrogen.

These three genetic deficiencies together with other data suggest that the effect of sex steroids in man is as important for sexual differentiation of the brain as in the rodent. Clearly, behavior and psychosocial attitudes may be permanently influenced by the nature of steroid exposure of the brain during early development. Further intensive genetic studies are warranted in this important psycho-social area.

8.2.2. Neural Plasticity: Long-Term Reversible Effects of Steroids

The bed nucleus of the stria terminalis (BNST) provides a dramatic example of the effects of sex steroids on neural plasticity. The stria terminalis is the main nerve fiber tract that connects the hypothalamus with the amygdala, an important component of the limbic system concerned with emotion, olfaction, aggression and the control of related behaviors, and central neuroendocrine systems. Equivalent to a junction box within the stria terminalis, the BNST contains neurons, concerned with olfactory or "social" memory, that project to the lateral habenula and lateral septum. These neurons utilize AVP as a neurotransmitter. The expression of AVP in the BNST, but not in the PVN or SON of the hypothalamo-neurohypophysial system is dependent on normal levels of testosterone or estrogen. Thus, the AVP concentrations in the BNST neurons fall to undetectable levels after castration or ovariectomy, and can be restored to normal by the administration of either of these two sex steroids. The action of testosterone is dependent upon its conversion to estrogen, and the action of estrogen or testosterone is mediated by AVP gene transcription (Figs. 15 and 16). The reason for the exquisite sensitivity to sex steroids of the BNST, but not the PVN or SON, remains to be established, but perhaps one relevant difference is that the BNST contains high concentrations of both alpha and beta estrogen receptor and aromatase whereas the AVP and SON contain only relatively low concentrations of the beta estrogen receptor and little if any aromatase.

8.3. Effects of Sex Steroids on Neurotransmission (Relevance for Mood and Mental State)

Many studies have demonstrated that adrenal and gonadal steroids affect the turnover of monoamine neurotransmitters by actions on the enzymes that synthesize and metabolize monoamines. Recent studies carried out by Fink and associates on rodents have shown that estrogen increases the expression of the genes for the serotonin 2A receptor and serotonin transporter in the dorsal raphe nucleus of the midbrain with a concomitant increase in the density of serotinin 2A receptors and serotonin transporter sites in higher forebrain centers which in man are concerned with the control of mood, cognition, mental state, emotion, and memory. Specifically, acute (32 h) treatment with estradiol or testosterone, but not 5α-dihydrotestosterone, induces a significant increase in the density of serotonin 2A receptors in frontal, cingulate and piriform cortex, and in nucleus accumbens. The lack of effect of the potent androgen, 5α-dihydrotestosterone, suggests that the action of testosterone is mediated by its conversion to estradiol-17β through the action of aromatase. This may also explain why estradiol, but not testosterone, increases the density of serotonin 2A receptors in the striatum which has little or no aromatase enzyme. Our findings on the acute effect of estradiol on the serotonin 2A receptor in cerebral cortex have been confirmed by T. di Paolo et al. who studied the long-term (14 d) effects of estradiol. The estradiol-induced changes in density of the serotonin transporter sites occurred in the basolateral amygdala, lateral septum and ventromedial nucleus of the hypothalamus—three areas noted for their role in emotion, and especially aggression. The possible clinical relevance of the latter is that aggression in man is sometimes amenable to therapy with selective serotinin reuptake inhibitors such as fluoxetine ("Prozac") which target the serotonin transporter.

Because the serotonin 2A receptor has been implicated in depression and schizophrenia and the serotonin transporter in depression, our findings offer a rational biological basis for the depressive symptoms which occur in some women premenstrually, around the time of the menopause or after childbirth, times at which estrogen levels fall precipitously. The effect

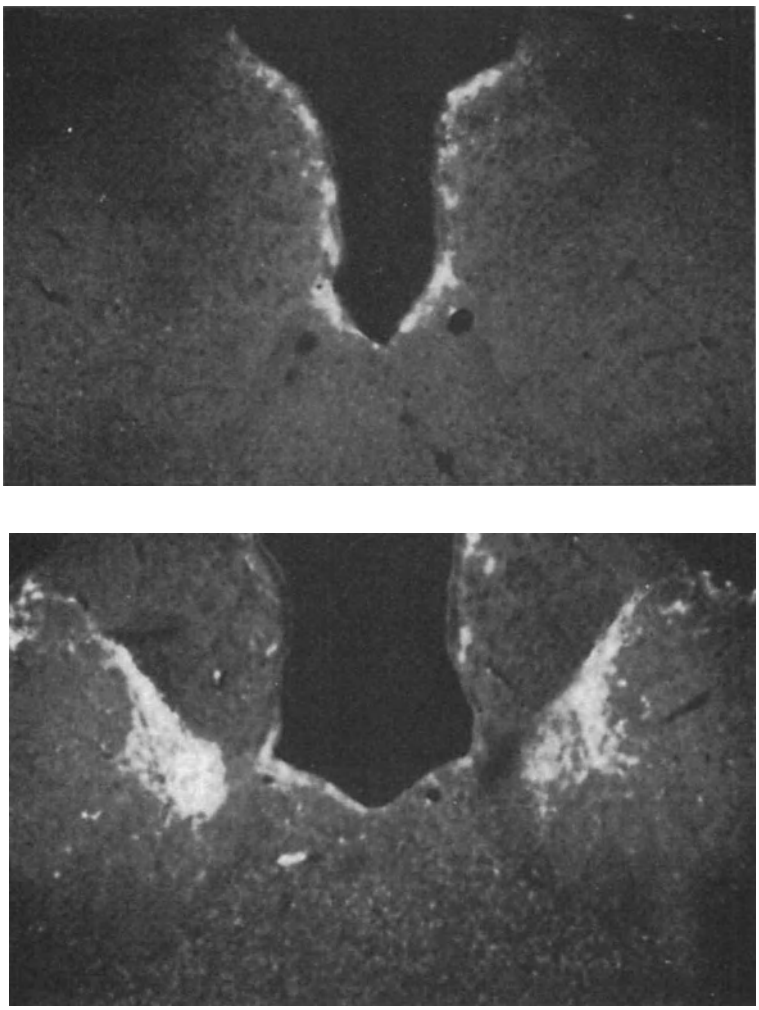

Fig. 15. Photomicrographs of coronal sections taken through the habenula of the hypogonadal (*hpg*) mouse, a mutant deficient in GnRH and therefore in estrogen or testosterone. The lower section was taken from an *hpg* mouse treated with testosterone while the upper section comes from an untreated *hpg* mouse. Note the high density of AVP-containing fibers in the lateral habenula of the mouse treated with testosterone. Similar results were obtained by treatment with estrogen and by the transplantation of a hypothalamic graft from a normal mouse into the third ventricle of an *hpg* mouse. The development of the dense plexus of AVP terminals in the lateral habenula of the *hpg* mouse treated with testosterone is due to stimulation of AVP gene transcription shown in Fig. 16. (Data modified with permission of Mayes et al., *Neuroscience* 1988; 25:1013–1022.)

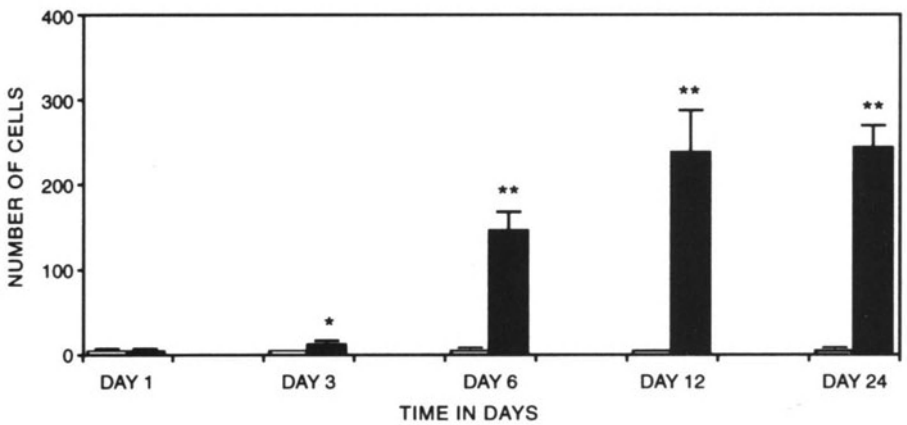

Fig. 16. Shows the dramatic stimulatory effect of testosterone on expression of the AVP gene in the bed nucleus of the stria terminalis (BNST). Mean (±SEM) number of cells expressing AVP mRNA in the BNST of hypogonadal mice at different times after implanting either empty (open bars) or testosterone-propionate containing (closed bars) silicone elastomer capsules. Significance of differences (Mann-Whitney *U* test): $*P < 0.05$; $**P < 0.01$. (Reproduced with permission from Rosie et al., *Mol Cell Neurosci* 1993; 4:121–126.)

of estrogen on serotonin 2A receptors may also help to explain the sex differences in schizophrenia.

The effect of estrogen on the serotonin transporter and the serotinin 2A receptor may have relevance for hormone replacement therapy (HRT). Furthermore, because the serotonin 2A receptor is the major target for atypical antipsychotics such as clozapine and the serotonin transporter is the target for the selective serotinin reuptake inhibitors (above) which are potent antidepressants, the findings outlined above may provide a basis for the use of sex steroids, or their analogs, as adjunct therapy for depression and psychoses.

8.4. Gonadal Steroid Action: Classical Versus Membrane Effects

The "classical" mechanism of steroid action involves transport of the steroid into the cell cytoplasm where it binds to and activates steroid receptors which in turn bind to a specific binding site (steroid response element) of the promoter region of a gene. The activated steroid receptor thereby stimulates or suppresses gene expression. In addition to this "classical" mechanism of steroid action, some steroid effects are likely to involve rapid effects on membranes and membrane receptors. Progesterone and its cogeners, for example, activate the $GABA_A$ receptor and thereby potentiate the influx of chloride ions that results in marked hyperpolarization of the cell. This action of progesterone and its derivatives explains its anesthetic/sedative effect which, first noted by H. Selye, led to the development of alphaxalone (3α-hydroxy-5α pregnane-11,20 dione), a progesterone derivative, which is a potent anesthetic. The sedative effect of progesterone may explain why some women remark on the fact that they feel remarkably calm during the second and third trimesters of pregnancy when progesterone levels are elevated. Similarly, progesterone may also exert an anxiolytic-like effect during the luteal phase of the menstrual cycle. Electrophysiological and other studies show that some of the effects of estrogen may also be mediated by actions at the cell membrane as well as through its cytoplasmic receptors. This point is underscored by our findings and those of Di Poalo and her associates that estradiol increases the density of serotonin 2A and dopamine 2 receptors in the striatum, which appears to be bereft of "classical" cytoplasmic estradiol receptors.

9. PSYCHONEUROENDOCRINOLOGY

The recognition that the secretion of pituitary hormones reflects the activity of hypothalamic neurons which in turn reflects neurotransmitter activity in the brain, provided the basis for the use of pituitary hormone secretion as a marker of disordered central neurotransmission that may underlie or be associated with mental disorders. The growth of this discipline, "psychoneuroendocrinology," has been impeded by our lack of fundamental knowledge about the precise neurotransmitter regulation of hypothalamic neurons that regulate the anterior pituitary gland. Nonetheless, several robust findings have been made and these provide important clues for further research that might optimally be carried out in conjunction with human genetics and sophisticated brain imaging.

Thus, for example, it has long been known that plasma cortisol concentrations are abnormally high in patients with psychosis, and especially severe depression. Furthermore, as shown by the ground breaking work of Carroll and Rubin et al., in many psychotic patients it is not possible to suppress these high cortisol concentrations by injecting a glucocorticoid agonist such as dexamethasone (the "*dexamethasone suppression test*"). Although it cannot accurately discriminate between psychoses, failure of dexamethasone to suppress cortisol levels is a robust method for providing collateral biological evidence that a patient is suffering from a severe psychosis. The inability of dexamethasone to suppress cortisol levels in many patients with depressive psychosis points to a significant abnormality in the regulatory mechanism for ACTH release. Elucidation of the precise nature of this abnormality may help to explain the central neurotransmitter dysfunction that leads to severe depression or other psychoses.

The growth hormone and prolactin responses to pharmacological challenge are also abnormal in certain psychotic states. In anorexia nervosa, for example, a life-threatening condition, which occurs predominantly in young women, the normal growth hormone surge that can be induced by infusion of L-tryptophan, the precursor for serotonin synthesis, is completely absent. This cannot simply be attributed to a lack of food intake because the GH response to L-tryptophan infusion is enhanced, rather than suppressed, in women of a similar age who have been placed on a low calorie diet. Again the precise explanation for this phenomenon, shown first by G. Goodwin et al., is not established, but point to an abnormality in central serotonin function that underlies or is associated with anorexia nervosa.

The advantages of the psychoneuroendocrine investigation of mental disorders is that it is cheap, only mildly invasive, and can be carried out in

severely ill patients. Its power will depend upon further fundamental studies to enable us accurately to interpret neuroendocrine responses to pharmacological challenges and correlative studies with sophisticated brain imaging and human genetics.

SELECTED READINGS

Antoni F. Vasopressinergic control of pituitary adrenocorticotropin secretion comes of age. Front Neuroendocrinol 1993; 14:76–122.

Baulieu EE. Neurosteroids: of the nervous system, by the nervous system, for the nervous system. Recent Prog Horm Res 1997; 52:1–32.

Behan DP, Grigoriadis DE, Lovenberg T, Chalmers D, Heinrichs S, Liaw C, De Souza EB. Neurobiology of corticotropin releasing factor (CRF) receptors and CRF-binding protein: implications for the treatment of CNS disorders. Mol Psychiatry 1996; 1:265–277.

Buggy JJ. Binding of α-melanocyte-stimulating hormone to its G-protein-coupled receptor on B-lymphocytes activates the Jak/STAT pathway. Biochem J 1998; 331:211–216.

Cone RD, Lu D, Koppula S, Vage DI, Klungland H, Boston B, Chen W, Orth DN, Pouton C, Kesterson RA. The melanocortin receptors: agonists, antagonists, and the hormonal control of pigmentation. Recent Prog Horm Res 1996; 51:287–317.

Crowley WF, Jr., Filicori M, Spratt DI, and Santoro NF. The physiology of gonadotropin-releasing hormone (GnRH) secretion in men and women. Recent Prog Horm Res 1985; 41:473–531.

Cyr M, Bosse R, and Di Paolo T. Gonadal hormones modulate 5-hydroxytryptamine$_{2A}$ receptors: emphasis on the rat frontal. Neuroscience 1998; 83:829–836.

Fan W, Boston BA, Kesterton RA, Hruby VJ, Cone RD. Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. Nature 1997; 385:165–168.

Fink G. The GW Harris Lecture. Steroid control of brain and pituitary function. QJ Exp Physiol 1988a; 73:257–293.

Fink G. Gonadotropin secretion and its control. In: Knobil E, and Neill J, eds.). The Physiology of Reproduction. New York: Raven, 1988b: pp. 1349–1377.

Fink G. The self-priming effect of LHRH; A unique servomechanism and possible cellular model for memory. Front Neuroendocrinol 1995; 16:183–190.

Fink G. The psychoprotective action of oestrogen is mediated by central 5-hydroxytryptamine as well as dopamine receptors. In: K Fuxe, T Hökfelt, L Olson, D Ottoson and A Bjorklund, eds. Molecular Mechanisms of Neuronal Communication. Oxford, UK: Pergamon, 1996: pp. 177–204.

Fink G. Mechanisms of negative and positive feedback of steroids in the hypothalamic-pituitary system. In: Bittar E, and Bittar N, Eds. Principles of Medical Biology. Greenwich, CT: JAI, 1997; pp. 29–100.

Fink G and Sumner BEH. Oestrogen and mental state. Nature 1996; 383:306.

Fink G, Dow RC, Casley D, Johnston CI, Bennie J, Carroll S, Dick H. Atrial natriuretic peptide is involved in the ACTH response to stress and glucocorticoid negative feedback in the rat. J Endocrinol 1992; 135:37–43.

Fink G, Sumner BEH, McQueen J, Wilson H, Rosie R. Sex steroid control of mood, mental state and memory. Clin Exper Pharmacol Physiol 1998; 25:764–775.

Gaylinn BD, Harrison JK, Zysk JR, Lyons CE, Lynch KR, Thorner MO. Molecular cloning and expression of a human anterior pituitary receptor for growth hormone-releasing hormone. Mol Endocrinol 1993; 7:77–84.

Georgopoulos NA, Pralong FP, Seidman CE, Seidman JG, Crowley WF, Jr., Vallejo M. Genetic heterogeneity evidenced by low incidence of KAL-1 gene mutations in sporadic cases of gonadotropin-releasing hormone deficiency. J Clin Endocrinol Metab 82:213–217.

Guillemin R. Control of adenohypophysial functions by peptides of the central nervous system. Harvey Lect 1978; 71:71–131.

Harris GW. Hormonal differentiation of the developing central nervous system with respect to patterns of endocrine function. Phil Trans R Soc Lond B Biol Sci 1970; 259:165–177.

King DP, Zhao Y, Sangoram AM, Wilsbacher LD, Tanaka M, Antoch MP, Steeves TD, Vitaterna MH, Kornhauser JM, Lowrey PL, Turek FW, Takahashi JS. Positional cloning of the mouse circadian *clock* gene. Cell 1997; 89:641–653.

Klein DC, Coon SL, Roseboom PH, Weller JL, Bernard M, Gastel JA, Zatz M, Iuvone PM, Rodriguez IR, Begay V, Falcon J, Cahill GM, Cassone VM, Baler R. The melatonin rhythm-generating enzyme: molecular regulation of serotonin N-acetyltransferase in the pineal gland. Recent Prog Horm Res 1997; 52:307–357.

Knobil E. The neuroendocrine control of the menstrual cycle. Recent Prog Horm Res 1980; 36:53–58.

Kozicz T, Yanaihara H, Arimura A. Distribution of urocortin-like immunoreactivity in the central nervous system of the rat. J Comp Neurol 1998; 391:1–10.

Kumar U, Laird D, Srikant CB, Escher E, Patel C. Expression of the five somatostatin receptor (SSTR1-5) subtypes in rat pituitary somatotrophes: quantitative analysis by double-label immunofluorescence confocal microscopy. Endocrinology 1997; 138:4473–4476.

Legouis R, Cohen-Salmon M, Del Castillo I, Petit C. Isolation and characterization of the gene responsible for the X chromosome-linked Kallmann syndrome. Biomed Pharmacother 1994; 48:241–246.

Lincoln GA and Short RV. Seasonal breeding: nature's contraceptive. Recent Prog Horm Res 1980; 36:1–52.

McQueen JK, Wilson H, and Fink G. Estradiol-17β increases serotonin transporter (SERT) mRNA levels and the density of SERT binding sites in female rat brain. Mol Brain Res 1997; 45:13–23.

Mayo KE, Godfrey PA, Suhr ST, Kulik DJ, Rahal JO. Growth hormone-releasing hormone: synthesis and signaling. Recent Prog Horm Res 1995; 50:35–73.

Moss RL, Gu Q, Wong M. Estrogen: nontranscriptional signaling pathway. Recent Prog Horm Res 1997; 52:33–69.

Petersenn S, Rasch AC, Heyens M, Schulte HM. Structure and regulation of the human growth hormone-releasing hormone receptor gene. Mol Endocrinol 1998; 12:233–247.

Rees JL, Healy E. Melanocortin receptors, red hair, and skin cancer. J Invest Dermatol Symp Proc 1997; 2:94–98.

Rominger DH, Rominger CM, Fitzgerald LW, Grzanna R, Largent BL, Zaczek R. Characterization of [125I]sauvagine binding to CRH2 receptors: membrane homogenate and autoradiographic studies. J Pharmacol Exp Ther 1998; 286:459–468.

Schally AV, Arimura A, and Kastin AJ. Hypothalamic regulatory hormones. Science 1973; 179:341–358.

Schindler M, Holloway S, Humphrey PP, Waldvogel H, Faull RL, Berger W, Emson PC. Localization of the somatostatin sst2(a) receptor in human cerebral cortex, hippocampus and cerebellum. Neuroreport 1998; 9:521–525.

Schwanzel-Fukuda M, Pfaff DW. Origin of luteinizing hormone-releasing hormone neurons. Nature 1989; 338:161–164.

Schwanzel-Fukuda M, Crossin KL, Pfaff DW, Bouloux PM, Hardelin JP, Petit C. Migration of luteinizing hormone-releasing hormone (LHRH) neurons in early human embryos. J Comp Neurol 1996; 366:547–557.

Shughrue PJ, Lane MV, Merchenthaler I. Comparative distribution of estrogen receptor-alpha and -beta mRNA in the rat central nervous system. J Comp Neurol 1997; 388:507–525.

Sumner BEH, and Fink G. Estrogen increases the density of 5-hydroxytryptamine$_{2A}$ receptors in cerebral cortex and nucleus accumbens in the female rat. J Steroid Biochem Mol Biol 1995; 54:15–20.

Sumner BEH, Fink G. The density of 5-hydroxytryptamine$_{2A}$ receptors in forebrain is increased at pro-estrus in intact female rats. Neurosci Lett 1997; 234:7–10.

Sumner BEH, Fink G. Testosterone as well as estrogen increases serotonin$_{2A}$ receptor mRNA and binding densities in the male rat brain. Mol Brain Res 1998; 59:205–214.

Takahashi JS. The biological clock: it's all in the genes. Prog Brain Res 1996; 111:5–9.

Yates FE, Maran JW. Stimulation and inhibition of adrenocorticotropin release. In: Greep RO, Astwood EB, eds. Handbook of Physiology: Endocrinology, vol. IV, Washington, DC: American Physiological Society, pp. 367–404.

8 Neuroendocrine Regulation of Reproductive Cyclicity

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CONTENTS

1. INTRODUCTION

A reproductive cycle is a recurring set of events that culminates in the ability to reproduce; that is, to ovulate eggs, mate, achieve fertilization, proceed through pregnancy, and deliver and nurture young. The time interval between consecutive reoccurences of an event within the nonpregnant cycle defines the cycle length. Although the cyclicity of reproduction has been recognized in both humans and domesticated animals for centuries, it has been only recently that the sequence of events responsible for the recurrence of events has been understood.

Seasonal breeders show annual cycles, but may show, within the breeding season, sequential bouts of ovulations. For seasonal breeders, the ratio of light to dark, food availability, or the amount of rain acts a signal for an increase in GnRH (gonadotropin-releasing hormone) pulses, which in turn stimulate gonadotropin secretion. Conspecifics can act as inhibitors or stimulators of reproduction—in the vole the appearance of males aids in the process of ovarian follicular recruitment into a pool susceptible to gonadotropin stimulation. The laboratory rodents such as mouse, rat, and hamster show an ovulation to ovulation interval of about four days, and do not have a functional corpus luteum phase (i.e., progesterone secretion adequate for implantation) unless mating occurs. In contrast, every cycle in primates includes a luteal phase (when the follicle ovulates, turning into a corpus luteum) during which progesterone secretion dominates and implantation can occur. Many species such as the domestic cat and the rabbit show cycles in ovarian follicular growth and estradiol secretion, but do not have "spontaneous" luteinizing hormone (LH) surges. Instead, the estradiol induces mating behavior, which leads to cervical stimulation and reflexive GnRH release leading to the provulatory LH surge. These "coitus-induced" ovulators thus show only the follicular phase (low progesterone) in the absence of a male.

Whereas cycle lengths vary between four days to one year, they share several fundamental features. All cycles include a period of ovarian follicular growth—

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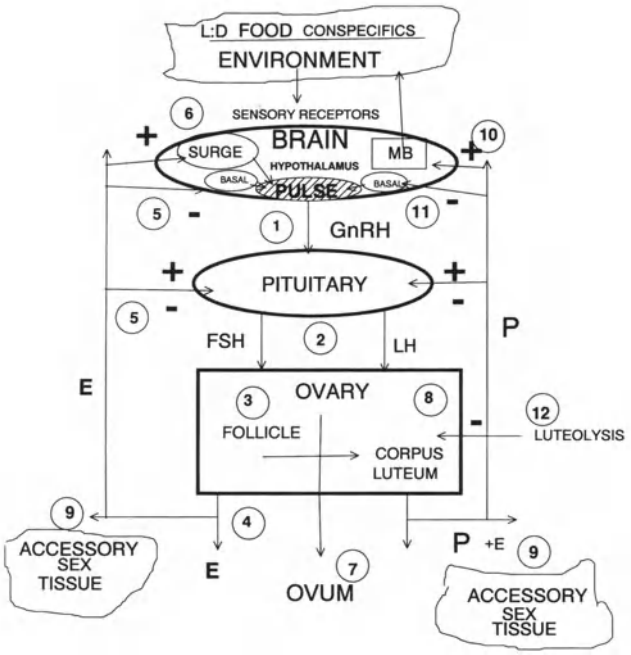

Fig. 1. The overall system that participates in reproductive cycles in female mammals. L:D = ratio of light to dark. FOOD = availability of food in the environment. CONSPECIFICS = presence of males or other females of the same species. GnRH = gonadotropin releasing hormone secretion from the hypothalamus. FSH and LH = follicle-stimulating hormone and luteinizing hormone from the anterior pituitary gland. P = progesterone. E = estradiol. MB = Mating behavior.

the increase in numbers of granulosa or somatic nurse cells surrounding an oocyte. This follicular growth results in increasing secretion of estradiol, which is responsible, in all species, for the triggering of the preovulatory surge of GnRH and LH, which will trigger ovulation. Another common event is that in the process of ovulating, follicles are converted into corpora lutea (CL), which secrete less estradiol and higher levels of progesterone. Ovulation abolishes the microorgan (follicle) that has provided estradiol, the positive feedback trigger, thus removing that stimulus. If pregnancy ensues, the accompanying high levels of progesterone from the CL block the surge mechanism. If pregnancy does not occur, the CL, which have a limited life span, cease secreting progesterone and follicular growth is permitted as the steroid block on baseline LH and follicle-stimulating hormone (FSH) secretion is removed.

The "cycle" does not exist as an independent entity within any one organ or tissue, but in the interrelationships among organs and environmental inputs. Fig. 1 illustrates the players involved in the "cycle." Evidence suggests that signals among the environment, brain, anterior pituitary gland, ovary, and accessory sex tissue drive the repetitive nature of the cycle. The crucial involvement of the brain in regulating the anterior pituitary, in producing behavior, and in monitoring sensory inputs from the environment makes the cycle a "neuroendocrine" process.

The sequence of events is essentially similar regardless of the cycle length (Fig. 1). An intact hypothalamus secretes GnRH in a pulsatile manner, which causes the anterior pituitary gland to secrete FSH and some LH. Within the ovary, some follicles begin to grow because of granulosa cell mitosis and secrete estradiol. Rising levels of estradiol increase granulosa cell mitoses, acting with FSH, and exert tonic negative feedback effect on LH and FSH secretion. Eventually, estradiol levels reach a critical threshold value and trigger a preovulatory surge of GnRH and/or alter the pituitary gland's sensitivity to GnRH resulting in the LH (and FSH) surge. The surge not only causes ovulation, producing an ovum or ova in the oviduct ready for fertilization and reduces follicular estrogen secretion, but additionally converts the follicle(s) into CL, which secrete progesterone (and some estradiol) in large quantities. The luteal steroids act on sex accessory tissue, preparing the uterine endometrial lining for implantation of a fertilized egg a few days after fertilization if mating has occurred, triggered by estradiol and progesterone. Progesterone exerts a suppression of LH and GnRH. If there is no mating or implantation the corpus luteum dies, progesterone drops and stops inhibiting GnRH, LH, and FSH secretion. If GnRH pulses are still occurring, the next cycle starts. If successful mating occurs, the dividing fertilized egg eventually implants into the uterine lining and prevents luteolysis; the maintenance of high progesterone levels blocks further ovulation and maintains a quiescent uterus for the pregnancy.

2. EVENTS THAT OCCUR IN THE OVARY (FIG. 2)

The ovary contains oocytes encased within follicles. The smallest follicles contain only one layer of thecal cells and one layer of granulosa cells and are called "primordial" (or primary) follicles. They do not increase in size until serum FSH and LH rise above a certain level. Follicles become bigger by virtue of granulosa cell division under the influence of basal levels of FSH and LH and the estradiol produced locally in the granulosa cells. At this time, ovarian physiologists do not know the local conditions

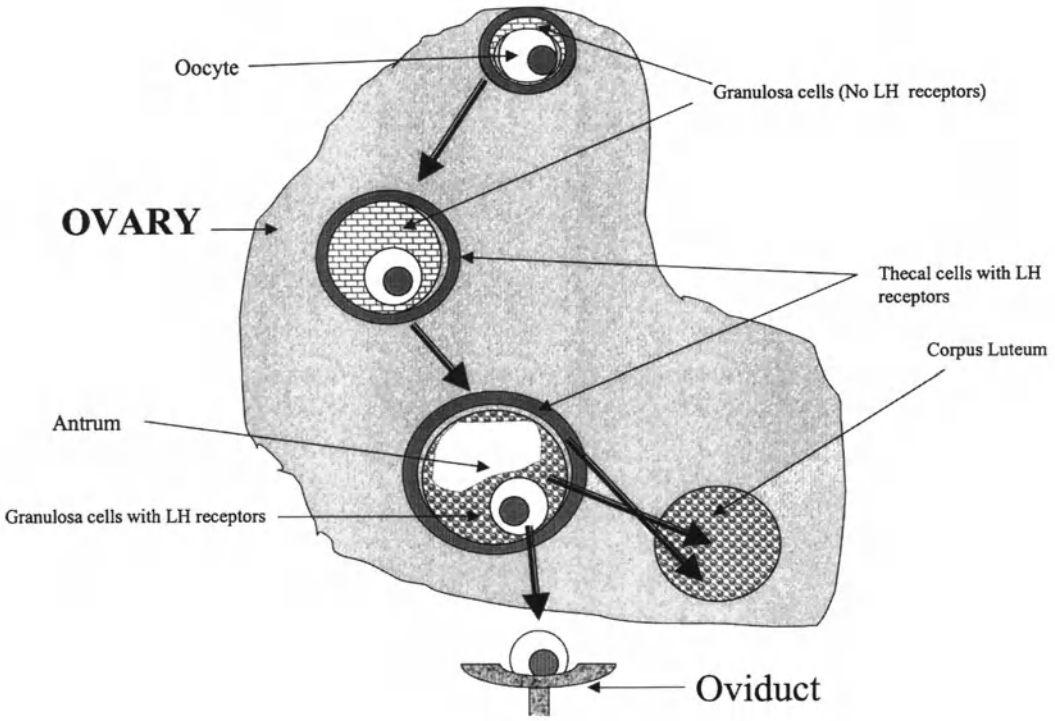

Fig. 2. Cartoon of ovarian follicular development: a single follicle is followed as it matures and ovulates. Granulosa cells have FSH-R throughout their lifespan.

that cause a *given* primordial follicle or follicles to begin to grow and respond to FSH.

Granulosa cells have membrane receptors for FSH (FSH-R). Cells in the thecal layer on the outside of the follicle synthesize androgens (androstenedione or testosterone) under the influence of serum LH, which acts on membrane LH receptors (LH-R). [LH and FSH receptors are both members of the seven-membrane receptor family, which is linked inside the cytoplasm to adenylate cyclase, which triggers the intracellular cascade resulting in hormonogenesis]. The androgens diffuse across the granulosa cells, which synthesize estradiol from testosterone. Thus, thecal cells contain the enzymatic machinery for testosterone synthesis from cholesterol, and granulosa cells contain the enzyme aromatase, which converts testosterone to estradiol under the influence of FSH. This is known as the "two-cell" hypothesis of estradiol synthesis. Estrogens serve as a paracrine mitotic signal for granulosa cell growth as well as an endocrine signal to the hypothalamus, pituitary, and other target tissues like the uterus (*see* Fig. 1).

The maturation of follicles, once growth has started, is characterized by acquisition of LH-R (under the influence of FSH secretion) by the granulosa cells—with this comes the enzymatic potential to secrete progesterone (*see* Table 1). Of the group of follicles that starts to mature in each cycle, most fail to continue and die in a programmed cell death called "atresia." Only those follicles (or *single* follicle in the case of single ovulators) that are mature at the time of the preovulatory LH surge can ovulate.

The preovulatory LH surge initiates a set of events within the follicle that alters its morphology and its enzymatic machinery (*see* Table 1) resulting in a number of events. (1) There is a switch from estradiol to progesterone as the principal steroid secreted; (2) resumption of oocyte meiosis occurs, resulting in throwing off of the first polar body (containing half the chromosomes); (3) degrading of the follicular/ovarian wall so that the oocyte can leave the ovary and enter the oviduct. Blood vessels invade the follicle, thecal, and granulosa cells intermingling to form the corpus luteum (Fig. 1). The corpus luteum has a limited lifespan, and this lifespan is prolonged by events associated with pregnancy.

Table 1 (modified from Richards et al., 1995) summarizes the changes in mRNAs and translated proteins during folliculogenesis for a number of important hormones and enzymes. The combination of FSH and the estradiol secreted by the granulosa cells is responsible for upregulating (1) the P450 aromatase, which synthesizes estradiol from androgens; (2) LH-R receptors necessary for conferring responsivity within these cells for LH; (3) the RIIb regulatory subunit, which is part of the PK-A (protein kinase A) necessary for adenyl cyclase production and internal signaling for LH; (4) inhibin subunits made by the granulosa

Table 1
Genes Regulated in the Follicle by Gonadotropic Hormones

Gene/Protein	*Function*	*Regulation*
P450 Aromatase	Estradiol synthesis	upregul by E and FSH/downregul by surge
LH receptor	LH transduction	upregul by E and FSH/downregul by surge
RIIβ	Regulatory subunit of type IIβPK-A	upregul by E and FSH/downregul by surge
Inhibin subunits	Lowers FSH	upregul by E and FSH/downregul by surge
PGS-2	Prostaglandin synthesis	upregul by LH surge
P450 scc	Progesterone synthesis	upregul by LH surge
PR	Progesterone transduction	upregul by LH surge

[Adopted from Richards et al.] (See text for discussion)

cells necessary to maintain FSH at correct levels by inhibiting FSH secretion by the anterior pituitary. Inhibin also acts as a local paracrine signal.

When the preovulatory LH surge occurs, the preceding genes are downregulated (Table 1) and concomitantly, a number of other genes are upregulated. Among them is (1) prostaglandin synthetase (PGS-2) which increases prostaglandin that weakens the follicle wall in preparation for ovulation; (2) P450 side-chain cleavage enzyme, which leads to progesterone synthesis and secretion; and (3) progesterone receptor necessary for progesterone secretion and luteinization.

3. SYNTHESIS AND SECRETION OF THE GONADOTROPINS BY THE ANTERIOR PITUITARY GLAND

The anterior pituitary gland contains gonadotropes, cells that can synthesize and secrete the gonadotropic hormones LH and FSH. These cells contain membrane receptors for GnRH and activin (probably inhibin as well). They have cytoplasmic or nuclear receptors for estradiol and progesterone. In addition to synthesizing LH and FSH, these cells make activin and follistatin. A model of the significant input signals and output hormones is shown in Fig. 3.

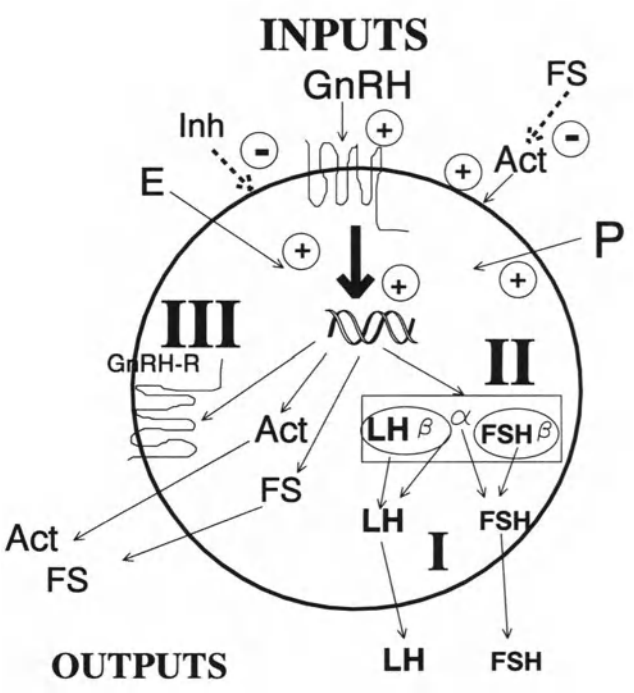

Fig. 3. Regulation of gonadotropin (FSH and LH) secretion in the gonadotrope. GnRH = gonadotropin releasing hormone. Act = activin. FS = follistatin. P = progesterone. E = estradiol. G = glucocorticoid. Inh = inhibin. GnRH-R = GnRH receptor. LHβ, FSHβ, α = respective gonadotropin subunits.

Each gonadotropin consists of two peptide subunits or chains: the alpha subunit (about 92 amino acid residues) is the same whereas the beta subunits for FSH and LH (about 112 residues) are different and confer hormone specificity. These subunits are glycoproteins with internal disulfide bridges; the MW of mature LH and FSH is about 28,000. Virtually every gonadotrope cell synthesizes all three subunits and can secrete free-alpha subunits while secreting heterodimeric LH and FSH. The gonadotropes have membrane receptors for GnRH—the typical seven transmembrane domain type.

Three aspects of gonadotrope function are regulated. The rates of *secretion* of LH and FSH, and their relative amounts can be altered (Fig. 3-I), the rates of *synthesis* of the three subunits can shift (Fig. 3-II) (as well as synthesis of activin and follistatin), and the *numbers* of GnRH receptors can be changed (Fig. 3-III).

The intracellular mechanism(s) by which GnRH

(amplitude and pulse frequency) leads to synthesis and secretion of gonadotropins has been intensively studied. In spite of this, we are still not sure of the specific function of the several pathways and mechanisms because of the heterogeneity of primary pituitary-cell cultures and the lack of cell lines expressing all three subunits. GnRH receptors (GnRH-R) couple to G proteins of the Gq/11 family, leading to phosphoinositide turnover, with resultant increase in intracellular calcium and protein kinase C (PKC) activation. Extracellular calcium is involved, as is cAMP and diacylglycerol (DAG). In the short term, movement of extracellullar calcium into the gonadotrope can cause release of LH hormone (*see* Fig. 3-I). In the presence of increases in GnRH signaling, cAMP and DAG rise. Phospholipase C induces the production of DAG and IP3. The DAG acts on PKC, which directly admits calcium ions through a membrane channel. Although calcium via calmodulin is responsible for LH release, the action of PKC is necessary to increase subunit transcription and synthesis (*see* Fig. 3-II).

In primary-cell cultures, calcium appears to be more important in altering LH-β subunit synthesis and PK-C in regulating alpha subunit production. GnRH pulse interval is clearly linked both to subunit synthesis and hormone release, with long pulse intervals diminishing LH and enhancing FSH synthesis and release. Utilizing GH3 cells (a lactotrope derived line) transfected with GnRH-R and the gonadotropin subunits it was shown that the higher the number of GnRH-R expressed, the lower the relative stimulation of FSHβ subunit. Because low levels of GnRH-R result from infrequent pulses, it is suggested that GnRH-R numbers (*see* Fig. 3-III) regulate relative FSH-LH production by altering the intracellular signaling pathways with low frequencies of GnRH secretion favoring FSH secretion and synthesis. Additionally, low frequencies have been directly shown to favor a low number of GnRH receptors per gonadotrope (Fig. 3-III), and low rates of synthesis of follistatin in the pituitary.

The extracellular signals that influence relative gonadotropin synthesis and secretion rates are summarized in Table 2. Inhibin is a protein heterodimer of an alpha subunit (20-kd) and a β subunit (15-kd) in the TGF-β family. Inhibin is synthesized in ovarian follicles and secreted into the blood. Inhibin suppresses FSH synthesis and secretion, having virtually no effect on LH. Activin is a dimer of two β subunits and stimulates FSH synthesis and secretion. Activin is synthesized in both the ovary and the anterior pituitary. Follistatin is a protein, not homologous to the inhibin family. It is also synthesized in both the ovary and pituitary. Follistatin suppresses FSH by combining with activin. This complex trio profoundly influences FSH secretion.

Table 2
A Summary of Factors That Alter Gonadotrope FSH to LH Secretory Ratio

Increase FSH/LH Ratio	*Increase LH/FSH Ratio*
GnRH Low Frequency	GnRH High Frequency
Low GnRH Receptor No.	High GnRH Receptor No.
Low Inhibin	Ovariectomy
High Activin	Castration
Progesterone	
Low Follistatin Level	
Glucocorticoids	
GnRH Antagonists	

In vivo, estradiol suppresses gonadotropin secretion rates, LH more than FSH. In vitro, pituitary-cell culture estradiol can increase FSH secretion or decrease it. Estradiol can also increase the number of GnRH-R in the anterior pituitary.

4. REGULATION OF GnRH SYNTHESIS AND SECRETION DURING THE CYCLE

GnRH is synthesized by a group of neurons with cell bodies in the anterior hypothalamus projecting dorsally to terminals in the medial basal hypothalamus where they can secrete into the hypothalamic-hypophyseal portal system. GnRH is secreted in a pulsatile manner to act directly on membrane receptors in the gonadotrope cells of the anterior pituitary gland. GnRH must be released in pulses in order to maintain gonadotrope responsivity. The aspect of the GnRH neurons that is responsible for secretion is called the "pulse generator." The pulse generator appears to be located within the preoptic area, although aspects of it could also emanate from the terminals near the portal system. Lesions of either the preoptic area or within the medial basal hypothalamus can block ovulation.

The pulsatile nature of GnRH secretion was initially inferred by the presence of LH pulses, but it has now become possible to directly measure these pulses in portal vein blood. Additionally, hypothalamic multiunit electrical action potentials correlate with LH pulses. A number of neurohormones act on the GnRH neuron, including catecholamines, neuropeptide Y, neurotensin, GABA, and the opiates.

A major issue in the hypothalamic regulation of GnRH is whether negative feedback of gonadal steroids such as estradiol acts at different anatomical sites from the positive feedback, which leads to the preovulatory GnRH surge. The evidence in general suggests that the two feedbacks are at two different anatomical loci. Estradiol and progesterone also act directly at the gonadotrope level, where their action is generally to increase gonadotropin secretion.

GnRH pulse frequency and amplitude vary during the cycle. GnRH pulse frequency can be changed by gonadal steroids. At low levels, estradiol exerts a negative feedback on GnRH secretion. This action of estradiol is probably exerted in the medial basal portion of the hypothalamus. A specific manifestation of the preovulatory rise in estradiol is synaptic retraction in the arcuate nucleus, which has been proposed to disinhibit the negative feedback signal. The GnRH neurons release the preovulatory GnRH surge, presumably by positive feedback of estradiol at the level of the anterior hypothalamus. Separation of the anterior hypothalamus from the medial basal hypothalamus blocks the GnRH and LH surge and ovulation, but permits some negative feedback.

Can the partially differential release of LH and FSH (see below) be wholly explained on the basis of the factors listed in Table 2, or is there a hypothalamic FSH releasing factor (FSH-RF) independent of GnRH? This issue has been argued back and forth, but there is as yet no definitive evidence for a separate FSH-RF.

5. EXAMPLES OF CYCLES: RAT, SHEEP, AND PRIMATE

We will now describe three species in detail: rat, sheep, and primate. These animals have been the major species studied and much of the foregoing general discussion is based on data obtained in them. In Figs. 4, 5, and 6 the hormonal changes occurring during a single cycle for the rat, ewe, and monkey are displayed. In Fig. 7, the three cycle types are depicted again, emphasizing the causative sequence of events. As can be seen, there is a surge or spike of blood LH (and FSH) followed by ovulation and progesterone secretion. In all three species, the surges are preceded by a rise in blood estradiol, which decreases following the surge of LH. Despite these very important congruences among these cycles, there are some important species differences. Most obvious from these figures is the wide range of ovulation-to-ovulation intervals.

5.1. Rat—Circadian Driven (Fig. 4)

The estrous cycle of the female laboratory rat has been an excellent model for reproductive studies. (1) The follicular cycle is short, (2) the animal is small and does not occupy a lot of space, and (3) the rat is cheaper to buy than other animals.

The secondary FSH surge (seen in Fig. 4 during the night between proestrus and estrus) recruits a group of follicles (about 30 in each ovary of which about seven ovulate) into a class that can then grow and be ready to ovulate four days later when the next LH surge will occur if mating has not occurred. These follicles begin to secrete some estradiol (and inhibin) under the influence of basal levels of FSH and LH, leading to increasing estradiol levels (Fig. 4). On the day of metestrus, the corpora lutea from the most recent ovulation secrete progesterone for several hours. In some rats, this progesterone secretion is prolonged, which leads to a five-day rather than a four-day cycle. The rising estradiol induces increased numbers of GnRH-R in the pituitary gland (Fig. 4).

Every day in the female rat, there is a circadian signal to the hypothalamus, which leads to a brief time of day when rising estradiol, if above threshold, can trigger a surge of GnRH from the median eminence. That critical combination of events occurs on the day of proestrus. On a lighting schedule of lights on at 0500 h, off at 19:00 h, the critical period for GnRH release starts at 1400 h and runs for about 2 h. This causes LH to be released in a surge, and FSH secretion as well. As can be seen from Fig. 4, GnRH-R number is at its peak at proestrus—the rising estradiol has induced these receptors.

The critical period signal is known to occur every day because if GnRH release is blocked at proestrus by an injection of an anesthetic such as a barbiturate, LH release is delayed by exactly 24 h. Rats that have been ovariectomized and treated with high doses of estradiol release LH every day during the same critical time period. This repetitive release does not occur in the intact rat because progesterone secretion, which follows the LH surge in the intact rat, wipes out the circadian signal so that the LH surge is restricted to one day.

When the primary surges of LH and FSH are secreted on proestrus they cause progesterone to rise as estradiol secretion and inhibin drop. The LH surge also causes resumption of oocyte meiosis and weaken-

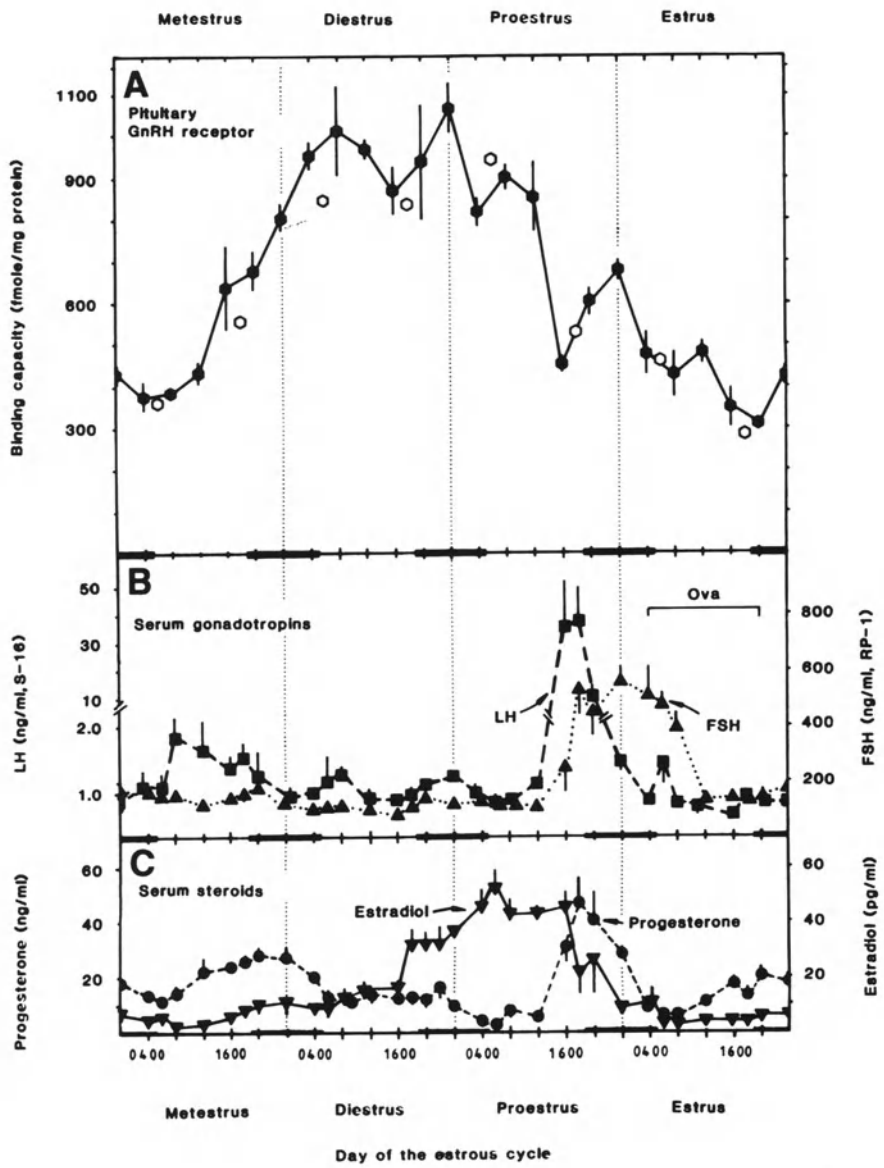

Fig. 4. Hormone changes during the rat estrous cycle. **(A)** The binding capacity of D-Ala^6 analog to pituitary plasma membrane preparations from rats decapitated at 4-h intervals during the estrous cycle. Means ± S.E. Open symbols indicate the binding capacity calculated by Scatchard analysis of competition curves using membranes from rats decapitated at 0600 and 1800 h. **(B)** Serum LH (■) measured in units of NIH LH S-16 and serum FSH (▲) measured in units of NIH FSH RP-1. Note the scale break and scale change for LH values. Means ± S.E. are plotted. Black bars on the time scale indicate periods of darkness, and the light vertical dotted lines divide the days of the cycle. **(C)** Serum estradiol (▼) and progesterone (●) are represented. (Reprinted with permission from Savoy-Moore et al. Pituitary gonadotropin-releasing hormone receptors during the rat estrous cycle. *Science* 209:942–944. Copyright 1980 American Association for the Advancement of Science.)

ing of the wall surrounding the expanding follicles and finally ovulation (*see* Table 1). The progesterone released, acting on estradiol priming, causes mating behavior. If mating occurs, the resultant cervical stimulation causes twice daily surges of prolactin from the pituitary gland which "rescues" the CL and maintains progesterone secretion for 12 d. Implantation occurs on the fifth day and the placenta takes over the necessary progestational functions. In the absence of mating and prolactin release, the CL of the rat cycle undergo spontaneous luteolysis and progesterone secretion is withdrawn.

The secondary surge of FSH occurs as a result of a drop in circulating inhibin, and perhaps a drop in pituitary follistatin. This prolonged secretion of FSH "recruits" a group of follicles within the ovaries into a size, which can then respond to FSH and LH and grow up for the next cycle.

5.2. Primate—Pelvic vs Hypothalamic Clock (Fig. 5)

The hormonal changes that occur during the primate menstrual cycle are summarized in Fig. 5. During the follicular phase, estradiol is secreted from the growing follicles. The initially elevated FSH recruits follicles into growth. Eventually one follicle (from either ovary) becomes dominant—other follicles become atretic because of negative feedback of the increasing estradiol levels on gonadotropin secretion. When estradiol reaches a threshold level, an increase

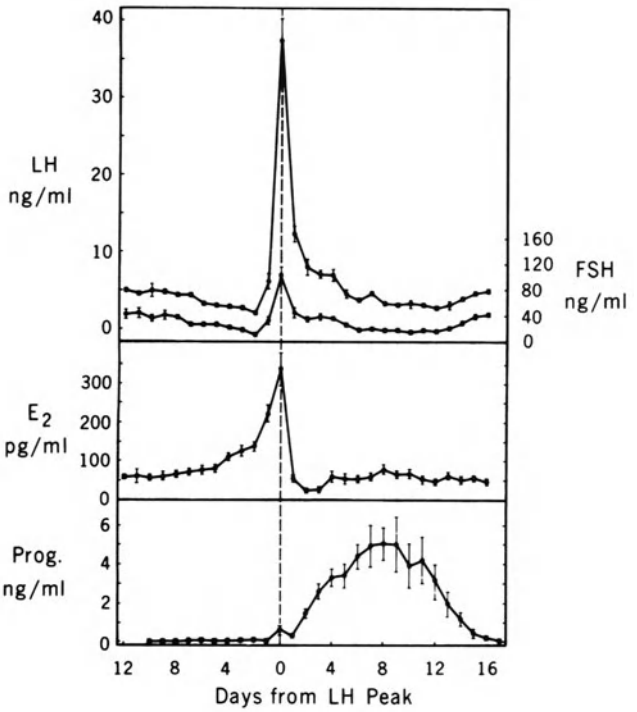

Fig. 5. Hormone changes during the monkey estrous cycle. Concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol (E_2), and progesterone (Prog) in plasma samples taken daily throughout the menstrual cycle of rhesus monkeys (*Macaca mulatta*). The data are normalized to the day of the LH peak (day 0). (From Hotchkiss and Knobil, 1994, with permission.)

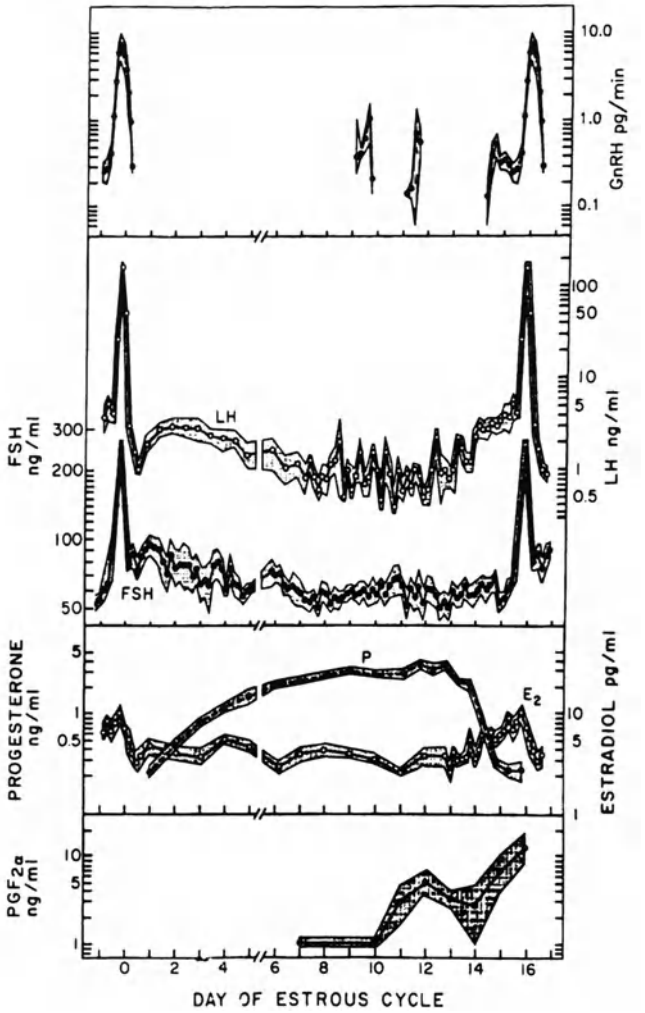

Fig. 6. Changes in hypophyseal portal vein GnRH, mean peripheral concentration of LH, FSH, progesterone (P), and estradiol (E_2), and uterine vein concentrations of $PGF_{2\alpha}$ throughout the ovine estrous cycle. *Shaded areas* depict ±SEM for each hormone. Values from days −1 to 5 normalized to the first LH surge (on day 0); values from days 6 to 17 normalized to the second LH surge (on day 16). (From Goodman, 1994, with permission.)

in GnRH secretion occurs and the preovulatory surges of LH and FSH occur on about day 14. Ovulation takes place within 24 h. The CL that remains in the ovary secretes progesterone and some estradiol. The CL has a secretory lifespan of about 14 d. If implantation of an embryo takes place, about five days after ovulation the embryonic chorion layer begins to secrete chorionic gonadotropin—a modified form of LH with a longer half-life. This gonadotropin rescues the CL and permits it to secrete progesterone for another 60 d or so, after which the placenta secretes progesterone. As far as is known, there is no active luteolytic substance that terminates CL function, but the CL have an intrinsic lifespan.

GnRH secretion rates during the monkey (and human) cycle change from phase to phase as inferred from LH pulses. During the follicular phase, LH pulses are more frequent than they are during the luteal phase. If the hypothalamus and pituitary are disconnected, all hormonal changes cease. If the monkey (or human lacking GnRH pulses) is provided with a pump that injects GnRH at unvarying hourly intervals ("clamped"), pituitary and ovarian hormone secretion resumes and ovulation still occurs at approximately a 28-d interval. Hotchkiss and Knobil (1994) have described this as the "pelvic" clock and interpret the data as indicating that the ovary, by secreting estradiol, sensitizes the gonadotropes to release the LH surge. However, whereas this may be true, as seen in the human female, there is in fact an increase in GnRH secretion in the monkey that precedes the LH surge.

Removal of the ovary in the monkey or human causes a rise in circulating LH and FSH, which can be lowered by estradiol injection, demonstrating negative feedback. This LH suppression occurs even in

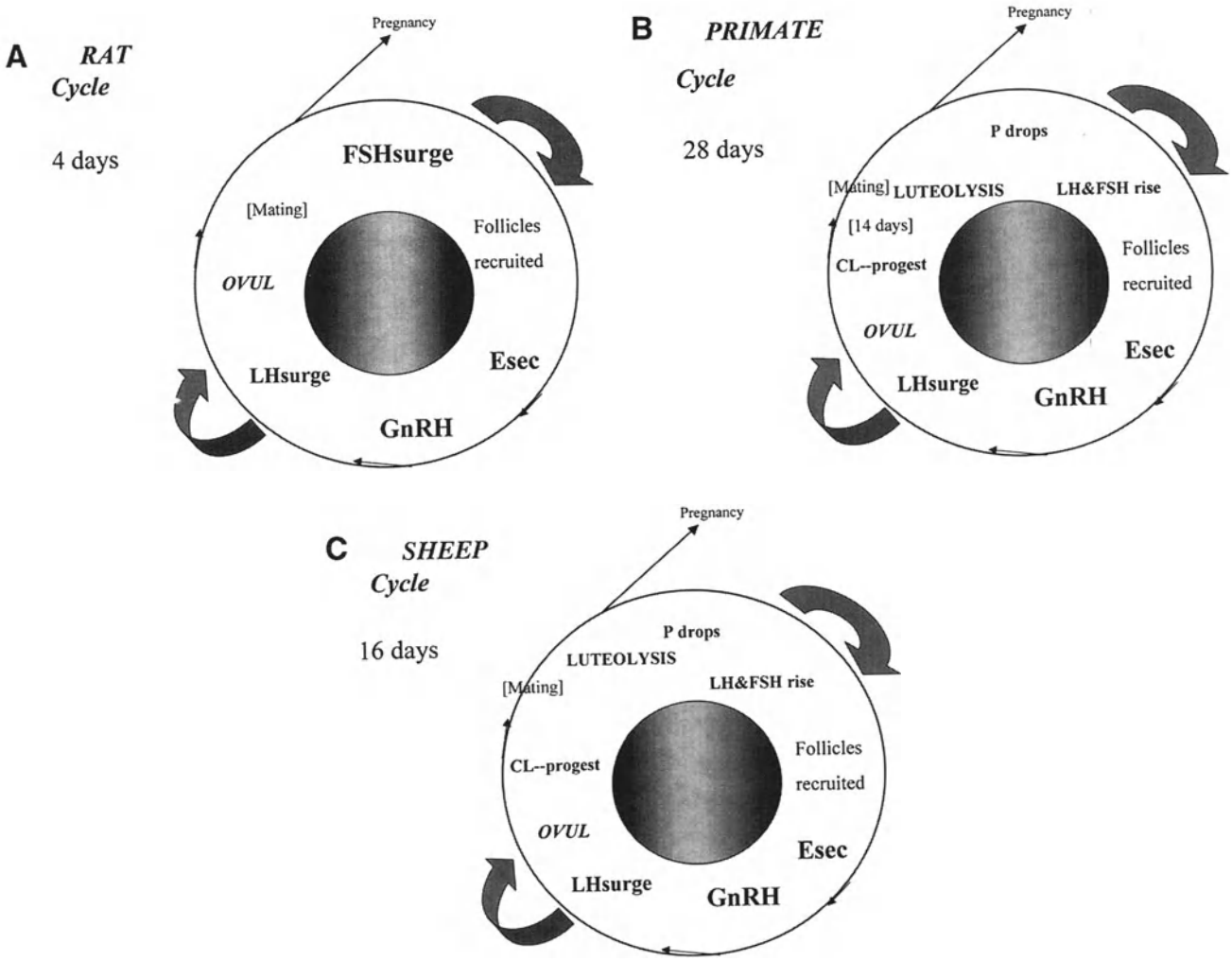

Fig. 7. Sequence of events during rat, primate, and sheep cycles, emphasizing the similarity of sequential events.

the GnRH "clamped" monkey. Injection of estradiol in ovariectomized monkeys can lead to an LH surge. Adult males can also release an LH surge in response to estradiol after castration and estrogen priming (Hotchkiss and Knobil, 1994); in contrast to the rodent, in which adult males cannot respond to estrogen with an LH surge. Therefore, the hypothalamic regulation of the primate reproductive cycle is less important than in the rodent (or in the sheep). Pentobarbital, which blocks the GnRH surge leading to the LH surge in rodents and sheep, does not work in the primate. However, the primate cycle is not totally devoid of neural regulation. For example, it is known that menstrual cycles become synchronized among women living in dormitories. Synchronization is probably because of pheromonal exchange.

Removal of a corpus luteum during the cycle lowers serum progesterone and causes the next cycle to start prematurely. Thus progesterone exerts a negative feedback on gonadotropin secretion in the primate, as can be seen by suppression of follicular development during the luteal phase. This negative feedback is the basis of the contraceptive action of the oral contraceptives in humans.

5.3. Sheep—Seasonally Driven (Fig. 6)

When English sheep farmers sailed to Australia, their sheep, which had bred in England in the early fall months, reversed their breeding cycle and began breeding in the spring months. In both cases, the breeding occurred as the hours of daylight were waning. A major advantage of studies in the sheep has been the relative ease of sampling GnRH directly over a long time period.

Fig. 6 displays the hormone levels seen during the ovine cycle. Day one is the day on which behavioral estrus occurs, which usually coincides with the day of the LH surge. Progesterone is close to zero at this

time, and rises gradually from day 3 to day 8. It then remains relatively constant until day 14 when it falls. Prostaglandin F2α rises in the uterine vein, from which it goes to the ovary. It is this action that terminates luteal function in a nonpregnant cycle. As progesterone levels fall, estradiol levels rise. This estradiol comes from the growing follicle. The follicular phase is short in the ewe. Serum FSH remains relatively constant except during the preovulatory surge, but as in the rat there is a secondary FSH rise. There is an increase in portal vein GnRH, which precedes the gonadotropin surges.

Mating behavior in the ewe requires a precedent exposure to progesterone, followed by estrogen working with the progesterone. As in the other species we have discussed, rising estradiol levels trigger the GnRH-LH cascade. GnRH pulse frequency is greater during the follicular phase than during the luteal phase, as in the primate. Progesterone is always inhibitory in the sheep; the luteal progesterone secretion blocks the effects of estradiol in triggering GnRH release, acting in the brain. Negative feedback during the cycle is predominantly because of progesterone, not estradiol—removal of the corpus luteum during the cycle allows tonic gonadotropin secretion to rise, leading to follicular growth and estradiol secretion.

The nature of the seasonality in the ewe is interesting. During the nonbreeding season, (anestrus, high light-to-dark ratio) GnRH and LH pulse frequency is low, as the result of low (but not zero) estradiol levels, which exert a strong negative influence. Thus, the hypothalamus is not quiescent at this time, but under stringent negative feedback. As the transition to the breeding season takes place, estradiol negative feedback becomes less effective, and GnRH and LH pulse frequency increase, which elicits more and more estradiol, which can then reach the threshold for a surge.

The pineal gland mediates the effects of photoperiod in the sheep. Melatonin is secreted during the dark period, and treatment with long durations of melatonin acts like long dark photoperiods. Pentobarbital can block the LH surge in the sheep, as it does in the rat. The nervous system in the sheep, as in the rat, ultimately controls the cycle.

6. WHY MALES DO NOT CYCLE

Males do not show the kind of cycles we have described for the females. Fundamentally, this is because the testis does not demonstrate a cataclysmic event, represented by the conversion of an estrogen-secreting follicle into a corpus luteum that secretes progesterone, which has very different effects on the pituitary and brain. The testicular "cycle" of spermatogenesis is the length of time for a given gonocyte to mature into a spermatozoan. But this process is continuous and overlapping in different tubule segments during the breeding season. Males, which are annual breeders, are influenced like females of these species so that the hypothalamic-pituitary axis is relatively quiescent until changing environmental conditions increase GnRH secretion leading to gonadotropin release inducing testosterone secretion. Increased circulating androgens then alter territorial behavior, external and internal morphological changes such as horn growth, and of course, mating behavior.

But is the male hypothalamic-pituitary unit capable of demonstrating experimentally both negative and positive feedback to exogenous estrogen? The answer is different for different species. In the laboratory rodent, secretion of androgen on the first postnatal day in male pups alters the hypothalamus irrevocably, resulting in the inability of adult males to show a surge in LH after estrogen pretreatment. Conversely, female rodents treated with exogenous androgen within the first five days of birth never show an estrogen-induced LH surge; males castrated on day 1 can show an LH surge as adults if treated with estrogen. Thus, the rodent is "hard-wired" with respect to the LH surge. Early androgen permanently prevents the ability of hypothalamic neurons to respond to estradiol. Male sheep, like male rats, show a critical developmental period when the capacity to show an LH surge is naturally abolished under the action of testosterone.

In the primate, this is not the case. This has been shown in the human. Men or women seeking sex-change operations have been tested before treatment. Men show no LH surge with estrogen, but women do. After treatment with the opposite sex hormone and gonadectomy, estrogen-treated men can show the LH surge, unlike the rodent.

7. SUMMARY AND CONCLUSIONS

The cycle is located in a distributed fashion among the organs and relationships and signals depicted in Fig. 1, and removal of any of the signals, whether environmental, neural, pituitary, or gonadal hormonal prevents further cycles unless replacement therapy is carried out.

Females of all mammalian species (Fig. 7) demonstrate ovarian follicular growth under the influence

of basal levels of FSH and LH, driven by basal levels of GnRH pulses. The estradiol secreted by growing follicles triggers a surge of GnRH from the hypothalamus, which triggers a surge of LH (and smaller amounts of FSH) from the pituitary gland. The surge of LH causes a series of changes in the mature follicle(s), including cessation of estradiol secretion, ovulation, and formation of a corpus luteum. Progesterone is secreted by the corpora for varying lengths of time and must remain elevated if implantation is to take place.

The major differences among species in the neuroendocrine regulation of reproductive cycles are: (1) the degree to which environmental inputs are critical; (2) the degree to which mating behavior is dependent on circulating steroids; (3) the number of ova ovulated; and (4) the regulation and timing of luteal function.

SELECTED READINGS

Bowen JM, Dahl GE, Evans NP, et al. Importance of the gonadotropin-releasing hormone (GnRH) surge for induction of the preovulatory luteinizing hormone surge of the ewe: dose-response relationship and excess of GnRH. Endocrinology 1998; 139:588.

Conn PM. The molecular mechanism of gonadotropin-releasing hormone action in the pituitary. In: Knobil E, Neill JD, eds. The Physiology of Reproduction, 2nd ed., New York: Raven, 1994:1815.

Freeman ME. The neuroendocrine control of the ovarian cycle of the rat. In: Knobil E, Neill JD, eds. The Physiology of Reproduction, 2nd ed., New York: Raven, 1994:613.

Goodman RL. Neuroendocrine control of the ovine estrous cycle. In: Knobil E, Neill JD, eds. The Physiology of Reproduction, 2nd ed., New York: Raven, 1994:659.

Gooren L. The neuroendocrine response of luteinizing hormone to estrogen administration in the human is not sex specific but dependent on the hormonal environment. J Clin Endocrinol Metab 1986; 63:589.

Herbison AE. Multimodal influence of estrogen upon gonadotropin-releasing hormone neurons. Endoc Rev 1998; 19:302.

Hotchkiss J, Knobil E. The menstrual cycle and its neuroendocrine control. In: Knobil E, Neill JD, eds. The Physiology of Reproduction, 2nd ed., New York: Raven, 1994:711.

Kaiser UB, Conn PM, Chin WW. Studies of gonadotropin-releasing hormone (GnRH) action using GnRH receptor-expressing pituitary cell lines. Endoc Rev 1997; 18:46.

McClintock MK. Pheromonal regulation of the ovarian cycle: enhancement, suppression and synchrony. In: Vandenbergh JG, ed. Pheromones and Reproduction in Mammals, New York: Academic, 1983: 113.

Naftolin F, Mor G, Horvath TL, Luquin S, Fajer AB, Kohen F, Garcia-Segura LM. Synaptic remodeling in the arcuate nucleus during the estrous cycle is induced by estrogen and precedes the preovulatory gonadotropin surge. Endocrinology 1996; 137:5576.

Richards JS, Fitzpatrick SL, Clemens JW, Morris JK, Alliston T, Sirois J. Ovarian cell differentiation: a cascade of multiple hormones, cellular signals, and regulated genes. Rec Prog Horm Res 1995; 50:223.

Savoy-Moore RT, Schwartz NB, Duncan JA, Marshall JC. Pituitary gonadotropin-releasing hormone receptors during the rat estrous cycle. Science 1980; 209:942.

Schwartz NB. Follicle stimulating hormone and luteinizing hormone: a tale of two gonadotropins. Can J Physiol Pharmacol 1995; 73:675.

Shupnik MA. Gonadotropin gene modulation by steroids and gonadotropin-releasing hormone. Biol Reprod 1996; 54:279.

Xia L, Van Vaugt D, Alston EJ, Luckhaus J, Ferin M. A surge of gonadotropin-releasing hormone accompanies the estradiol-induced gonadotropin surge in the Rhesus monkey. Endocrinology 1992; 131:2812.

9 Neuroendocrine Regulation of Pregnancy and Parturition

John R.G. Challis, PHD, DSC

CONTENTS

1. INTRODUCTION

Premature delivery, defined as birth before 37 wk of completed pregnancy, occurs in approximately 10% of all pregnancies in North America. This figure may be even higher in certain population groups. Preterm birth is associated with 70% of all neonatal deaths and up to 75% of neonatal morbidity. Infants born preterm have increased incidence of neurologic, metabolic, and respiratory disorders. In the United States, the cost of caring for babies born preterm has been estimated at $5–6 billion annually. However, the incidence of this condition has remained relatively unchanged over the past 20 to 30 yr. The methods of treating preterm birth are of limited effectiveness. Mainly, this reflects the inadequacies of our understanding of the endocrine mechanisms during pregnancy, and our ability to extrapolate from the available information to better diagnose women at risk of preterm delivery. It is these patients for whom treatment will afford benefit for the mother and for the enhanced maturation of the fetus.

From: *Neuroendocrinology in Physiology and Medicine*, (P. M. Conn and M. E. Freeman, eds.),

2. PHASES OF UTERINE ACTIVITY

In most animal species, the *uterus* remains relatively quiescent for most of pregnancy. The contractions which occur are poorly synchronized, of low amplitude, and develop relatively small increases in uterine pressure. This pattern of activity termed *contractures* has been described in the sheep, baboon, and rhesus monkey. In women, it has been speculated that contractures are equivalent to Braxton–Hicks contractions. This pattern contrasts with the high frequency, high amplitude contractions of the *myometrium* that occur at the time of labor. Thus, the uterine phenotype can be divided into distinct phases (Fig. 1). During 95% of *pregnancy* the myometrium is relatively quiescent, corresponding to Phase 0 of parturition. In late gestation, the myometrium undergoes a transition from a state of quiescence to one of activation, which corresponds to Phase 1 of parturition. Stimulation of the uterus leading to contractions of labor is termed Phase 2 (*see* Fig. 1). Involution of the uterus post partum is termed Phase 3. In this sequence of events, the "initiation of parturition" corresponds to the transition from Phase 0 to Phase 1.

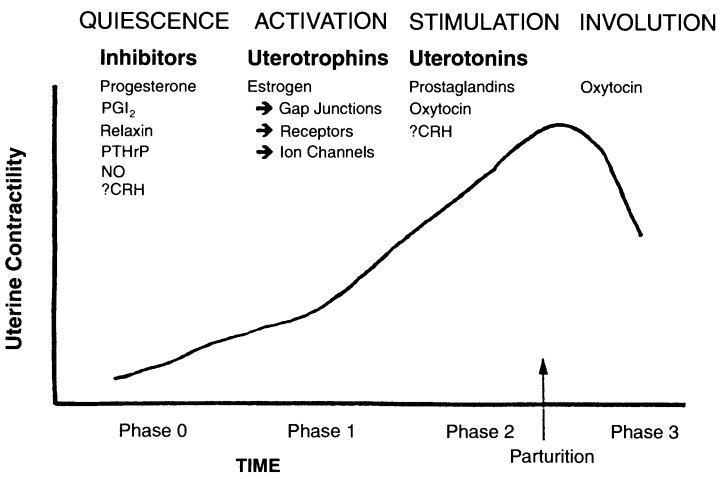

Fig. 1. Phases of uterine contractility during pregnancy and parturition. PGI2, prostacyclin; PTHrP, parathyroid hormone-related peptide; NO, nitric oxide; CRH, corticotrophin-releasing hormone.

3. PREGNANCY—PHASE 0 OF PARTURITION

During pregnancy, the myometrium is acted upon by a variety of inhibitors including *progesterone, relaxin, prostacyclin (PGI2), parathyroid-related protein (PTHrP), and nitric oxide* (Fig. 1). These agents act in different ways, but in general increase the intracellular levels of cyclic nucleotides, cAMP or cGMP. Increases in cAMP or cGMP levels in turn inhibit the release of calcium (Ca^{2+}) from intracellular stores or reduce the activity of the enzyme myosin light chain kinase (MLCK). Contractility of the myometrium depends upon conformational changes in the actin and myosin filaments, allowing them to slide over each other resulting in shortening of the myocytes. This change requires ATP, generated by myosin after phosphorylation of the myosin light chains by MLCK. MLCK is activated by interacting with the Ca^{2+} binding protein, *calmodulin*, which in turn requires four Ca^{2+} ions for its own activation. Thus, agents that inhibit release of Ca^{2+} from intracellular stores, or reduce the level of MLCK, result in reduction of uterine contractility. Suppression of uterine activity is clearly important to avoid premature expulsion of the *fetus,* and to allow the normal developmental maturation of the fetus to occur *in utero.* In addition, the maintenance of uterine quiescence during pregnancy allows symmetrical development of the fetus and its growth within the fluid-filled environment of the amniotic cavity. It ensures that blood flow to the placenta is not impaired, thereby maintaining oxygen and nutrient transfer to the fetus.

Some hormones have multiple activities. For example, both progesterone and estrogen are required for increased uterine growth during pregnancy, which is necessary to accommodate the developing fetus. Estrogen has long been recognized as one of the stimulants to increased uteroplacental blood flow during gestation. It also promotes many of the maternal cardiovascular adjustments that occur in pregnancy. Estrogen is regarded as one of the agents leading to myometrial activation at term. While we consider effects of individual hormones, it must be recognized that none operates in isolation, and that activities are modulated by the sum of the prevailing endocrine environment.

3.1. Inhibitors of Myometrial Activity

Progesterone has a major role during pregnancy in suppressing spontaneous myometrial contractility and in reducing stimulated uterine activity. In species where the ovary continues as a major source of progesterone throughout pregnancy, ovariectomy results in increased myometrial contractility. This effect can be reversed by administration of exogenous progesterone, and is the most obvious example of withdrawal of the progesterone block to the myometrium. In many species such as, the sheep where the placenta is the major source of progesterone during the second half of pregnancy, a decrease in placental progesterone is reflected in a decrease in peripheral plasma progesterone concentrations. In *primates*, however, there is little evidence in support of systemic progesterone withdrawal occurring in late gestation. Indeed, in some subhuman primates such as the rhesus monkey, maternal peripheral plasma progesterone concentrations clearly rise before parturition. Thus, it has been speculated or postulated that local, intrauterine changes in progesterone production or action occur in these species at the time of labor.

Ovarian progesterone production predominates during the first 5 to 6 wk of human pregnancy, and

ovariectomy or administration of a progesterone receptor antagonist such as RU486 during this time leads to the evolution of myometrial contractility. The placenta becomes the major site of progesterone production after the sixth week of human pregnancy, although ovarian progesterone production continues. It is generally considered that placental progesterone production depends on the availability of LDL-associated cholesterol, and adequate uteroplacental blood flow. Trophoblast tissue contains LDL receptors. Human placental syncytiotrophoblast expresses the key enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) Type 2 which is responsible for conversion of pregnenolone to progesterone. The uptake of LDL by trophoblasts is enhanced by estrogen, an effect owing to upregulation of LDL receptor expression. In the chorion, one of the membranes that surrounds the fetus inside the uterus, progesterone is also produced by trophoblast cells, but predominantly from pregnenolone or pregnenolone sulphate derived from amniotic fluid or from the maternal circulation. Maternal plasma progesterone concentrations are lowered in patients with homozygous *hypobetalipoproteinemia*, showing the importance of substrate availability for placental production. Progesterone output in the placenta and fetal membranes can be stimulated by analogs of cAMP and activators of protein kinase A including β-agonists. Levels of mRNA encoding the cholesterol side chain cleavage enzyme (P450scc) and adrenodoxin in cultured trophoblasts were all increased by the addition of a cAMP analog to the cells. In the baboon, maternal progesterone concentrations falls in animals treated with an antiestrogen. This drug results in a reduction in placental mitochondrial P450scc activity, and impairs the usage of LDL.

Parathyroid hormone-related peptide (PTHrP) is produced in intrauterine tissues, and in the rat there are very high concentrations of PTHrP mRNA in the myometrium during pregnancy. PTHrP mRNA levels were increased in uterine horns occupied by fetuses, and increased in nonpregnant rats treated with estrogen. Progesterone, and distension of the uterine cavity also increases levels of PTHrP mRNA. Receptors for PTHrP are expressed in myometrial tissue. It has been suggested that this peptide can act in an autocrine/paracrine fashion through specific receptors to activate the GαS subunit of G proteins and increase intracellular levels of cAMP. Thus, PTHrP activates adenylate cyclase activity in myometrial cells. Expression of the gene encoding PTHrP can be regulated in a number of ways. This protein clearly has the potential to act as an endogenous inhibitor of uterine activity.

Nitric oxide (NO) is also a potent endogenous relaxant of smooth muscle. Nitric oxide is synthesized from L-arginine by different isoforms of nitric oxide synthase (NOS). The constitutive form of NOS is expressed in human amnion, chorion, decidua and placenta. The inducible form of NOS is also expressed in smooth muscle cells of the pregnant myometrium, but levels of expression decreased by up to 75% before parturition, either preterm, or at term. NO acts through cGMP and alters the levels of intracellular calcium. Therefore, several authors have suggested that NO might act in a paracrine fashion, potentially in conjunction with progesterone to effect myometrial quiescence during pregnancy. In species such as the rat, the fall in NO production by the uterus in late gestation is accompanied by an increase in NO production by inflammatory cells in the cervix. This observation suggests that NO might be involved in promoting cervical effacement and dilation at the time of birth, events that are essential for the fetus to pass through the birth canal. This activity obviously increases as the inhibitory influence of NO on the myometrium is diminished.

Relaxin might also have a dual role in the inhibition of myometrial contractility and in the regulation of the connective tissue changes that occur in the cervix. Relaxin suppresses spontaneous uterine contractility in the rat and the guinea pig, although sensitivity to uterine stimulants such as oxytocin is maintained. Relaxin acts by elevating myometrial cAMP, and by inhibiting oxytocin-induced turnover of phosphoinositide (PI) by the action of cAMP-dependent protein kinase. In several species, including the human, relaxin is also expressed in the fetal membranes, placenta and decidua. Relaxin expression is upregulated dramatically in patients with preterm rupture of the membranes. It is interesting that relaxin increases levels of matrix metalloproteinases (MMP), especially MMP-1, MMP-3, and MMP-9. These enzymes are involved in the normal mechanisms of remodelling cervical connective tissue, and suggest a possibility by which hyperelaxinemia may be associated with prematurity.

It is evident that withdrawal of one or more of the above compounds, progesterone, PTHrP, NO, or relaxin, from the myometrium may occur in relation to labor at term. It is apparent that premature withdrawal of one or more of these compounds may predispose to premature delivery. The concept of withdrawal of the progesterone block to the myometrium at term is well established in animals and is demonstrable in early human pregnancy. There is consider-

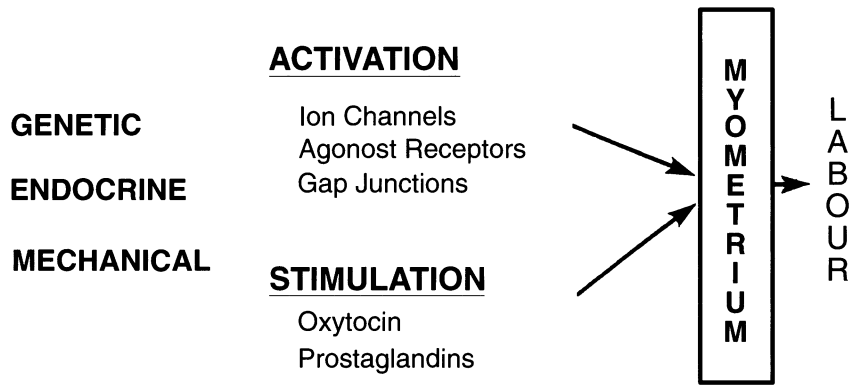

Fig. 2. Activation and stimulation of myometrial activity.

able debate whether the process of labor in women results from withdrawal from the influence of progesterone on the myometrium, or from the active imposition of factors which stimulate myometrial contractility. An obvious possibility is that it results from a combination of these factors.

4. ACTIVATION OF MYOMETRIUM—PHASE 1 OF PARTURITION

Activation of the myometrium (Phase 1 of parturition) can be considered as an active process that reflects the influence of the fetal genome (Fig. 2). This can be exerted through activation of a fetal endocrine axis, the *fetal hypothalamic pituitary adrenal (HPA) axis* or through processes that determine fetal growth, or through combinations of these. Phase 1 activation is manifest by upregulation of a cassette of *contraction-associated proteins (CAPs),* including *connexin-43 (CX-43),* the major protein of gap-junctions, and *receptors for oxytocin* and *stimulatory prostaglandins.* Expression of increased gap-junction proteins permits cell–cell coupling, whereas expression of proteins that are constituents of ion channels determine the resting membrane potential and hence excitability of uterine myocytes (Fig. 2).

4.1. Uterine Stretch and Activation

In an elegant series of experiments S. Lye et al. in Toronto, ON., Canada, have shown that CAP activation can be induced by *uterine stretch,* but the magnitude of this action is determined by the prevailing endocrine environment. Progesterone given to rats at term (thereby preventing the normal prepartum fall in plasma progesterone concentrations), blocked the increase in CX-43, oxytocin receptor (OTR), and F prostaglandin receptor (FP) mRNA in the myometrium, and blocked the onset of labor. Administration of the progesterone receptor antagonist RU486 or ovariectomy to remove the source of progesterone, caused a premature increase in OTR and CX-43 and induced premature labor. Although estrogen treatment leads to increased transcription of CX-43, there are no sequences resembling typical estrogen response elements (ERE) in the proximal promoter region of the CX-43 gene. One current idea is that effects of estrogen are modulated through other transcription factors, including *c-fos.* Uterine stretch is also a potent stimulus to increase CAP expression at the time of labor. When nonpregnant ovariectomized rats had a small (3 mm) inert tube placed within one uterine horn, there was a significant increase in mRNA levels encoding CX-43 compared to the contralateral horn after 48 h. Control studies showed that this effect was not simply a response to a foreign body. Stretch-induced expression of CX-43 could be blocked in ovariectomized rats by the administration of progesterone. It did not occur at day 20 of pregnancy, presumably because the endogenous progesterone concentration is still high at that time. In contrast, on day 23 at the time of labor, inert tubing placed into the nongravid horn of unilaterally pregnant animals was able to increase CX-43 and OTR mRNA levels to values similar to those seen in the gravid horn. Lye et al. have interpreted these data as showing that the increase in CAP expression and activation of the myometrium during labor requires endocrine changes, and tension within the uterine wall. The mechanism of this effect remains to be explained, although there is a putative shear stress response element within the CX-43 promoter region.

4.2. Estrogen and Myometrial Activation

Several studies have also implicated *estrogen* as mediator of Phase 1 activation events. In this setting, estrogen may be regarded as a *uterotrophin.* During human pregnancy estrogen production depends upon biochemical complementation between the placenta and the adrenal gland of the fetus. The human placenta

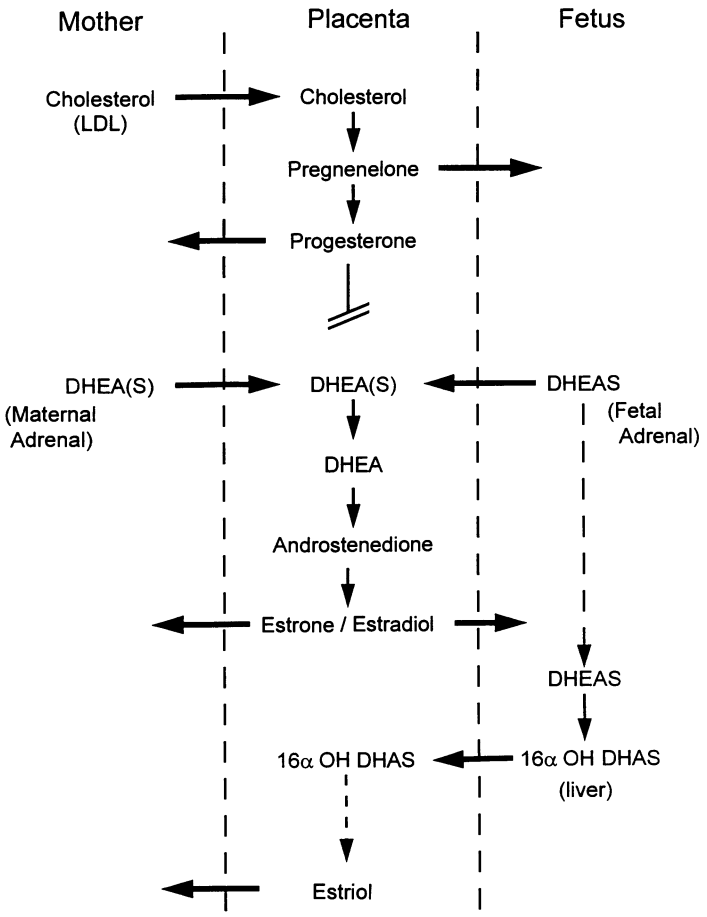

Fig. 3. Steroidogenic pathways in human pregnancy. Note particularly the interdependence of mother, placenta and fetus in the biosynthesis of estrogen.

lacks P450C17 activity and is unable to convert C21 steroids such as progesterone to C19 steroids, the androgens that are estrogen precursors. However, the placenta expresses abundant *aromatase* activity and can utilize C19 precursor steroids derived from other sources in the synthesis of estrogen (Fig. 3). The fetal *zone of the fetal adrenal gland*, which occupies approximately 85% of the fetal adrenal cortex is deficient of the enzyme, 3β-hydroxysteroid dehydrogenase (3β-HSD) Type-2. Therefore, the fetal zone secretes predominantly steroids with a double bond in the B-ring between carbons 5 and 6 (Δ5 steroids). The major one of these is dehydroepiandrosterone (DHEA) secreted as a sulphoconjugate (DHAS). Fetal adrenal DHAS can be converted to estrone and estradiol in the placenta, and 50% of maternal estrogen is derived from placental aromatization of fetal DHAS. The rest of maternal circulating estrogen is derived from maternal adrenal precursors. Fetal adrenal DHAS can also undergo 16α-hydroxylation in the fetal liver to form 16-hydroxy DHAS, which is converted in the placenta by aromatization to the 16α hydroxylated estrogen, estriol. Approximately 90% of estriol in the maternal circulation is derived from precursors of fetal origin. Activation of the pituitary-adrenal axis of the fetus occurs in late gestation. In subhuman primate species, it is known that there is an increase in the concentration of DHAS in the fetal circulation at this time. This mirrors an increase in maternal plasma estriol concentration. Production of estrogen depends on the level of drive to the fetal adrenal from the fetal pituitary. It is reduced in circumstances such as fetal pituitary atrophy, i.e., fetal anencephaly, adrenal atrophy, or in the absence of placental enzymes such as sulphatase that is required to hydrolyze the DHAS arriving from the fetus into DHEA. Maternally administered glucocorticoids also suppress feto-placental estrogen production, because they are transferred across the placenta from mother to fetus and exert negative feedback on the fetal HPA axis. The recent observation that human pregnancy can proceed to term in the absence of the aromatase enzyme has raised questions about the fundamental importance of estrogen in regulatory mechanisms of gestation. However, it is clear that there must still be some maternal estrogen production, even in these

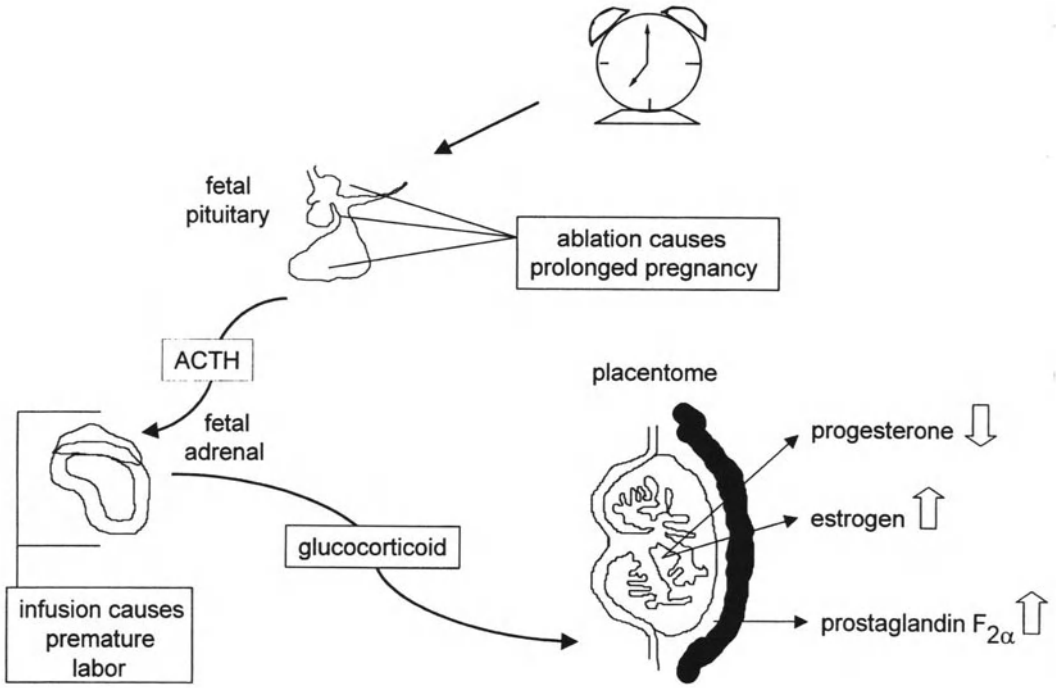

Fig. 4. Events leading to the onset of parturition in sheep showing activation of the fetal HPA axis resulting in changes in progesterone, estrogen and PGF2α from the placenta.

cases. Moreover, the redundancy of biologic systems may allow emergence of alternate pathways to affect estrogen action.

Animal experiments have suggested clearly that the activation of myometrial function at term depends on the balance of activity between estrogen and progesterone. As discussed above, increased tension within the myometrium due to stretch of the uterine wall from the growing fetus (or experimentally induced stretch) can increase the expression of myometrial CAP's. This effect is modulated by the endocrine environment. During pregnancy, stretch-induced expression of CAP's is blocked, probably due to the inhibitory action of progesterone. However, in the endocrine environment of labor, with an increase in the estrogen to progesterone ratio, stretch-induced CAP expression is activated. The mechanism of these interactions remain to be determined. The synchronous expression of CAP's in association with the onset of labor, suggests a role for a small number of transcription factors acting as master control genes. Because estrogen activates the CX-43 promoter, there are no consensus ERE elements in the proximal promoter region. The effect of estrogen on CX-43 requires *de novo* protein synthesis. Estrogen-induced upregulation of CX-43 mRNA is associated with increases in *c-fos* and *c-jun* mRNA levels. Progesterone treatment of rats reduces expression of these genes and blocks labor. Thus, it can be suggested that in the uterus under the tension of stretch, an increase in the estrogen:progesterone (E:P) ratio triggers a regulatory cascade in which one or more transcription factors (such as *c-fos*) orchestrates the expression of a cassette of CAPs in the myometrium. Activation is manifested as increased spontaneous activity, responsiveness, and cell–cell coupling. Thus, the altered E:P ratio contributes both directly and indirectly to the upregulation of stimulatory uterotonins, and to the increased ability of uterotonins to generate high amplitude, high frequency contractions of labor.

4.3. The Fetal Input to the Process of Parturition—Animal Studies

It is now recognized that the changes in *uterotrophin* (estrogen) and uterine inhibitor (progesterone) described above depend in large part upon endocrine activities within the fetus, particularly within the fetal HPA axis. More than 30 yr ago, Professor Sir Graham (Mont) Liggins and Geoffrey Thorburn, showed conclusively in the sheep and goat that the fetus triggered the onset of parturition through activation of the fetal HPA axis, with increased output of *cortisol* from the fetal adrenal gland. Lesions of the paraventricular region of the fetal hypothalamus, hypophysectomy of the fetus or adrenalectomy of the fetus *in utero* resulted in prolonged pregnancy, whereas infusion of adrenocorticotrophic hormone (ACTH) or cortisol into the fetal lamb resulted in premature birth. Fetal cortisol acts on the placenta initiating a sequence of events that results in decreased placental progesterone

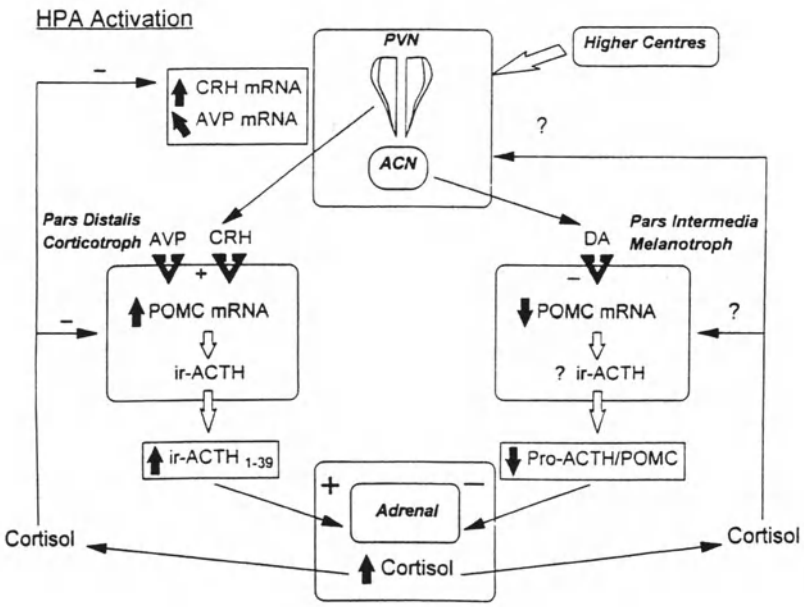

Fig. 5. Summary of factors concerned with activation of HPA function in the fetus during late pregnancy. PVN, paraventricular nucleus; ACN, arcuate nucleus; CRH, corticotrophin-releasing hormone; AVP, arginine vasopressin; POMC, proopiomelanocortin; ACTH, adrenocorticotrophic hormone; DA, dopamine.

output, increased estrogen output, and a later increase in production of the uterotonin, prostaglandin F2α, by uterine tissues (Fig. 4).

There is now extensive knowledge of the factors associated with fetal HPA activation in species such as the sheep (Fig. 5). It is clear that this knowledge may be applied to corresponding mechanisms in the primate fetus. Parturition in the sheep is associated with a progressive increase in the concentrations of cortisol and of ACTH in the circulation of the fetal lamb. The rise in ACTH results from an increase in fetal pituitary *proopiomelanocortin* (POMC) synthesis, and levels of POMC mRNA in the fetal pars distalis increase progressively through gestation reaching their highest values at the time of birth. Pituitary POMC is driven in turn by *corticotrophin-releasing hormone* (CRH) and arginine vasopressin (AVP) synthesized in parvocellular neurons of the paraventricular nucleus (PVN) of the *fetal hypothalamus*. Levels of CRH mRNA and of CRH protein in the fetal PVN increase during late gestation. CRH added to fetal pituitary cells in vitro provokes an increase in ACTH output, and an increase in levels of POMC mRNA (Fig. 5). In the fetus, the hypophyseal-portal system is functional by at least one-third of the way through gestation, implying that an intact hypothalamic-pituitary link is established by that time. POMC is processed through activities of proconvertase enzymes in the fetal pituitary gland. Recent studies have shown that proconvertase-1 (PC-1) and proconvertase-2 (PC-2) are both expressed in the pars distalis of the *fetal pituitary*, although levels of PC-2 in the pars intermedia are very much higher than those of PC-1, consistent with processing of ACTH to smaller molecular weight peptides in this region of the gland.

ACTH appears to be the major trophic factor for the fetal adrenal gland. ACTH increases expression of its own receptor in fetal adrenal cortical cells. With advancing gestation, there is enhanced coupling of ACTH receptor to adenylate cyclase, and increased capacity for cAMP generation. There is also increased expression of key steroidogenic enzymes in the fetal adrenal cortex, including P450C17, which allows production of 17-hydroxylated corticosteroids such as cortisol. Levels of mRNA encoding P450C17, P450C21, and P450C11 are increased following ACTH administration to the fetal lamb *in utero*. The low levels of ACTH in the circulation of the fetus at midgestation are apparently responsible for the diminished steroidogenic activity of the fetal adrenal gland at that time.

During the last 2–3 wk of gestation there are concurrent increases in concentrations of ACTH and cortisol in the circulation of the fetal lamb. These occur despite the potential for negative feedback of cortisol at the level of the fetal hypothalamus and pituitary. The fetus appears to have evolved mechanisms that diminish the ability of cortisol to exert negative feedback. These include (1) inactivation of circulating cortisol to cortisone by the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) 1 in the fetal pars distalis (2) decreased numbers of glucocorticoid receptors in the fetal hypothalamus (preventing glucocorticoid negative feedback on CRH synthesis and release), and (3) increased synthesis from the fetal

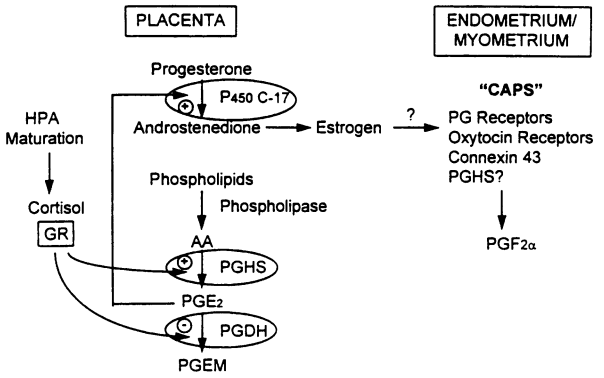

Fig. 6. Summary of events at parturition in sheep showing potential sites of cortisol action on enzymes to the prostaglandin synthetic and metabolic pathways. GR, glucocorticoid receptors; AA, arachidonic acid; PGHS, prostaglandin H synthase; PGDH, prostaglandin dehydrogenase; CAPS, contraction-associated proteins.

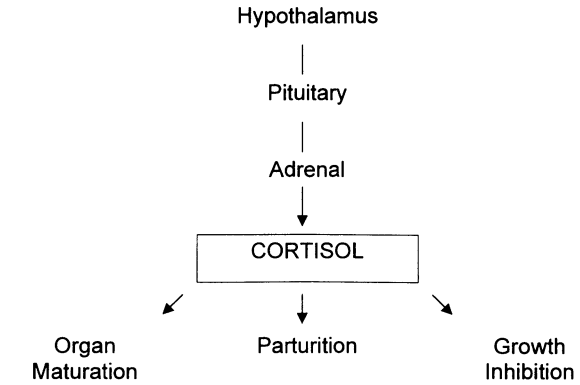

Fig. 7. Activation of the fetal HPA axis in late gestation results in increased cortisol production. Fetal cortisol promotes fetal organ maturation and initiates parturition. These activities are balanced by potential adverse effects on fetal growth.

liver of corticosteroid binding globulin (CBG). By stimulating production of its own circulating binding protein, cortisol maintains relatively low concentrations of free steroid in the circulation, which diminishes negative feedback potential.

Fetal cortisol acts on the placenta to alter the pattern of *steroidogenesis* such that progesterone output falls and estrogens increase. A current view is that cortisol itself induces expression of the enzyme P450C17 in placental trophoblast cells. This diverts Δ5 C21 steroids away from progesterone, and through the Δ5 pathway leading to dehydoepiandrosterone (DHEA), and into androstenedione. The sheep placenta expresses aromatase, and can utilize C19 steroids, generated *de novo*, for estrogen synthesis. However, this view of cortisol actions has been challenged recently. An alternative proposal is that fetal cortisol increases expression of prostaglandin synthesizing enzymes in the ovine placenta (Fig. 6). Increased levels of these enzymes result in increased output of prostaglandins, particularly prostaglandin E2, from fetal placental *trophoblast* into the fetal circulation. Prostaglandins can serve as systemic hormones in the fetus, since the proportion of cardiac output passing through the lungs is small (about 7%), and the rate of pulmonary inactivation of prostaglandins is substantially less than in the adult. Placental PGE2 can feedback in the fetus to stimulate pituitary ACTH and adrenal cortisol production, thereby accelerating a feedforward cascade. In the adrenal gland, prostaglandins increase expression of the enzyme P450C17. If they exerted a similar activity in the sheep placenta, this would also effect the switch from progesterone to estrogen output that is seen at term.

Importantly, activation of the fetal HPA axis can take place in response to an adverse intrauterine environment, for example *hypoxemia*. Short-term hypoxemia results in increases in plasma ACTH and cortisol concentrations. Hypoxemia prolonged for up to 6 h results in significant increases in levels of CRH mRNA and POMC mRNA in the fetal hypothalamus and pituitary respectively. If mild hypoxemia is maintained over 48 h, there are further increases in pituitary POMC mRNA levels, and rises in fetal plasma cortisol concentrations. This results from upregulation of steroidogenic enzymes in the fetal adrenal gland. The elevation of cortisol then has the potential to promote premature delivery. At the same time, increases in fetal cortisol output may promote fetal organ maturation or inhibit the growth of many fetal organ systems, in part by suppressing the expression of locally produced insulin-like growth factors (IGFs) (*see* Fig. 7). One can envisage a similar sequence of events occurring in human pregnancy complicated by maternal hypertension, preeclampsia, and conditions of compromised uteroplacental perfusion.

4.4. The Fetal Input to the Process of Parturition—Human Studies

At the present time, the evidence linking the primate fetus to the onset of parturition is less convincing than in the sheep. In human anencephaly, the mean length of gestation is similar to that of the control population, although there is a marked increase in the numbers of premature and postmature births. Similar results have been reported in the monkey with experimental fetal exencephaly. Removal of the fetus (fetectomy) in the rhesus monkey, leaving only a placental pregnancy clearly delays the delivery of that placenta.

However, this experiment is difficult to interpret because uterine volume ("stretch") has not been maintained. As indicated above, levels of C19 estrogen precursors rise in the circulation of the intact fetal monkey in late gestation, with a similar time course to the rise of cortisol in the circulation of the fetal sheep. When androstenedione is infused to pregnant rhesus monkeys at 0.8 of gestation, there is a significant increase in maternal plasma estrogen concentrations and premature delivery. This effect can be blocked by coadministration of an aromatase inhibitor, indicating that it depends on the conversion of the infused androgen into estrogen. However, systemic estrogen infusion is ineffective in precipitating premature delivery in the monkey. The difference between these two experiments may reside in the necessity to generate estrogen locally within the placenta or fetal membranes in order for it to exert autocrine/paracrine activities.

In the primate, it is clear that activation of the fetal pituitary occurs during late gestation and is associated with increases in levels of POMC mRNA. It is likely that POMC synthesis is upregulated by CRH as in the fetal sheep, although levels of CRH mRNA do not change in the fetal hypothalamus at least late in gestation. One reason for the apparent difference between the sheep and primate models for the onset of parturition lies in the functional zonation of the cortex of the fetal adrenal gland. In the primate, the fetal adrenal cortex is divided into a *fetal zone* that produces substantial amounts of C19 estrogen precursor steroids (DHEA), a *transitional zone* that produces cortisol, and an outer *definitive cortex*. In the sheep, there is no analogous functional zonation of the fetal adrenal, and the primary steroid produced is cortisol. As in primates, ACTH drives the fetal adrenal to increase cortisol which facilitates *organ maturation* and secretes C19 steroids for estrogen synthesis in the placenta. In the sheep, ACTH stimulates the fetal adrenal to produce cortisol, with relatively low output of C19 steroids. These differences, however, serve to illustrate how different species utilize an assortment of mechanisms to achieve the same objective (*see* Fig. 8). In both the sheep and the primate, the major steroid produced in the placenta is estrogen. In the primate, the C19 steroids required for estrogen production are secreted directly from the fetal adrenal gland. In the sheep, however, the C19 steroids for placental estrogen production are generated within the placenta itself, but under the influence of cortisol from the fetal adrenal gland. Thus, in both species there is fetal adrenal–placental interaction in order to produce estrogen. The difference is simply the site at which the estrogen precursors are generated (*see* Fig. 8).

Recent studies, in primates have suggested that cortisol from the fetal adrenal gland could still influence the timing of parturition, at term or preterm, through two important mechanisms. First, fetal adrenal cortisol has the potential to stimulate output from the placenta of peptides such as CRH. Maternal CRH concentrations rise dramatically through the third trimester of normal pregnancy, and are elevated in preterm labor. Second, glucocorticoids appear to stimulate prostaglandin output in trophoblast tissues including the fetal membranes. Glucocorticoids have additional effects on the pathways of prostaglandin production. In myometrium they inhibit prostacyclin synthase, and the production of prostacyclin (PGI2), which as discussed above, is an inhibitor of myometrial contractility.

5. STIMULATION OF THE MYOMETRIUM— PHASE 2 OF PARTURITION

5.1. Prostaglandins

There is now extensive evidence to support a role for *prostaglandins* (PG) in the labor process at term and preterm, in animal species and in primates. Mice lacking the ability to generate prostaglandins *(PGHS-1 knockouts)* have protracted labors. There is increased capacity for prostaglandin production by intrauterine tissues prior to the appearance of labor-like myometrial contractions. Prostaglandin synthase inhibitors, such as *indomethacin,* effectively block uterine contractility and prolong gestation length. Finally, there are substantial increases in the concentrations of primary prostaglandins in amniotic fluid, and in levels of prostaglandin metabolites in maternal plasma and urine at the end of gestation, in virtually all species studied. In species where the placenta has become the major source of progesterone, the likely site of action for prostaglandins is directly on the myometrium. In species where the ovary remains the major source of progesterone, an additional role for uterine prostaglandin F2α production may be to cause luteolysis. Thus, mice lacking prostaglandin F receptor (FP knockouts) failed to deliver, because luteal regression did not occur. In the absence of a decline in maternal progesterone concentrations, there is no upregulation of the uterine oxytocin receptor, and oxytocin fails to stimulate uterine contractility and birth.

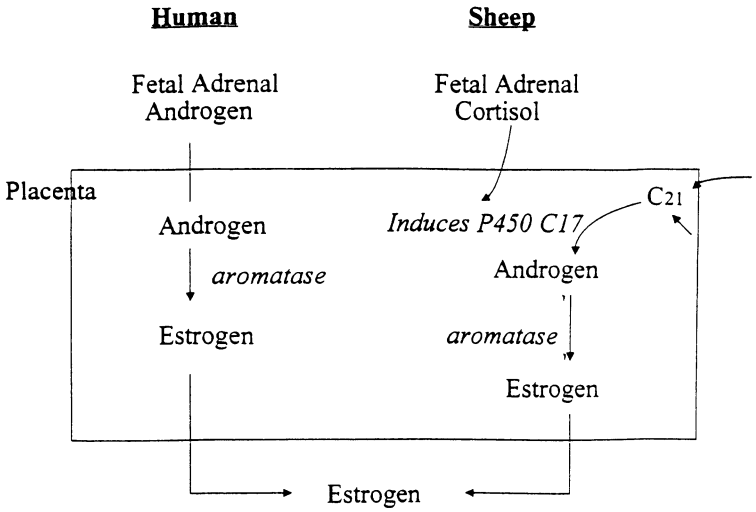

Fig. 8. Mechanism of estrogen biosynthesis in human and sheep pregnancy. In the human, C19 precursor steroids (androgen) are produced directly from the fetal adrenal gland and converted by placental aromatase to estrogen. In the sheep, cortisol is produced by the fetal adrenal gland and this induces the enzyme P450C17 in the placenta so that the placenta itself can make the C19 precursors (androgen) that can be aromatized to estrogen.

Primary prostaglandins, including PGE2 and PGF2α are formed from the obligate precursor *arachidonic acid* liberated from membrane phospholipids through one or more of the isozymes of phospholipase C or phospholipase A2 (Fig. 9). Arachidonic acid in turn is converted to prostaglandins through the activity of *prostaglandin H2 synthase* (PGHS). There are two forms of PGHS: PGHS-1 and PGHS-2. These have been described as constitutive and inducible forms of the enzyme, respectively. It is now clear that both forms may be upregulated in response to certain stimuli. Arachidonic acid may also be metabolized through one of at least four distinct lipoxygenase pathways. In human fetal membranes, this appears to be the preferred direction of arachidonic metabolism, with formation of leukotrienes for most of pregnancy. However, at term, lipoxygenase activity falls while prostaglandin H synthase activity increases, resulting in a relative increase in primary PG output.

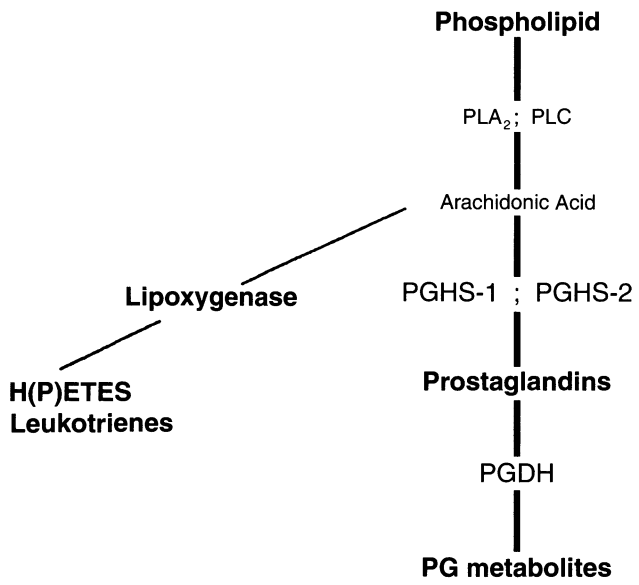

Fig. 9. Pathways of prostaglandin synthesis and metabolism. PLA2, phospholipase A2; PLC, phospholipase C; PGHS1, prostaglandin H2 synthase 1; PGHS2, prostaglandin synthase 2; PGDH, prostaglandin dehydrogenase.

Primary prostaglandins in turn are metabolized through an NAD-dependent *15-hydroxyprostaglandin dehydrogenase (PGDH),* which catalyzes oxidation of 15-hydroxy groups of the E and F prostaglandin series. Metabolites of PG's have reduced biological activity. It will be shown that the PGDH enzyme is present in very high activity in chorionic trophoblasts, and may be important through much of pregnancy in preventing the passage of amnion-derived prostaglandin to the underlying decidual tissue and myometrium.

Prostaglandin action is effected through specific receptors including the four main subtypes: EP1, EP2, EP3, and EP4 for PGE2 and FP for PGF2α. EP1 and EP3 receptors mediate contractions of smooth muscle in a number of tissues through mechanisms that include increased Ca^{2+} mobilization and inhibition of intracellular cAMP production. EP3 receptors exist as a number of isoforms produced after alternative splicing of a single gene product. Activation of EP2 and EP4 receptors increases cAMP formation and relaxes smooth muscle. These receptor subtypes are expressed in the human myometrium in late pregnancy. EP2 expression was higher before the time of parturition. In the rat, Myatt et al. have shown that parturition is associated with upregulation of myome-

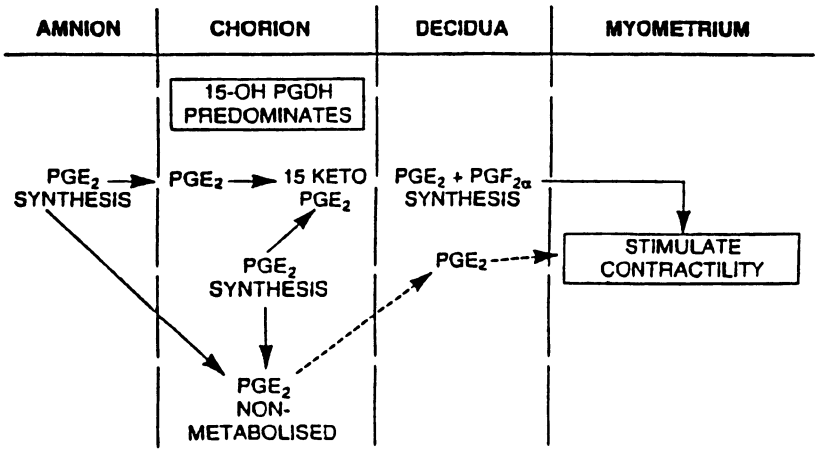

Fig. 10. Compartmentalization of prostaglandin synthesis and metabolism in human fetal membranes and intrauterine tissues during pregnancy.

trial FP receptors, and downregulation of EP receptor subtypes, effectively a switch from inhibition to stimulation of uterine contractility.

5.1.1. Prostaglandins and Labor

The sequence of events associated with increased prostaglandin production and parturition has been studied most extensively in the sheep. In this species, the placenta is the major site of prostaglandin production, and there is a dramatic upregulation in PGHS-2 expression in placental trophoblast cells during the last 2–3 wk of gestation. PGHS-2 expression also increases in the luminal epithelium of the endometrium, although it is not clear whether this is directly involved in the process of parturition. Levels of mRNA encoding PLA2 also increase in intrauterine tissues. These changes can be induced by cortisol infusion to the fetus and by administration of the antiprogestin RU486. An understanding of the molecular events associated with PGHS2 activation will require full sequencing of the proximal promoter region of the PGHS-2 gene. Recognition of NFκB, glucocorticoid receptor and cAMP response elements provide clues as to the trophic factors involved.

In human pregnancy prostaglandin generation is compartmentalized discretely within the *fetal membranes* (Fig. 10). PGHS activity predominates in *amnion*, and PGE2 is the major prostaglandin produced. Levels of PGHS2, but not PGHS1 mRNA, increase in amnion tissue at preterm and term labor. Output of prostaglandins and PGHS2 activity is also higher in *chorion* obtained from patients at spontaneous labor than at elective term Caesarean section. Although in preterm labor chorion both PGHS1 and PGHS2 mRNA levels are increased. PGHS1 and PGHS2 enzymes are both expressed in *decidua*, although there is little change in levels of mRNA or in enzyme activity at the time of labor.

In human amnion PGHS2, mRNA levels are upregulated in response to glucocorticoids. This response occurs predominantly in the subepithelial fibroblast cells rather than in the epithelial cell layer, however, both of these cell types express glucocorticoid receptors. It has been suggested that the epithelial cells might contribute to changes in PGHS expression by producing locally acting neuropeptide effectors such as CRH in response to glucocorticoids. The addition of CRH to human fetal membranes maintained in tissue culture leads to an increase in PGE2 output, and upregulation of PGHS2 mRNA levels. It is also well established that cytokines such as IL-1 exert a similar activity, and increase levels of PGHS2 mRNA and ir-PGHS2 within fibroblast cell preparations of amnion primary cultures.

Many other compounds acting locally or systemically can affect prostaglandin synthesis within the human fetal membranes. The importance of Ca^{2+} for prostaglandin synthesis was demonstrated in earlier studies with intact dispersed cells in which PGE2 output was reduced in the presence of Ca^{2+} channel blockers, and increased in the presence of the calcium ionophore A23187. Epidermal growth factor (EGF) increases the rate of PGHS synthesis, and potentiates arachidonic acid-stimulated PGE2 output from amnion. For some time, it was considered that a peptide such as EGF might be secreted or excreted by the fetus into the amniotic cavity, thereby gaining access to the amniotic membrane, and providing the trigger to the stimulation of prostaglandin synthesis and birth. This has been an attractive hypothesis, but is largely superseded by more recent information. Early studies also showed that prostaglandin output

by amnion could be increased in response to activators of cAMP-dependent protein kinase. Amnion cells possess β2 receptors and respond to catecholamines with increased cAMP production. Activators of adenylate cyclase also increase prostaglandin output by these cells, an effect that can be mimicked by addition of the cAMP analog dibutyryl cAMP or the phosphodiesterase inhibitor methylxanthine to amnion cells. These observations may help explain the rather disappointing lack of efficacy of β2 sympathomimetic drugs in sustaining inhibition of uterine activity in women in preterm labor. Although these compounds are effective in the short term, their continuous administration is associated with a return of uterine contractility. In part, this has been attributed to downregulation of the β2 receptor. However, it is also clear that these compounds provoke output of stimulatory prostaglandins, and in fact promote enhanced production of the compounds that their usage is intended to antagonize.

5.1.2. Prostaglandins and Infection

A substantial proportion of human preterm labor, associated with increased prostaglandin production, occurs in the presence of an underlying infective process. Some studies have suggested that *infection* may cause up to 30% of preterm labor, although others have maintained that infection-driven prostaglandin release is a result of rather than a cause of preterm birth. In patients with infection-associated preterm labor there are generally increased concentrations of *cytokines* including IL-1β, IL-6 and TNF in the amniotic fluid. Administration of cytokines such as IL-1, or of bacterial endotoxin to pregnant mice provokes premature delivery. In vitro studies have shown increased output of PGE2 by amnion and by chorion-decidua obtained from patients in preterm labor. Studies with cultured cells have demonstrated clearly that different cytokines increase expression of phospholipase A2 and PGHS, and increase the output of prostaglandins in a dose- and time-dependent fashion. A widely accepted model is that in the presence of an ascending bacterial infection, organisms pass between the fetal membranes and later reach the amniotic cavity. Bacterial organisms may release phospholipases which in turn stimulate prostaglandin production. They may also release endotoxins such as lipopolysaccharide (LPS) which act on macrophages to cause prostaglandin or cytokine release. Cytokines might include IL-1, TNF, and IL-6. In turn, these act on amnion or decidual stromal cells to increase expression of PGHS2, and to decrease expression of the prostaglandin-metabolizing enzyme 15-hydroxyprostaglandin dehydrogenase (PGDH). In addition, IL-1 also stimulates output of other cytokines including IL-6 and IL-8 from decidua, thereby establishing a positive cytokine-PG cascade. Cytokines such as IL-1 may also cause release of other uterotonins including oxytocin and CRH from the decidua membranes and/or placenta.

5.1.3. Prostaglandin Metabolism

Recent studies have suggested that the predominance of 15-hydroxyprostaglandin dehydrogenase (PGDH) in chorion trophoblast presents a metabolic barrier to the passage of prostaglandins generated within amnion or chorion during gestation towards the underlying decidua and myometrium (*see* Fig. 11). Recently, a group of patients presenting in *idiopathic preterm labor* without clinical or histologic evidence of infection was identified with decreased levels of PGDH activity, decreased immunoreactive PGDH protein, and decreased PGDH mRNA in chorion trophoblasts. There was a further reduction of ir-PGDH and PGDH activity in chorion of patients presenting in preterm labor with diagnosed infection. In the latter group, the loss of PGDH was accounted for by loss of the trophoblast cells that accompanies the infective process (*see* Fig. 11). The loss of PGDH from the trophoblast cells in idiopathic preterm labor without infection was specific to this tissue because placental PGDH was normal and unchanged in this group of patients. This observation suggests that PGDH activity in chorion trophoblast is regulated, and that factors which alter its expression may predispose to premature delivery. At full-term pregnancy, the activity of PGDH in chorion is normally sufficient to prevent transmembrane passage of most of the prostaglandin generated in the amnion or chorion. The likely source of the prostaglandin that stimulates myometrial contractility is therefore decidua and/or myometrium. In idiopathic preterm delivery, in the absence of infection, PGDH expression is reduced and so is the number of chorion trophoblast cells. In the presence of an inflammatory response, with loss of trophoblast cells, PGDH activity in chorion is further reduced dramatically. In these circumstances, if prostaglandin production is stimulated in amnion or chorion, those prostaglandins will not be metabolized and can easily reach the myometrium to provoke preterm birth.

In vitro studies with chorion trophoblast cells have indicated that regulation of PGDH occurs in response to steroid hormones as well as to cytokines. Progesterone stimulates and maintains PGDH activity whereas

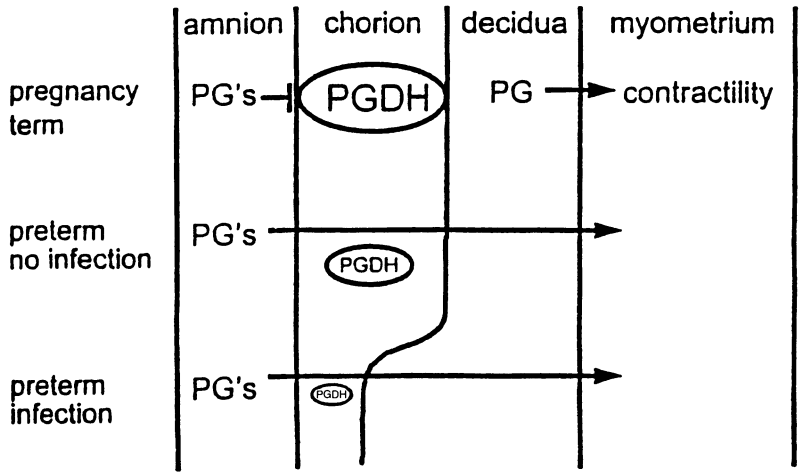

Fig. 11. The role of prostaglandin dehydrogenase (PGDH) in chorion in providing a metabolic barrier to the transmembrane passage of prostaglandins (PG's) from amnion to decidua and myometrium during normal term pregnancy is reduced in preterm labor without infection, and diminished further in preterm labor with infection.

glucocorticoids such as cortisol, decrease PGDH expression. The enzymes for progesterone synthesis from pregnenolone (3β-hydroxysteroid dehydrogenase) and cortisone-cortisol interconversion (11β-hydroxysteroid dehydrogenase Type 1) are localized within chorion trophoblasts indicating the potential for local regulation of production of these steroids, and the paracrine/autocrine regulation of PGDH. In support of this contention, activity of PGDH in chorion is inhibited in cells treated with cortisol, antiprogestins, or trilostane, a 3β-HSD inhibitor and is stimulated by synthetic progestins. Cortisone is as effective as cortisol in chorion, but ineffective in placenta. This finding is explained by the presence in chorion of 11β-HSD-1, with the potential to convert cortisone to the biologically active cortisol. Placenta contains much less 11β-HSD-1, and predominantly 11β-HSD-2, which oxidizes cortisol back to cortisone. Thus, regulation of PGDH in chorion trophoblast cells appears to involve a balance between effects of progesterone and cortisol, possibly acting through the same glucocorticoid receptor (Fig. 12). In addition, cytokines such as IL-1 inhibit expression and activity of the enzyme.

The levels of PGDH in chorion are not uniform throughout the uterus. At term, PGDH in chorion trophoblasts overlying the internal os to the *cervix* was dramatically reduced compared to other regions of the membranes. It is possible that glucocorticoids, or cytokines, present in the vagina or cervix, or generated from adherent decidual fragments, could specifically decrease expression of PGDH in this region of membranes. This observation suggests that prostaglandins generated within amnion and chorion in the lower segment might escape metabolism in chorion at the time of labor. These prostaglandins could then effect changes in effacement and dilation of the cervix and facilitate progression of the labor process.

5.2. Steroid Effects on the Myometrium

The failure to demonstrate systemic withdrawal of progesterone in human pregnancy has led many investigators to examine the possibility that loss of intrauterine progesterone action might occur locally at the level of the fetal membranes. One possibility is increased metabolism of progesterone within these tissues to an extent that is not reflected in systemic progesterone concentrations. Support for this idea is

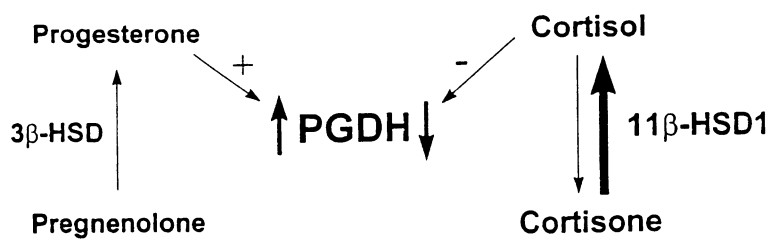

Fig. 12. Prostaglandin dehydrogenase (PGDH) activity in human chorion depends upon the balance of stimulatory influences from progesterone and inhibitory influences of cortisol. In turn, levels of progesterone are determined by activity of 3β-hydroxysteroid dehydrogenase (3β-HSD) in chorion trophoblasts, and levels of cortisol may be influenced by the ability of 11β-hydroxysteroid dehydrogenase Type 1 (11β-HSD1) to convert cortisone to cortisol within chorion. Progesterone and cortisol may also be derived from the maternal systemic circulation, or from amniotic fluid.

derived from measurements on *5α-reductase-1* knockout mice. These animals do not deliver at the expected time, although some uterine contractility develops late in gestation. It has been suggested that loss of 5α reduction in the knockout animal results in failure to complete tissue-specific progesterone withdrawal to the inactive 5α-dihydroprogesterone. Others have suggested that progesterone action may be antagonized in intrauterine tissues by endogenous antiprogestins such as transforming growth factor β (TGFβ). TGFβ acts on endometrial cells in culture to prevent expression of a spectrum of progestin-responsive genes including PTHrP and enkephalinase. TGFβ-1 mRNA is present in human myometrium during pregnancy, and the possibility remains that activation of the latent form of this protein in the myometrium occurs to allow local antagonism of progesterone action at the time of labor. A further persuasive option to bring about progesterone withdrawal is derived from studies showing that placental CRH output is inhibited by progesterone, and increased by corticosteroids, including dexamethasone. It has been argued that the inhibitory effect of progesterone on CRH transcription might be mediated through interaction with the glucocorticoid receptor. As cortisol levels increase at term, these compete with progesterone for binding to the glucocorticoid receptor, resulting in a diminished inhibitory influence, which appears in culture as a relative stimulation of CRH output. This intriguing possibility requires further experimental examination.

5.3. Uterine Peptides and Parturition

It is now well recognized that several peptides of pituitary or hypothalamic origin are also produced by the placenta and fetal membranes and may have important physiologic actions during pregnancy and at the time of birth. Specific examples include oxytocin and CRH as peptide modulators, and proteins structurally related to TGFβ. The latter include inhibin and *activin*. Both are heterodymeric glycoproteins, produced in trophoblast from fetal membranes. Activin A levels rise progressively in maternal plasma during pregnancy, and at the time of preterm labor. Activin A stimulates prostaglandin release from cultured amnion cells and may contribute to uterotonin production of later pregnancy.

5.3.1. Corticotrophin-Releasing Hormone (CRH)

Prepro-CRH mRNA is present in placental tissue and decidua in increasing amounts during human pregnancy. Changes in CRH expression result in increases in CRH peptide within placental tissue and in an exponential rise in the concentration of CRH in the maternal peripheral circulation during the course of pregnancy. Maternal CRH concentrations are elevated in preterm labor in the absence of infection. This elevation may occur quite early in pregnancy, and has recently been alluded to as the "*placental clock*" of gestation length. At 28–36 wk of gestation elevations in maternal CRH levels may be used to discriminate patients presenting in threatened preterm labor who deliver within 24–48 h from those patients who do not deliver within that time. In normal pregnancy, the bioactivity of circulating CRH may be diminished by the presence in maternal blood of a high affinity CRH-binding protein (CRHBP). CRHBP attenuates the output of ACTH from pituitary cells or prostaglandin output from intrauterine tissues in response to CRH. In the last 4-5 wks of pregnancy, CRHBP concentrations fall, presumably increasing concentrations of free CRH in the circulation.

Immunoreactive (ir-) CRH localizes to the syncytiotrophoblast in human pregnancy. It also localizes to amnion epithelial cells and to chorion trophoblast cells. CRH output from placenta is decreased by NO and progesterone, and stimulated by glucocorticoids, prostaglandins, cytokines, and vasopressin. Glucocorticoids increase levels of pro-CRH mRNA in placental cells. This effect appears to require the cAMP response element within the CRH proximal promoter region. Glucocorticoids also increase placental CRH output in vivo. As discussed earlier, CRH and corticosteroids increase PGHS2 expression by intrauterine tissues, and glucocorticoids decrease PGDH activity in human fetal membranes. These results, which are supportive of an interrelationship between glucocorticoids and CRH with prostaglandin production, might provide an explanation of the transient increases in uterine activity seen in patients with multiple gestations treated prenatally with glucocorticoids.

In the placenta, CRH stimulates output of POMC-derived peptides including ACTH and beta-endorphin. Thus, the placenta secretes peptides capable of stimulating fetal HPA function, in addition to having direct effects on the uterus. More recently, it has been shown that CRH may directly affect the output of steroids from the human fetal adrenal gland. Human fetal adrenal cortical cells in culture produced cortisol and DHAS in response to addition of CRH in a dose-dependent fashion. Thus, in normal gestation, a series of positive feedback loops are established, whereby cortisol from the fetal adrenal or from the maternal

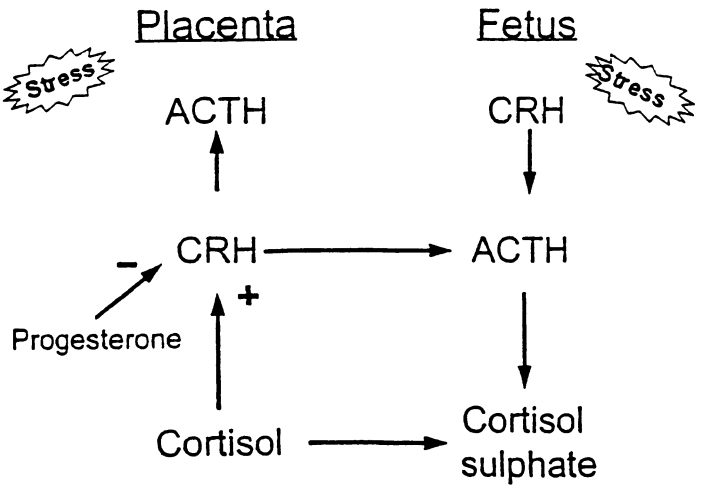

Fig. 13. Activation of placental CRH production in the presence of "stress," i.e., fetal hypoxemia. CRH, corticotrophin-releasing hormone; ACTH, adrenocorticotrophic hormone.

adrenal may stimulate placental CRH. This, in turn, directly or indirectly further increases fetal adrenal steroidogenesis. In response to an adverse uterine environment such as hypoxemia, we have seen already that the fetus activates HPA function with increased output of ACTH and of cortisol (Fig. 13). In the primate fetus, ACTH also increases DHAS production from the fetal zone of the fetal adrenal gland. It is likely that fetal cortisol reaches the placenta where it upregulates expression of placental CRH. In turn, placental CRH stimulates placental ACTH. Both peptides are secreted back into the fetal and into the maternal circulation. In the placenta, these peptides are vasodilators, acting through the NO-cGMP pathway to promote vascular relaxation. This action is perceived as an attempt to increase uteroplacental blood flow and correct an oxygen deficit. If it fails, then continued fetal hypoxemia sustains the stimulus for fetal cortisol production, resulting in further elevations of placental CRH output, and activation of the CRH-prostaglandin pathway, uterine contractility, and birth. Similar endocrine mechanisms may also underlie the association between maternal stress and preterm birth. There are dramatic increases in maternal CRH levels in patients with elevated scores for perceived stress and anxiety states as early as 20–24 wk of gestation.

The effects of CRH on the myometrium are both interesting and confusing. Human myometrium expresses predominantly *CRH receptor subtype 1* (CRH-R1). In the pituitary, this receptor couples to adenylate cyclase and leads to increases in cAMP. Activation of this receptor would be expected to result in uterine quiescence. It has been suggested recently that the effects of CRH may vary simultaneously in different areas of the uterus. In the lower segment at term, the role of CRH may be to facilitate relaxation, and passage of the fetus through the birth canal. An alteration in the binding affinity of myometrial CRH receptor subtypes in the fundal region of the uterus with labor, or binding to CRH-R2 with PLC activation may affect contractility. Further, CRH-induced increases in cAMP may have direct effects on PGHS2 in fetal membranes. Those prostaglandins could then stimulate uterine contractility through EP1, EP3 or FP receptors. The peptide urocortin, which is structurally related to CRH is now recognized as the natural ligand for CRH-R2. However, in human pregnancy expression of urocortin appears to be much lower than that of CRH. This may allow CRH accessibility to CRH-R2 subtypes. Further studies in other species with different ratios of CRH-R1 to R2 (i.e., the rat where the uterus expresses predominantly CRH-R2) will be crucial in resolving this issue.

5.3.2. Oxytocin

Oxytocin (OT) is present in maternal and fetal blood during late gestation. Secretion within the two compartments is relatively independent. There is high amplitude spurt release of oxytocin into maternal blood at the time of labor. Umbilical arterial concentrations exceed those in the umbilical vein implying that there is also fetal secretion of oxytocin. The modest changes, however, in oxytocin concentrations in systemic plasma are offset by the increased expression of oxytocin receptor in myometrial and decidual tissue in patients at term and at preterm labor. These changes can be induced experimentally by uterine stretch, by progesterone withdrawal, and by estrogen stimulation. As discussed above, estrogen is derived

in vivo from the systemic circulation after fetal placental production, or generated locally within chorio-decidual tissue from sulphoconjugated precursors derived from the maternal plasma, or from the amniotic fluid.

The chorio-decidual tissue itself also produces oxytocin. There is a three-fold increase in the levels of OT mRNA in human decidua at the time of parturition. Estrogen increases OT mRNA and peptide levels in decidual tissue in vitro. This effect is blocked in the presence of tamoxifen, an estrogen receptor antagonist. There are no changes in oxytocin metabolism in chorion or decidua in relation to labor, suggesting that changes in oxytocin peptide content reflect alterations in oxytocin synthesis. OT promotes myometrial activity by increasing the myometrial intracellular free calcium concentration secondary to increases in PI turnover and generation of IP3. The OT receptor couples to PLC through a GTP binding protein to promote calcium mobilization. These actions can be inhibited by protein kinase A indicating that cAMP-dependent phosphorylation at a step involving the GTP binding protein-PLC coupling can antagonize the stimulatory effect on IP3 formation by OT. Thus, the relationship between these two pathways may be critical in determining intracellular Ca^{2+} levels and myometrial activity patterns.

CONCLUDING COMMENTS

In this chapter, we have focused on endocrine regulation of myometrial activity during pregnancy and at the time of parturition. It is clear that the development and growth of the fetus is dependent upon the regulation of myometrial contractility. In turn, the myometrium is influenced by steroids, peptides, eicosanoids, and by the physical stimulus of uterine stretch itself. It has been suggested how aberrant expression of inhibitors, uterotrophins or uterotonins in the different phases of pregnancy or parturition may contribute to the etiology of preterm labor. It remains important to dissect actions of individual compounds, and then to consider these in the context of the total hormonal milieu. Recognition of these individual components will be critical in developing better tests for the diagnosis of preterm labor and for recognizing those patients to continue pregnancy in order to promote fetal maturation, and ultimately to treat this condition more rationally than current knowledge allows.

SELECTED READINGS

Challis JRG, Lye SJ, Gibb W. Prostaglandins and parturition. Ann NY Acad Sci 1997; 828:254.

Challis JRG, Mitchell BF. Steroid production by the fetal membranes in relation to the onset of parturition. In: McNellis D, Challis J, MacDonald P, Nathanielsz P, Roberts J eds. The Onset of Labor: Cellular and Integrative Mechanisms. Ithaca, NY: Perinatology, 1988; 233.

Chwalisz K, Garfield RE. Regulation of the uterus and cervix during pregnancy and labor. Role of progesterone and nitric oxide. In Bulleti C, de Ziegler D, Guller S, Levitz M, eds. The Uterus: Endometrium and Myometrium. Ann NY Acad Sci 1997; 828:238.

Karalis K, Majzoub JA. Regulation of placental corticotrophin-releasing hormone by steroids—possible implication in labor initiation. Ann NY Acad Sci 1995; 551.

Liggins GC, Thorburn GD. Initiation of parturition. In: Lamming, GE, ed. Marshall's Physiology of Reproduction, 4th ed. vol. 3, Pregnancy and Lactation Part 2 London, England; Chapman and Hall 1994; 863.

Lye SJ. Initiation of parturition. Anim Reprod 1997; 42:495.

MacDonald PC, Casey ML. The accumulation of prostaglandins (PG) in amniotic fluid is an after effect of labor and not indicative of a role for PGE2 or PGF2α in the initiation of human parturition. J Clin Endocrinol Metab 1993; 77:805.

Matthews SG, Challis JRG. Regulation of the hypothalamo-pituitary-adrenocortical axis in fetal sheep. Trends Endocrinol Metab 1996; 7:14.

Riley SC, Walton JC, Herlick JM, Challis JRG. The localization and distribution of corticotrophin-releasing hormone in the human placental and fetal membranes throughout gestation. J Clin Endocrinol Metab 1991; 72:1001.

Romero R, Avila C, Brekus CA, Morotti R. The role of systemic and intrauterine infection in preterm parturition. Ann NY Acad Sci 1991; 622:355.

Sangha RK, Walton JC, Ensor CM, Tai H-H, Challis JRG. Immunohistochemical localization, mRNA abundance and activity of 15-hydroxyprostaglandin dehydrogenase in placenta and fetal membranes during term and preterm labor. J Clin Endocrinol Metab 1994; 78:982.

Thorburn GD, Challis JRG. Endocrine control of parturition. Physiol Rev 1979; 59:863.

van Meir CA, Sangha RK, Walton JC, Matthews SG, Keirse MJNC, Challis JRG. Immunoreactive 15-hydroxyprostaglandin dehydrogenase (PGDH) is reduced in fetal membranes from patients at preterm delivery in the presence of infection. Placenta 1996; 17:291.

10 Neuroendocrine Regulation of Lactation and Milk Ejection

H. Allen Tucker, PHD

CONTENTS

1. INTRODUCTION

Milk is a secretion of the *mammary gland* that supplies all of the necessary nutrients for survival of mammalian species. Secretion of milk, or *lactation*, is the final stage of the reproductive cycle and is completely dependent on secretion of numerous hormones, many of which are, in turn, regulated by the neuroendocrine system. These hormones are responsible for *growth of the mammary gland*, for *initiating secretion of milk* at the time of *parturition* (lactogenesis), and for *maintaining secretion of milk* in the postpartum period *(galactopoiesis)* of the reproductive cycle. After milk is secreted from epithelial cells of the mammary gland, it is stored in the lumen of the *alveoli* and *ducts* of the gland and then removed in response to *suckling* or *milking*. Removal of milk from the mammary gland requires *milk ejection*, which is a classical neuroendocrine reflex. After a brief description of the anatomy of the mammary gland and the growth patterns of the mammary gland through the life cycle of a mammal, the major thrust of this chapter will be to describe hormonal and neuroendocrine regulation of mammary growth, lactogenesis, maintenance of lactation, and milk ejection.

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2. ANATOMY OF THE MAMMARY GLAND

The mammary gland is composed of a variety of cell types and contains a number of systems that are essential for its function. The secretory epithelial cells of the mammary gland are derived from ectoderm, whereas *connective tissue cells*, which include *fibroblasts* and *adipocytes*, are derived from mesoderm. The connective tissue cells form a matrix around the epithelial cells. The number of epithelial cells wax and wane with the physiological stages of the life cycle of female mammals.

2.1. Alveoli—Secretory Units of the Mammary Gland

Alveoli are composed of a one-cell layer of epithelium arranged in a hollow ball structure thereby forming a lumen (*see* Fig. 1). The function of the cells of

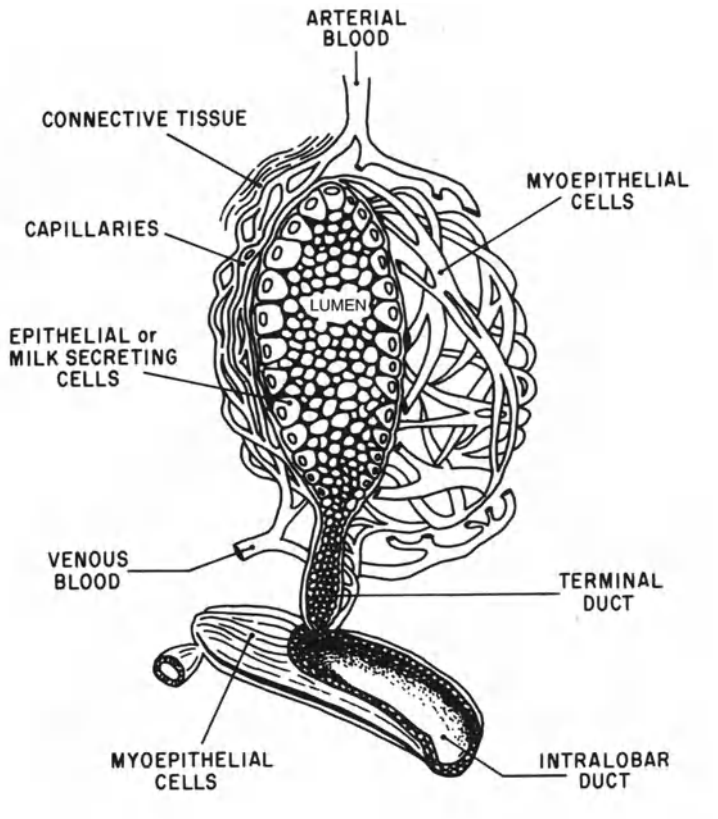

Fig. 1. Blood supply, myoepithelial cells, and connective tissue surrounding the epithelial cells that form the alveoli and ducts of the mammary gland. Modified from Turner CW. *The Mammary Gland.* Columbia: Lucas Brothers, 1952.

the alveoli is to remove nutrients from the blood and transform these nutrients into the components of milk. The major components of milk include water, *proteins* (casein, α-lactalbumin, and β-lactoglobulin), *fat* (triglycerides), *carbohydrates* (lactose), *minerals* (especially calcium and phosphorous), and *vitamins*. There is wide variation in concentrations of constituents amongst species; for example, protein in whole milk varies from 1 to 20%, fat from trace to 50% (marine mammals), lactose from 0 to 10%, and total mineral content from 0.2 to 2%. These components of milk are secreted into the lumen of the alveoli where they are stored until removed by suckling or milking.

2.2. Ducts—Structures that Transport Milk from Alveoli to Offspring

The mammary duct system is a series of drainage channels beginning with the alveolus and ending at the *teat* or *nipple* (Fig. 1). Thus, the ducts serve as a conduit for passage of milk from the alveoli to the offspring or milking machine. Ducts are composed of a two-cell layer of epithelium arranged in a branching motif. Branching of ducts is conducive to greater secretion of milk because more alveoli can grow out from the ducts. The ducts also store milk between bouts of suckling or milking.

2.3. Myoepithelium—Cells Responsible for Milk Ejection

Surrounding each alveolus and arranged along the surface of the ducts are specialized muscle-like cells, termed *myoepithelial cells*, that contract in response to *oxytocin* and force milk from the lumen of the alveoli, through the ducts to the surface of the nipple or teat (Fig. 1).

2.4. Stroma—Cells that Support the Mammary Gland

Ducts and alveoli are attached to connective tissue (Fig. 1). Adipocytes are also found throughout the mammary gland, but outside the basement membranes of ducts and alveoli. Connective tissue and adipocytes provide a framework into which the ducts and alveoli penetrate during periods of mammary growth.

2.5. Vascular System of the Mammary Gland

The mammary gland receives about 500 vol of blood for each volume of milk produced. Factors that control rate of blood flow affect the volume of milk secreted. The cells of each alveolus and the myoepi-

thelial cells are surrounded by, and in close contact with, an extensive capillary network (Fig. 1).

2.6. Innervation of the Mammary Gland

Nerve supply of the mammary gland consists of afferent sensory fibers that arise from receptors that are sensitive to touch, temperature, and pain, and are located in the skin, especially on the surface of the teat or nipple. These afferent fibers transmit signals from stimuli associated with suckling to the hypothalamus in the brain. Efferent fibers from the central nervous system (CNS) to the mammary gland are solely a part of the *sympathetic system*; there is no parasympathetic supply. Efferent fibers are restricted to innervation of the smooth muscle of arterioles, milk collecting ducts, and teat sphincters (if present). Efferent nerves control blood flow within the gland and are responsible for teat or nipple erection. Epithelial and myoepithelial cells are not innervated.

3. NORMAL MAMMARY GLAND GROWTH DURING VARIOUS PHYSIOLOGICAL STATES

Numbers of mammary ducts and alveoli, which constitute the parenchymal portion of the gland, are highly correlated with the volume of milk secreted. Thus, greater numbers of *parenchymal cells* increase the potential ability of the mother to rear more offspring in litter bearing species. In the case of single-birth mothers, size of the individual offspring may be greater at *weaning* if there are a large number of mammary parenchymal cells. In the dairy industry, the number of parenchymal cells has increased with selective breeding, and over time, this has led to greater amounts of milk produced per animal, which has economic importance because of the resultant increase in efficiency of milk production. Thus, studies of normal as well as abnormal growth (mammary cancer) have obvious practical importance.

3.1 Mammary Growth Before Birth

The first discernible rudiment of the mammary gland is a thickening of the *ectodermal cell* layer on the ventral surface of the embryo where the gland will be positioned in the adult. These ectodermal cells divide and aggregate into two lines on each side of the midline, and with further division, migration, and differentiation, these cells form a mammary bud. The *mammary bud* is the anlage of the epithelium of the ducts, alveoli, and myoepithelium. The number of buds determines the number of mammary glands characteristic for each species. The bud elongates above and below the surface of the fetus, and in those species that present a single duct through the teat, (e.g., *cattle*) the bud gives rise to a *primary sprout*, which is the anlage of the *teat* and *gland cisterns*. The primary sprout, in turn, divides and gives rise to several secondary sprouts, which eventually become the major ducts of the mature mammary gland. In those species that present multiple ductular openings on the nipple (e.g., dog, rabbit, human), the mammary bud differentiates into multiple sprouts characteristic of the number of such ducts found in the mature animal. At birth, the sprouts may have branched further, but the epithelial portion of the mammary gland is immature. In contrast, the connective tissue and fat pad portions, which differentiate from *mesenchyme*, are in mature form.

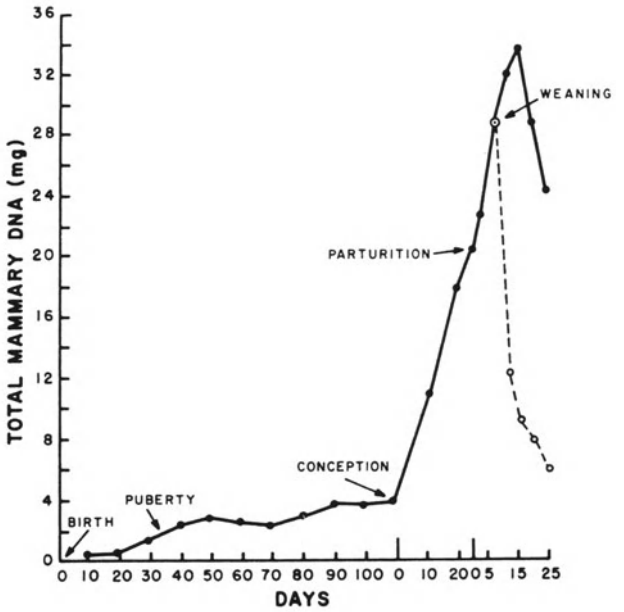

Fig. 2. Mammary development based on deoxyribonucleic acid (DNA) content of rats from birth through pregnancy, lactation and after weaning (involution). Reprinted from Tucker H.A. *J Dairy Sci* 1969; 52: 721.

The classical hormones that cause mammary growth in the adult female (*estradiol, progesterone, prolactin,* and *growth hormone*) are not essential for embryonic mammary growth. Rather, during embryonic development metabolic hormones and several growth factors promote growth of mammary tissue.

3.2. Mammary Growth from Birth through Puberty

Changes in total mammary cell numbers from birth until puberty are illustrated in Fig. 2. The ducts of

the mammary gland grow into the surrounding fat pad in proportion to increases in body size (*isometric growth*) until shortly before puberty when the gland begins to grow faster than the body (*allometric growth*), primarily in response to increased secretion of estrogens. After puberty, most of the growth of the ducts occurs during the estrogenic phase of the *estrous cycle*, and then the gland regresses slightly via *apoptosis* during the progestational phase of the estrous cycle; although the net gain in cell numbers remains allometric. However, after the first few estrous cycles, the net gain returns to an isometric pattern.

Growth of the mammary gland consists of differentiation of solid cords of cells derived from *cap cells* located at the ends of ducts. These cap cells are the precursors of future myoepithelial cells, ducts, and alveoli. Later, canalization of the solid core of cells forms the lumens typical of mature ducts and alveoli that make up the lactating mammary gland. As a general rule, the mammary gland does not contain alveoli until after conception. It should be noted that enlargement of the breasts of women at puberty is associated primarily with growth of the fat pad, not growth of the epithelial components of the breasts.

3.3. Mammary Growth During Pregnancy

With conception, mammary ducts begin to grow faster, and alveoli differentiate along the sides of the ducts. The epithelial components of the gland invade the stroma and begin to displace the adipose tissue of the fat pad. The fat pad determines the outer limits of the size of the mammary gland. Expansion of the parenchymal portion of the gland continues throughout pregnancy (*see* Fig. 2), but little secretory activity occurs until approximately the middle of the last trimester of pregnancy.

3.4. Mammary Growth During Lactation

After parturition, numbers of mammary epithelial cells continue to increase, with maximum numbers coinciding with the peak of secretory activity in early lactation (Fig. 2). Maintenance of mammary cell numbers and secretory activity is dependent on intensity and duration of the suckling or milking stimulus. This stimulus is transmitted from the teat or nipple via nerves to the spinal cord and then to the *hypothalamus* where appropriate *neurotransmitters* and *neuropeptides* induce release of galactopoietic peptide hormones from the anterior pituitary gland into blood (*see* Section 6). At the mammary gland, these hormones bind to *receptors* to maintain cell numbers and secretory activity. With advancing lactation the suckling stimulus gradually declines, which leads to a loss in the number of alveolar epithelial cells and their secretory activity.

3.5. Involution of the Mammary Gland

Loss of secretory activity and reduced numbers of alveolar epithelial cells in the mammary gland characterize *involution*. Involution begins after the peak of lactation and accelerates at weaning (*see* Fig. 2). Apoptosis accounts for the death of epithelial cells during involution. Although alveoli are lost during involution, myoepithelial cells and connective tissue of the stroma remain, and the adipocytes gain lipid. Several species may become pregnant during lactation, and under these circumstances loss of alveolar cell numbers may be reduced, and many alveoli may be carried over to a subsequent lactation.

4. HORMONAL REGULATION OF MAMMARY GROWTH

Allometric mammary growth occurs when secretion rates of various hormones increase in association with changing physiological status of the female. Without change in secretion rates of hormones, mammary growth would remain isometric.

4.1. Ovarian Hormones that Stimulate Mammary Growth

Hormones of the *ovary* stimulate mammary growth. For example, administration of *estradiol* stimulates mammary duct growth, whereas progesterone increases alveolar growth. A combination of estradiol and progesterone synergistically stimulates duct and alveolar growth. Conversely, ovariectomy prevents the allometric growth of the mammary gland in the peripubertal period. Furthermore, concentrations of estradiol and progesterone in blood are markedly and coincidentally increased during pregnancy (Fig. 3). During the estrous cycle, elevated concentrations of estradiol and progesterone are asynchronous (Fig. 3), which may explain why allometric mammary growth is relatively small and not sustained in this period. Thus, it seems likely that a coincidental increase in secretion of these steroids is a primary signal for mammary growth during pregnancy. In contrast, allometric growth of the mammary gland during early lactation is primarily dependent on hormones from glands other than the ovary because ovariectomy does not alter suckling-induced increases in mammary growth.

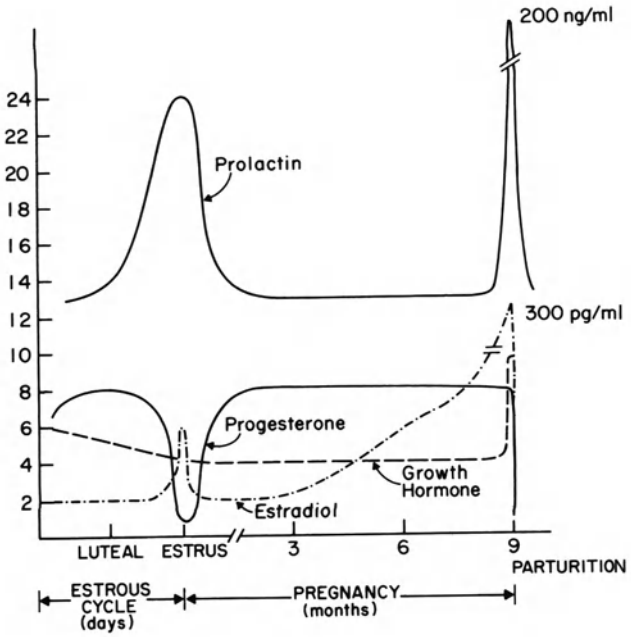

Fig. 3. Changes in concentration of estradiol, progesterone, prolactin and growth hormone in blood of cattle during the days of an estrous cycle and months of a pregnancy. Modified from Tucker HA. In: Larson BL, ed. Lactation, Ames: the Iowa State Univ. Press, 1985: 39.

Studies of neuroendocrine regulation of estrogen and progesterone directly related to mammary growth are lacking. The reader is referred to Chapters 8 and 9 for general discussions of this topic.

4.2. Neuroendocrine Regulation of Anterior Pituitary Hormones that Stimulate Mammary Growth

Hypophysectomy causes atrophy of the mammary gland, and the mammary growth-promoting activities of estradiol and progesterone are completely prevented in hypophysectomized animals. Many studies have confirmed that administration of prolactin, growth hormone, and *adrenocorticotropin* (acting via increased secretion of *glucocorticoids* from the adrenal gland) will induce varying degrees of mammary proliferation, depending upon the species.

Administration of prolactin increases alveolar growth of the mammary gland. Other evidence for the importance of prolactin's role in mammary growth has come from studies of its endogenous secretion. In most physiological states, secretion of prolactin is tonically suppressed. *Dopamine* is the major factor inhibiting release of prolactin. Dopamine originates from *tuberoinfundibular dopaminergic (TIDA)* neurons whose perikarya are in the *arcuate nucleus* of the hypothalamus and whose terminals are in the *median eminence*. Dopamine is tonically released from terminals in the median eminence into the *hypophysial-portal blood* vessels and carried to the anterior pituitary gland where it binds to D_2 receptors on *lactotropes* and suppresses secretion of prolactin. However, during the *proestrus* phase of the estrous cycle increased secretion of estrogen stimulates the lactotropes of the *anterior pituitary gland* to secrete prolactin,which subsequently feeds back to increase activity of TIDA neurons. It is likely that increased secretion of estrogen and prolactin causes the small growth spurt of the mammary gland during proestrus.

Injection of growth hormone stimulates mammary duct development. Indeed, within a species, growth hormone is often a more potent stimulator of mammary growth than prolactin. Growth hormone may directly stimulate the mammary epithelial cells to grow, or growth hormone may stimulate increased secretion of *insulin-like growth factor-1* (IGF-1), which stimulates growth of the mammary epithelial cells. But, it is not known whether growth hormone normally stimulates mammary growth during the proestrus phase of the estrous cycle.

A major portion of mammary growth occurs during pregnancy (Fig. 2). In rats during early pregnancy there are two daily surges in secretion of prolactin, which are associated with reduced TIDA neuronal activity. But, upon appearance of *placental lactogens* before midpregnancy, these daily surges in prolactin cease and concentrations of prolactin in blood remain low. Similarly, in cattle during pregnancy, endogenous concentrations of prolactin in blood do not change markedly until very late in pregnancy when they increase (Fig. 3). Prolonged elevation of estrogens, such as observed for the greater part of pregnancy, leads to increased activity of TIDA neurons, which may explain why prolactin secretion is relatively low during this period. Similar to prolactin, concentrations of growth hormone in blood of rats and cows do not increase until late in pregnancy. Thus, the greatest magnitude of growth of the mammary gland during *gestation* occurs when concentrations of both prolactin and growth hormone are rather low and stable. It, therefore, seems probable that prolactin and growth hormone permit the elevated secretion of estradiol and progesterone to drive the large pregnancy-associated growth of the mammary gland (*see* Chapters 8 and 9 for discussion of the neuroendocrine regulation of estrogen and progesterone).

Allometric growth of the mammary gland during

the early stages of lactation (*see* Fig. 2) occurs in response to suckling-induced release of anterior pituitary hormones. Additional details of suckling-induced changes in the neuroendocrine system will be described later.

4.3. Placental Hormones that Stimulate Mammary Growth

The placenta secretes estradiol and progesterone as well as various forms of placental lactogen, peptide hormones that have many of the mammary growth-promoting properties of prolactin and growth hormone. Generally, as pregnancy advances secretion of placental lactogens into the maternal blood increase. Thus, the placenta contributes substantially to hormonal stimulation of mammary growth during pregnancy. It has been estimated that during the first half of pregnancy, most of the hormonal stimulation for mammary growth is derived from the ovaries and anterior pituitary gland of the mother; but during the second half of pregnancy, the mammary growth-promoting hormones come primarily from the maternal placenta. Although neuroendocrine regulation of secretion of the placental lactogens has not been definitively established, there is evidence that placental lactogens feed back at the hypothalamus to increase activity of TIDA neurons, which in turn suppresses release of prolactin.

When animals are concurrently pregnant and lactating, placental lactogen suppresses suckling-induced release of prolactin. However, lactation is not suppressed because placental lactogen can substitute for prolactin by binding to and activating the prolactin receptor in the mammary gland.

4.4. Nervous System Regulation of Mammary Growth

Innervation of the mammary gland plays little or no role in development of the mammary gland in the peripubertal and pregnancy periods. For example, the mammary gland can be completely denervated, or even transplanted to a remote site on the body, and mammary growth will occur provided blood vessels are reconnected and an appropriate hormonal milieu is present. Collectively, neuroendocrine control of mammary growth during the peripubertal period and pregnancy, beyond that of maintaining secretion of the anterior pituitary hormones, is probably limited. In contrast, during lactation the neural connections from the teat to the spinal cord and hypothalamus are essential to mediate suckling-induced increases in mammary development.

5. HORMONAL REGULATION OF LACTOGENESIS

Initiation of lactation, or lactogenesis, begins late in pregnancy, continues through parturition and ceases when *milk yield* reaches its peak in the early postpartum period. Lactogenesis consists of two phases. During the first phase, mammary alveolar cells differentiate histologically and enzymatically, whereas during the second phase the alveolar cells begin to secrete copiously the components of milk. An example of the large increase around the time of parturition in one of the components of milk, α-lactalbumin, is depicted in Fig. 4. In order to initiate a copious flow of milk, large numbers of alveolar cells must be present. As outlined below, changes in secretion rates of a variety of hormones are involved in lactogenesis.

5.1. Ovarian Hormones that Regulate Lactogenesis

Administration of estrogens to animals with well-developed mammary glands will initiate lactation. This response has been attributed to the fact that estrogens: 1) directly stimulate the lactotropes of the anterior pituitary gland to secrete prolactin, 2) increase secretion of a *prolactin releasing factor*, and 3) may act directly at the mammary gland to initiate lactation, perhaps by increasing the number of prolactin binding sites. In the late stages of gestation, secretion of estrogens increases sharply in some species (but not in women), coincident with lactogenesis (*see* Fig. 4).

Increased concentrations of progesterone, characteristic of pregnancy, inhibit lactogenesis. Conversely, marked reductions in secretion of progesterone in pregnant animals will promptly initiate lactation. But simply reducing progesterone is generally not sufficient to induce lactation. Rather, positive factors that initiate lactation also must be activated. For example, lactogenesis does not occur after reduction of progesterone in adrenalectomized or hypophysectomized animals. Administration of progesterone during late pregnancy retards lactogenesis, and progesterone blocks the lactogenic response of mammary tissue to prolactin. After conception, concentrations of progesterone rapidly increase, and these high levels of progesterone inhibit lactogenesis until concentrations decline with impending parturition (*see* Fig. 4),

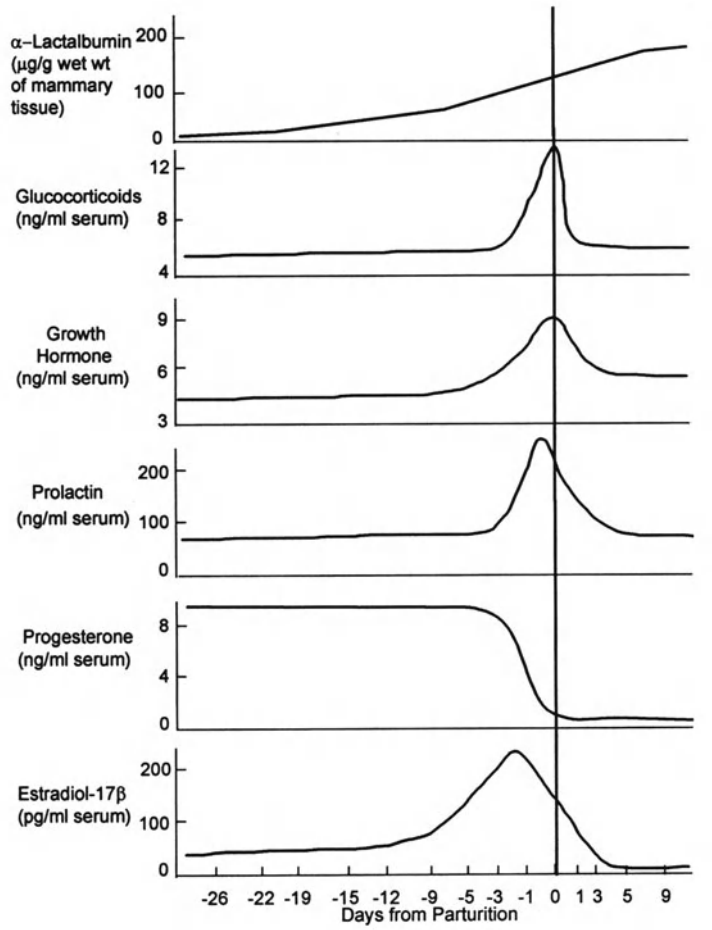

Fig. 4. Changes in concentrations of α-lactalbumin in mammary tissue and hormones in blood serum of cows during the periparturient period. Modified from Tucker HA. *Seminars Perinatal* 1979; 3: 199.

or in the case of women in the first day or two after parturition (Fig. 5). Indeed, the second phase of lactogenesis generally coincides with the decline in secretion of progesterone in the periparturient period. In sheep, increased secretion of fetal *cortisol* decreases maternal progesterone secretion in the periparturient period. Further discussion of regulation of progesterone secretion in the periparturient period is in Chapter 9.

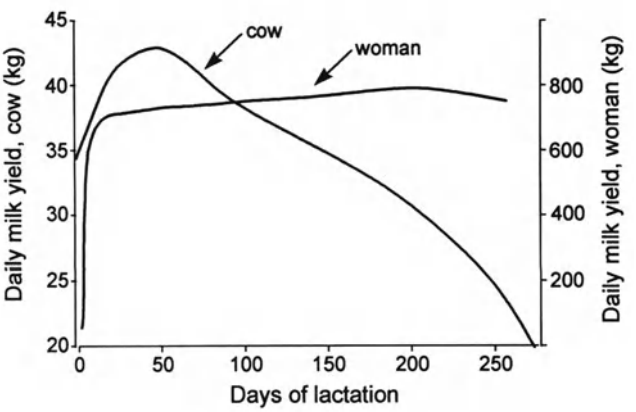

Fig. 5. Milk yield curves of the dairy cow and woman.

5.2. Neuroendocrine Regulation of Anterior Pituitary Hormones that Induce Lactogenesis

Hypophysectomy during pregnancy prevents occurrence of lactogenesis despite normal delivery of offspring at parturition. Thus, hormones secreted from the anterior pituitary are involved in lactogenesis. Indeed, the initial observation that administration of aqueous extracts of the anterior pituitary gland into individual mammary ducts of pseudopregnant rabbits initiated lactation in only those ducts injected eventually led to the isolation and chemical identity of prolactin in the anterior pituitary gland. There is a major surge in secretion of prolactin immediately after the precipitous decline in concentrations of progesterone in serum before parturition (Fig. 4). If this surge in prolactin is blocked with the dopamine agonist, *bromocriptine*, as shown in Fig. 6, lactogenesis is delayed

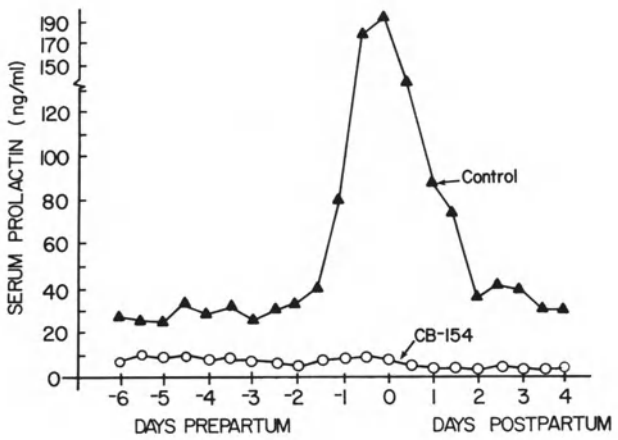

Fig. 6. Bromocriptine CB-154, a dopamine agonist, suppression of concentrations of prolactin in serum of cows. Bromocriptine was administered from approximately 12 days before parturition through 10 days after parturition. From Akers RM, Bauman DE, Capuco AV, Goodman GT, Tucker HA. Prolactin regulation of milk secretion and biochemical differentiation of mammary epithelial cells in periparturient cows. *Endocrinology* 1981; 109(1) 23–30. © The Endocrine Society.

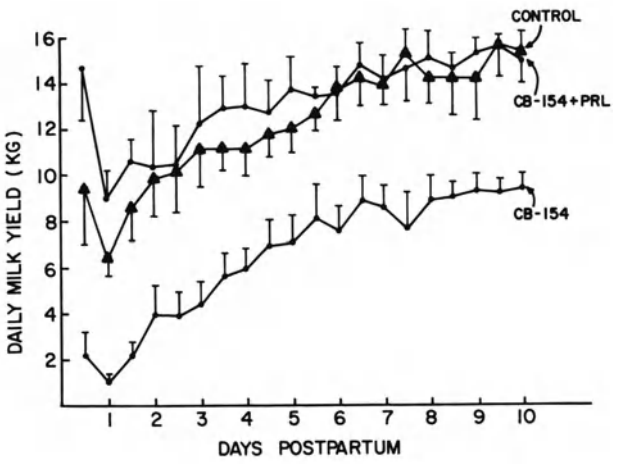

Fig. 7. Daily milk yields of untreated control cows (filled circles), cows treated with the dopamine agonist, bromocriptine CB-154 (bottom line), or cows treated with bromocriptine plus exogenous prolactin (PRL) (filled triangles). Bromocriptine was injected from approximately 12 d before parturition through 10 d after parturition. In cows administered bromocriptine plus prolactin, the prolactin was infused intravenously beginning 6 d before expected parturition at doses designed to mimic the normal periparturient surge in prolactin. From Akers RM, Bauman DE, Capuco AV, Goodman GT, and Tucker HA. Prolactin regulation of milk secretion and biochemical differentiation of mammary epithelial cells in periparturient cows. *Endocrinology* 1981; 109(1) 23–30. © The Endocrine Society.

and lactation suppressed (Fig. 7). Thus, there is little doubt that prolactin plays a key role in stimulating mammary differentiation and initiation of the biochemical steps involved in synthesis of milk in the later periparturient period.

In rats, neuroendocrine involvement in the late periparturient surge of prolactin involves suppression of activity of the TIDA neurons in the hypothalamus, which reduces concentrations of dopamine in hypophysial-portal blood. This may be due to the "*stress*" of impending parturition. The "stress sensitive" central *adrenergic* system is probably also involved because blockade of either α_1- or β_1-adrenergic receptors with appropriate antagonists reduces the periparturient-induced release of prolactin. In many endocrine states, increased secretion of prolactin (and placental lactogen) feeds back to stimulate activity of the TIDA neurons and secretion of dopamine, which suppresses secretion of prolactin. However, during the periparturient period TIDA neurons become resistant to the *feedback* effects of prolactin and placental lactogen. Thus, with reduced feedback on activity of TIDA neurons, elevated concentrations of prolactin are maintained during the periparturient period. In cattle, where the sequence of events can be more easily discerned, increased secretion of estrogen coupled with the relative absence of progesterone causes the surge in secretion of prolactin. In addition to reduced activity of TIDA neurons, there is a concomitant increase in secretion within the mediobasal hypothalamus of *thyrotropin-releasing hormone* (TRH), a hormone that increases release of prolactin. Thus, it is likely that the combination of inhibition of TIDA neurons and stimulation of releasing factors from the hypothalamus cause the periparturient surge in prolactin.

There is evidence that growth hormone increases the lactogenic response of mammary tissue to prolactin and glucocorticoids, and growth hormone will substitute for prolactin in initiating lactation in some strains of mice. Human growth hormone readily induces secretion of the products of milk in a variety of test systems, but bovine growth hormone is not lactogenic in either goat or bovine mammary culture systems. Binding to the prolactin receptor most likely explains the lactogenic properties of human growth hormone. There is a periparturient surge in secretion of growth hormone, with the peak corresponding to birth of offspring (*see* Fig. 4). Moreover, there is a concurrent decrease in secretion of *somatostatin* (the neuropeptide that normally suppresses secretion of growth hormone) from the hypothalamus without change in *growth hormone-releasing hormone* (GHRH) [the neuropeptide that normally increases

secretion of growth hormone]. However, it is unclear whether the stress of parturition or some other factor causes the changes in secretion of somatostatin and growth hormone during late pregnancy.

5.3. Neuroendocrine Regulation of Adrenal Hormones that Induce Lactogenesis

Exogenous glucocorticoids or *adrenocorticotropin*, especially in combination with prolactin, stimulate lactogenesis in females with appropriately developed mammary glands. Glucocorticoids induce differentiation of the organelles of the secretory epithelial cells, which permits prolactin to subsequently stimulate *gene expression* and ultimately the synthesis of milk proteins, especially casein. This action of the glucocorticoids normally occurs during the first phase of lactogenesis, well before parturition, and is likely mediated by a reduction in *corticoid binding globulins* in the blood rather than an increase in concentration of the hormone. A reduction in binding to the corticoid binding globulins makes more glucocorticoid available at the mammary gland. Blood concentrations of corticotropin-releasing hormone of placental origin increase gradually during late pregnancy in humans and culminate with increased secretion of adrenocorticotropin and glucocorticoids at the time of delivery of the offspring (Fig. 4), which is well into the second stage of lactogenesis. Thus, there is adrenal involvement in both the first and second stages of lactogenesis.

5.4. Nervous System Induction of Lactogenesis

There is no evidence for the existence of nerves at the level of the mammary gland directly controlling lactogenesis. Suckling is not required for occurrence of lactogenesis. On the other hand, application of the milking stimulus to the teats before parturition hastens initiation of lactation in pregnant heifers, probably by suppressing release of dopamine and increasing release of *corticotropin-releasing hormone* from the hypothalamus thereby stimulating release of prolactin and adrenocorticotropin (and glucocorticoids). Prolactin and glucocorticoids, in turn, initiate lactation. Several nonspecific stresses, such as prolonged exposure to cold, heat, intense light, and restraint, will initiate milk secretion in rats with well-grown mammary ducts and alveoli. The stress that normally accompanies parturition may play a role in promoting a copious flow of milk. Again, it is very likely that stress-induced lactogenesis is associated with a neuroendocrine-mediated increase in secretion of prolactin and adrenocorticotropin (and glucocorticoids). Lesions of the medial basal hypothalamus decrease TIDA neuronal function, thereby increasing release of prolactin, which will induce milk secretion in animals that possess a large amount of mammary alveolar tissue.

6. HORMONAL REGULATION OF MILK SYNTHESIS

In most species, the shape of the lactation curve parallels the intensity of the offspring's demand for milk. Thus, the suckling stimulus is an important force that contributes to lactational performance. As noted previously, lactation in women is delayed for about 2 d after parturition (*see* Fig. 5). The shape of the lactation curve of a dairy cow commences at a relatively high level immediately after parturition, rises to a peak, and then declines despite an unchanged frequency of milking (*see* Fig. 5). In dairy cattle, where machine milking is practiced, the lactation curve is extended beyond that needed for survival of the calf. In the case of both suckling and milking stimuli, a neural signal is sent from the nipples or teats to the hypothalamus where the signal is integrated, and appropriate neurosecretory hormones are released into the hypophysial-portal vessels and delivered to the anterior pituitary gland where prolactin, and depending upon the species, adrenocorticotropin, *thyrotropin*, and growth hormone are released into blood to maintain and/or increase secretion of milk. Effects of these hormones may be exerted as the level of the mammary gland or on organs/tissues outside the mammary gland such as liver or adipocytes.

6.1. Neuroendocrine Regulation of Anterior Pituitary Hormones that Stimulate Milk Synthesis

Hypophysectomy of lactating females leads to an immediate failure of milk secretion. Therefore, some hormones of the anterior pituitary gland are essential for lactation. Hypophysectomy-induced failure of lactation is associated with a decline in metabolic activity of the mammary gland and subsequent loss of mammary parenchyma.

The anterior pituitary hormone that has received the most study is prolactin. In hypophysectomized rabbits, supplementation with prolactin will completely restore milk secretion. In hypophysectomized rats, in contrast, prolactin supplementation only partially restores the capacity to secrete milk; more com-

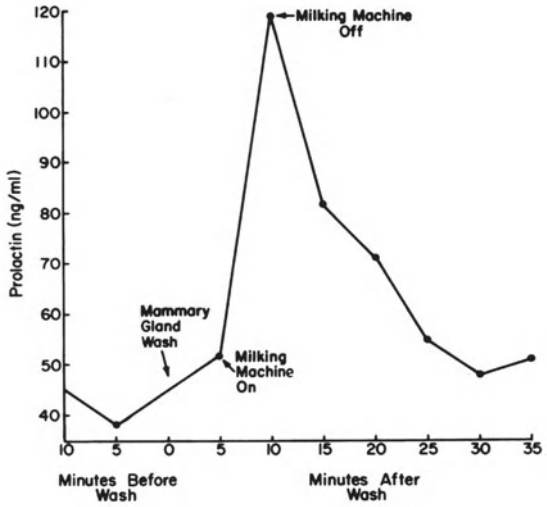

Fig. 8. Milking-induced release of prolactin in dairy cows. At time 0, the mammary gland and teats were washed for 15 s, a milking machine was attached 5 min later and removed 10 min later. Drawn from Tucker HA. *J Anim Sci*: 1971; Suppl. I, 32:137.

plete restoration requires additional supplementation with adrenocorticotropin (or a glucocorticoid) and growth hormone. Suppression of secretion of prolactin with bromocriptine in rats during midlactation decreases milk yield approximately 50%. Prolactin is an antiapoptotic factor because it maintains milk yield, at least in part, by inhibiting epithelial cell loss and by maintaining cellular differentiation. In goats, complete restoration of lactation to prehypophysectomy levels will occur following administration of a combination of prolactin, growth hormone, a glucocorticoid, and *triiodothyronine*. But once lactation is restored, prolactin is no longer required for maintenance of lactation in goats. Similarly in cattle, exogenous prolactin has no effect on maintenance of lactation. In *nonruminants*, in contrast, prolactin is continuously required for maintenance of lactation, although exogenous prolactin is more effective in stimulating milk secretion in early lactation as compared with the later phases when intensity of lactation is declining. The current concept is that prolactin is not limiting to maintenance of intense lactation in ruminants, but normal prolactin secretion rates limit milk secretion rates in nonruminants such as rabbits, rats, and women.

Other evidence regarding the importance of prolactin comes from studies showing that the neural stimulus of suckling (and in the case of *ruminants*, milking) causes release of prolactin from the anterior pituitary gland (Fig. 8). In rats, acute suckling induces a rapid disappearance of measurable concentrations of pro-

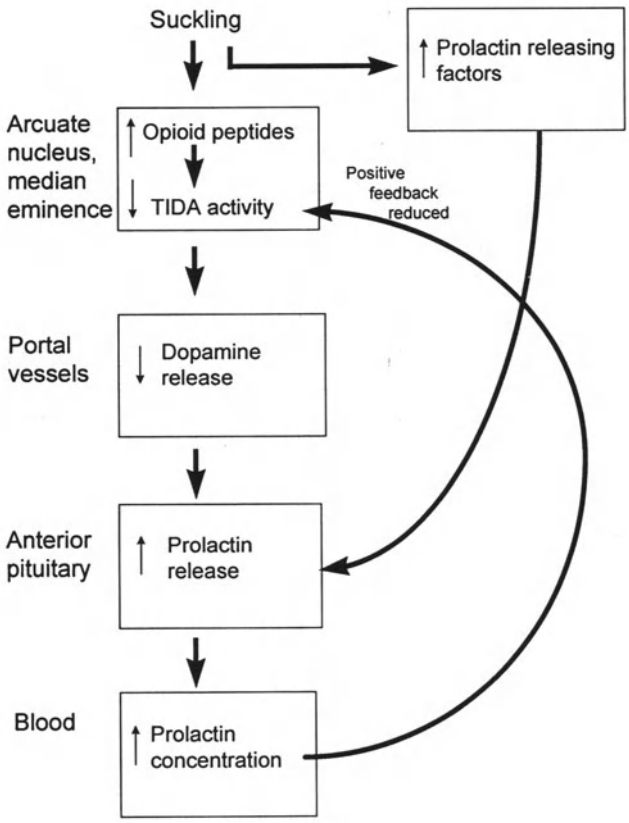

Fig. 9. Suckling-induced changes in hypothalamic factors that inhibit or stimulate release of prolactin from the anterior pituitary gland.

lactin stored in the pituitary gland, but prolactin is not immediately released into blood. Rather, prolactin must be transformed into a releasable form, which is then secreted into blood several minutes later.

Control of suckling-induced release of prolactin is integrated within the hypothalamus (Fig. 9). Acute suckling temporarily increases secretion of the *opioid peptide, endorphin*, in the arcuate nucleus, which, via the μ receptor subtype, suppresses activity of TIDA neurons, and thereby reduces dopamine release from terminals in the median eminence into the hypophysial portal vessels. During early lactation higher concentrations of prolactin feedback to increase activity of TIDA neurons. By midlactation, however, the feedback is greatly diminished, which contributes to maintenance of elevated secretion of prolactin. Lactation, via the suckling stimulus, also suppresses expression of *tyrosine hydroxylase* (the rate limiting enzyme catalyzing synthesis of dopamine) in the arcuate nucleus, which likely contributes to the reduced dopamine tone during lactation. As previously discussed, suppressed secretion of dopamine leads to increased release of prolactin.

Prolactin-releasing factors in the hypothalamus also contribute to suckling-induced release of prolactin (*see* Fig. 9). For example, thyrotropin-releasing hormone, *vasoactive intestinal peptide*, an unidentified neurointermediate lobe prolactin-releasing factor, oxytocin, *neuropeptide Y, enkephalin, neurotensin,* and *histamine* have all been implicated in suckling-induced release of prolactin. Most recently, a prolactin-releasing peptide found in the hypothalamus has been discovered to cause release of prolactin from rat anterior pituitary cells. Although the degree of involvement of these putative releasing factors is not well understood, it seems likely that the temporary suppression of dopamine at the onset of suckling enhances the action of one or more of the prolactin-releasing factors. More specifically, suppression of activity of TIDA neurons likely controls the pituitary transformation phase of prolactin secretion in response to acute suckling. TRH or other releasing factors control release of prolactin into blood. However, thyrotropin-releasing hormone is involved in acute suckling-induced release of prolactin only during the very early days of lactation. During lactation enkephalin, neuropeptide Y, and neurotensin are colocalized with dopamine in the TIDA neurons, which may diminish the inhibitory dopaminergic tone on lactotropes. Furthermore, it has been suggested that enkephalin maintains the elevated secretion of prolactin during the intervals between suckling bouts. Also, at the level of the anterior pituitary gland lactation increases the number of lactotropes to approximately 50% of total pituitary cell numbers, and the proportion of lactotropes responsive to prolactin-releasing factors increases whereas the proportion of lactotropes most susceptible to dopamine inhibition decreases.

Concentrations of prolactin in blood remain elevated as long as the suckling or milking stimulus is applied to the teats. When the suckling stimulus is prolonged, then the amount of prolactin secreted into blood is approximately the same as the amount transformed in the anterior pituitary gland.

Intensity of suckling or milking is a variable that plays a major role in maintenance of lactation. For example, increasing the suckling stimulus either by increasing the number of suckling offspring or daily frequency of milking increases production of milk. Long intervals between nursing or milking leads to a decline in the quantity of prolactin released into blood. In rats, suckling-induced release of prolactin is maximal when the nonsuckling interval is limited to 4–8 h. So frequent suckling promotes maintenance of suckling-induced release of prolactin. However, magnitude of the suckling-induced release of prolactin gradually declines after the peak of lactation despite maintenance of a strong suckling stimulus. This decline in suckling-induced release of prolactin with advanced lactation is most likely associated with a regain in sensitivity of the TIDA neurons to the feedback effects of prolactin, as well as a decline in responsiveness of lactotropes to prolactin-releasing factors.

Concurrent with the decline in suckling-induced release of prolactin in rats is an increase in concentrations of prolactin in the pituitary gland, which is associated with *exteroceptive signals*, primarily *olfaction*, emanating from the pups. This exteroceptive reflex may serve as a mechanism to ensure adequate release of prolactin as lactation advances.

Activation of dopamine receptors at the anterior pituitary gland with various D_2 agonists, e.g., bromocriptine, markedly reduces concentration of prolactin in blood of lactating females (*see* Fig. 7), and in the case of nonruminants, this treatment markedly suppresses milk synthesis. This suppression can be reversed with exogenous prolactin. Thus, dopamine agonists have been used clinically to suppress lactation in women who do not wish to nurse their children. Administration of *antisera* raised against prolactin also suppresses milk synthesis. Although dopamine agonists suppress secretion of prolactin in goats and cows, they have no effect on secretion of milk. This result supports the concept that prolactin is of lesser importance for maintenance of lactation in ruminants than in nonruminants.

Long-day *photoperiods* (16 h of light per 24 h) increase secretion of milk and increase concentrations of prolactin in sera of cattle, but most evidence suggests that changes in these variables are not causally related. Furthermore, long-day photoperiods have no affect on neuronal activity of dopaminergic neurons that terminate in the *infundibulum/pituitary* stalk of cattle. However, effects of long-day photoperiods on prolactin releasing factors cannot be ruled out. Collectively, it seems doubtful that dopamine or prolactin mediate long-day induced increments in lactational performance in cattle. Although long-day photoperiods do not affect secretion of growth hormone, they increase concentrations of insulin-like growth factor-1 (IGF-1) in plasma, which may explain the increased secretion of milk in response to long-day photoperiods.

To compensate for the increased energy requirements for secretion of milk, *food intake* increases dramatically during lactation, and this has been asso-

ciated with increased activity of neuropeptide Y neurons in the arcuate nucleus and median eminence. Indeed, intracerebroventricular administration of neuropeptide Y stimulates appetite. Acute suckling increases activity of NPY neurons in the arcuate and *dorsomedial nucleus* of the hypothalamus. Furthermore, during lactation immunoreactive neuropeptide Y is dramatically increased in TIDA neurons. Because increased secretion of prolactin during lactation has also been implicated in increasing food intake during lactation it would seem reasonable to speculate that neuropeptide Y, prolactin, and the TIDA neurons are associated in some way with stimulation of food intake during lactation.

Prolactin has been touted as the primary anterior pituitary hormone controlling secretion of milk, but growth hormone is also important. For example, in rats antiserum against growth hormone reduces milk yield 25%. As stated in section 5.2., suppression of prolactin secretion with bromocriptine alone suppresses lactation 50%, but if antiserum against growth hormone is combined with bromocriptine milk yield is reduced 90%. Growth hormone action may be mediated within the mammary gland, possibly via a direct effect on the mammary epithelial cells although definitive evidence of a growth hormone receptor within the mammary gland is presently lacking. Other evidence suggests that growth hormone acts indirectly on mammary epithelial cells by inhibiting lipogenesis in adipose tissue, which makes more energy available to the mammary gland. There is also evidence that increased secretion of IGF-1 from liver or locally from within the mammary gland mediates the galactopoietic effects of growth hormone in rats.

Although basal concentrations of growth hormone in serum during lactation are not different from those of nonlactating rats, suckling causes a transitory release of growth hormone. Suckling activates opioid and α_2-*adrenergic receptor* mechanisms within the arcuate nucleus to increase secretion of GHRH and subsequently, growth hormone. Suckling-induced release of growth hormone and prolactin are distinct mechanisms. Thus, secretion of growth hormone is important for maintenance of lactation, but under normal circumstances secretion of growth hormone does not limit secretion of milk, because administration of growth hormone has little effect on milk yield in rats.

In sharp contrast to the rat, growth hormone is the primary hormonal regulator of milk secretion in cattle. The first data on this topic were gathered in 1937 when it was observed that administration of aqueous

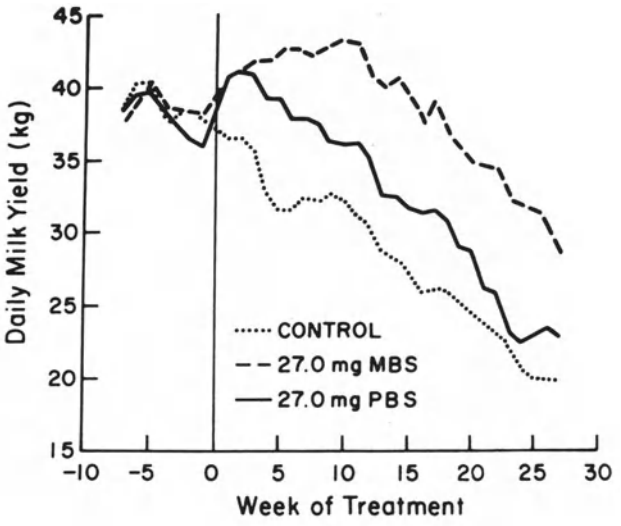

Fig. 10. Growth hormone (somatotropin) stimulation of milk yield in dairy cows. Cows were injected daily with diluent (control, . . .), 27 mg of recombinant methionyl bovine somatotropin (MBS, – – –), or 27 mg of bovine somatotropin extracted from anterior pituitary glands (PBS, _____). From Tucker HA. In: Knobil E, Neill JD, ed. *The Physiology of Reproduction*, 2nd ed., New York: Raven, 1994. Originally modified from Bauman DE, Eppard PJ, DeGeeter MJ, Lanza GM. *J Dairy Sci* 1985; 68: 1352.

extracts of the anterior pituitary gland stimulated milk yield in cows. Subsequent research showed that the active molecule was growth hormone, and injection of pituitary-derived growth hormone increased secretion of milk of 6–35%. Limited supplies, however, precluded extensive use of exogenous growth hormone. It was the advent of *recombinant DNA* technology in the early 1980's that permitted the use of large scale testing of the galactopoietic properties of growth hormone. Recombinantly derived growth hormone was at least equipotent with growth hormone extracted from anterior pituitary glands in terms of stimulating yield of milk (Fig. 10). Concentrations of growth hormone in milk from cows injected with growth hormone are not different from those of controls. Although concentrations of IGF-1 may increase slightly in growth hormone-treated cows, these concentrations are within the concentrations normally found in mother's milk. Moreover, these peptides do not possess biological activity when ingested orally. In addition, composition of the major components of milk from cows injected with recombinantly synthesized growth hormone is identical to that from controls. It is the quantity of milk that is increased; increments average 4–7 kg of milk per cow per day. Thus,

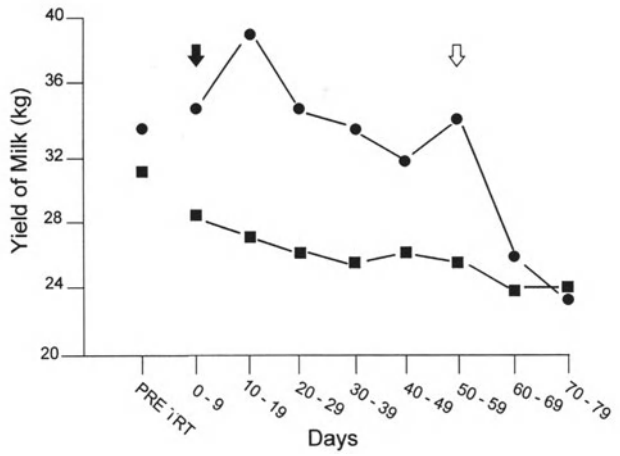

Fig. 11. Growth hormone-releasing hormone (GHRH) stimulation of milk yield in dairy cows. Cows were infused continuously for 60 d with 12 mg/day of recombinant bovine GHRH (•) or received no treatment. Beginning and end of infusion is indicated by the solid and open arrows, respectively. Modified from Dahl GE, Chapin LT, Zinn SA, Moseley WM, Schwartz TR, Tucker HA. *J Dairy Sci* 1990; 73: 2444.

based on safety and efficacy, recombinantly-produced growth hormone is approved by the *Food and Drug Administration* (FDA) for commercial use in lactating dairy cows in the United States.

In cattle, it is believed that growth hormone coordinates the partitioning of nutrients toward the mammary gland during lactation. Much of the action of growth hormone on lactation in ruminants is exerted outside the mammary gland, especially at the level of adipocytes and liver. Somewhat unexpectedly, milking does not cause an acute release growth hormone into the blood of cattle although basal concentrations are elevated during early lactation when milk production is most intense.

As previously discussed, GHRH from the hypothalamus is likely to be involved in the mechanism whereby growth hormone secretion and milk synthesis are maintained. Indeed, sustained administration of GHRH maintains an elevated secretion of growth hormone in cattle, which in turn, increases yield of milk an average of 8 kg per cow per day (Fig. 11). Activation of α_2-adrenergic receptors stimulates release of growth hormone-releasing hormone and increases concentrations of growth hormone in blood. Furthermore, dopamine and neuropeptide Y are colocalized in the growth hormone-releasing hormone neurons. But, whether these mechanisms are activated during lactation remains to be determined.

Activation of D_1 *dopamine receptors* increases secretion of somatostatin, which suppresses secretion of GHRH and growth hormone. However, the role of somatostatin in the regulation of growth hormone secretion during lactation has not been studied.

6.2. Neuroendocrine Regulation of Adrenal Hormones During Lactation

Adrenalectomy reduces milk production, and administration of a combination of gluco- and mineralocorticoids to adrenalectomized animals restores lactation better than either steroid alone. Paradoxically, administration of glucocorticoids to normal lactating females suppresses milk synthesis, but such suppression is associated with administration of pharmacological doses of the steroid. At least some of the suppression is associated with inhibition of the milk-ejection reflex. In rats, low doses of glucocorticoids prevent the late lactation decline in milk yield, but in other species, the results of administration of glucocorticoids on milk synthesis are mixed and controversial.

Suckling and milking stimuli induce release of adrenocorticotropin, which in turn, induces release of glucocorticoids into blood of lactating females of several species. Histaminergic neurons within the hypothalamus appear to mediate suckling-induced release of adrenocorticotropin. In contrast to the gradual decline in magnitude of the milking-induced release of prolactin, the reflex associated with milking-induced release of glucocorticoids persist throughout lactation in cows. Although glucocorticoid concentrations in serum are elevated in rats during intense lactation, suckling-induced release of glucocorticoids decreases as lactation progresses, which may explain why low doses of exogenous glucocorticoids are galactopoietic in this species.

Neuroendocrine and behavioral responses to a variety of stressors are attenuated in lactating animals, and this is conducive to high milk yields because intense stress can disrupt milk secretion and milk ejection. Indeed, intracerebroventricular administration of corticotropin-releasing hormone, which is released by stressors, reduces milk yield. The hyporesponsiveness of the hypothalamo-pituitary-adrenal axis to stressors during lactation is associated with suckling-induced suppression of *noradrenergic* inputs to corticotropin-releasing hormone neurons in the *paraventricular nucleus*. During late pregnancy gene expression of corticotropin-releasing hormone in the paraventricular nucleus becomes suppressed, which may contribute to the reduced responses to stress during the lacta-

tion. Central suppression of corticotropin-releasing hormone may explain the increased incidence of *affective disorders* such as *postpartum depression* in women.

6.3. Neuroendocrine Regulation of Thyroid Hormone Stimulation of Milk Synthesis

Supplementation with *thyroid hormones* stimulates lactation in several species. For example, administration of *thyroxine* to dairy cows increases milk yield up to 27%. Conversely, suppression of secretion of thyroxine reduces milk yield. In short, the thyroid hormones are essential for maximal secretion of milk. Administration of the hypothalamic peptide, TRH stimulates secretion of milk in hypogalactic women, but this response was associated primarily with increased release of prolactin, not thyroxine. In sows and cows, exogenous TRH stimulates milk yield simultaneously causing release of thyroxine, prolactin, and growth hormone. Thus, it has been difficult to assign the milk yield response to a single hormone because TRH causes release of several anterior pituitary hormones in many species.

Whether or not suckling or milking causes release of thyrotropin is unclear. In rats, suckling does not affect concentrations of thyrotropin in the anterior pituitary gland, but some authors report that suckling increases its concentration in blood. On the other hand, suckling has no effect on release of thyrotropin into blood of either sheep or women. Similarly in cows, milking does not affect concentrations of thyrotropin in blood.

6.4. Neuroendocrine Regulation of Estrogen During Lactation

Many species of mammals (with the notable exception of cattle) do not show estrous or menstrual cyclicity during intense lactation. Thus, secretion of estrogens is low during lactation so their effects on milk secretion are minimal under normal circumstances. Indeed, administration of estrogens suppresses milk synthesis. It is the suckling stimulus, not secretion of milk, that leads to *lactational anestrus* in women. Suckling suppresses release of *gonadotropin-releasing hormone* (GRH) from hypothalamic neurons, which in turn reduced secretion of *luteinizing hormone* (LH) thereby inhibiting cyclicity and ovulation. Worldwide, *breastfeeding* prevents more pregnancies than any other contraceptive method.

6.5. Nervous System Regulation of Milk Synthesis

Surgical sectioning of the nerve supply of the mammary gland does not inhibit secretion of milk-provided oxytocin is administered to elicit the milk-ejection reflex and thereby allow removal of milk from the gland. In goats, the gland can be surgically transplanted to a site remote from its normal position and after reestablishment of the blood supply the transplanted gland will secrete large volumes of milk. In short, direct innervation of the mammary gland does not affect secretion of milk; effects of the nervous system on lactation are mediated via the hypothalamus.

7. THE MILK-EJECTION REFLEX: A REQUIREMENT FOR REMOVAL OF MILK

In order to remove milk from the mammary gland of most species resistance of the major ducts or teat sphincter (present in ruminants) must be overcome, and the myoepithelial cells must be induced to contract and thereby force milk from the alveoli through the ducts to the exterior of the mammary gland. This process is termed milk ejection, and it requires release of oxytocin into the general circulation from neurosecretory neurons that terminate in the posterior pituitary gland. Indeed, mutant mice that do not possess a functional oxytocin gene have no oxytocin present in the posterior pituitary gland and show no milk-ejection reflex. Milk ejection occurs at discrete intervals usually at the time of milking or suckling, whereas milk secretion is a continuous process. Thus, neuroendocrine control of milk ejection and milk secretion are independent of each other. The milk-ejection reflex represents a classical neuroendocrine regulatory mechanism and is shown in Fig. 12.

7.1. Nerves Comprise the Afferent Pathway from the Mammary Gland to the Posterior Pituitary Gland

The milk-ejection reflex occurs in response to activation of densely packed nerve plexuses in the skin of the nipple or teat, which are sensitive to touch or temperature. In response to suckling or milking, nerve impulses are transmitted from the skin of the teat through the dorsal roots of the appropriate spinal nerves to the dorsal horns of the spinal cord (*see* Fig. 13). Within the spinal cord the signal from several

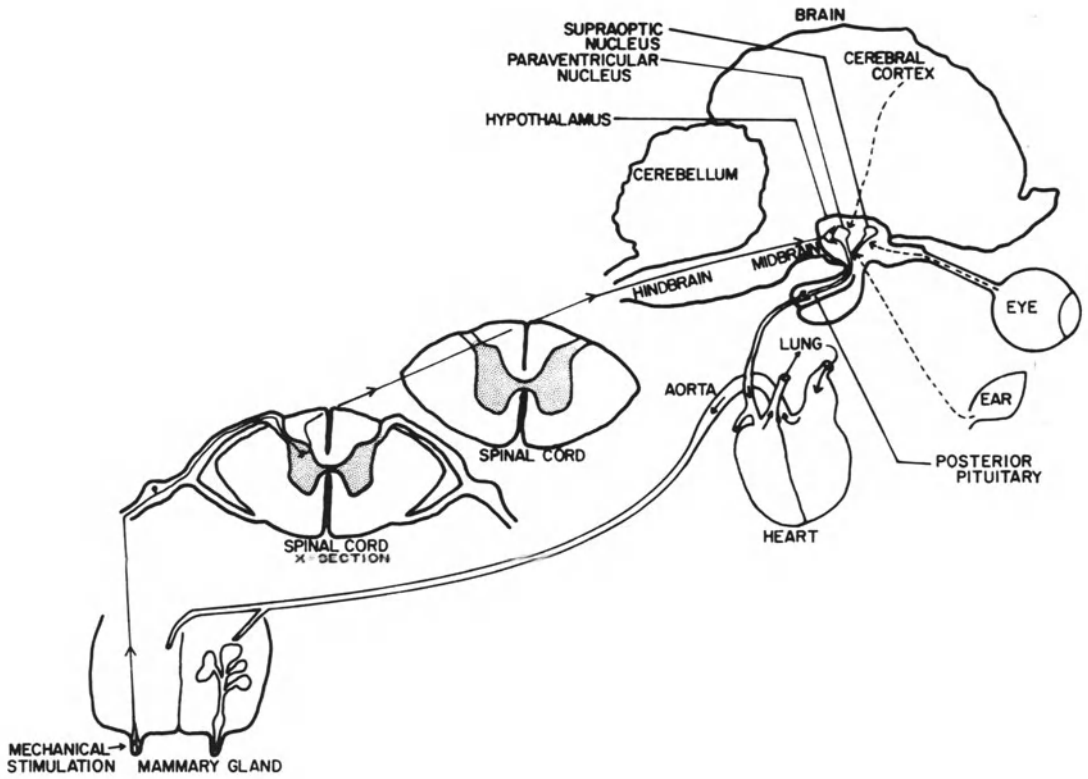

Fig. 12. The milk-ejection reflex. Mechanical stimulation of the teats initiates a neural reflex that travels from the teats to the spinal cord to the paraventricular and supraoptic nuclei of the hypothalamus. Because of space limitations the afferent neurons in the figure are depicted as innervating only the paraventricular nucleus. From the paraventricular and supraoptic nuclei the neural signal is transmitted to the posterior pituitary gland where oxytocin is released into blood. Oxytocin binds to and causes contraction of the myoepithelial cells thereby evoking ejection of milk from the mammary gland. Signals from the cerebral cortex, eyes, and ears may also elicit release of oxytocin. From Tucker HA. In: *Proc Int Symp on Milking Machine*, 21. 17th Annual Meeting National Mastitis Council, Inc. Washington: National Mastitis Council. 1978.

adjacent teats are summed and then transmitted uncrossed within the *lateral funiculi* to the *lateral cervical nucleus* where the nerves crossover and ascend in the *ventro-lateral medulla* to the *medial lemniscus*. Nerves carrying the signal from the suckling stimulus then traverse the pons to enter the *lateral tegmentum* via the *spinal lemniscus*. Above the midbrain, the signal is transmitted through the *peripeduncular area* and *posterior lateral hypothalamus* before reaching the paraventricular and *supraoptic* nuclei in the hypothalamus. A second pathway involves noradrenergic neurons that ascend from the *dorsal vagal complex* directly to the paraventricular and supraoptic nuclei. During suckling activation of the neuronal pathway from the lateral cervical nucleus and activation of the noradrenergic pathway cause release of oxytocin from the paraventricular and supraoptic nuclei.

It is within the cell bodies of the paraventricular and supraoptic nuclei that oxytocin is synthesized together with its associated peptide, *neurophysin*, to which it is weakly bound within the *hypothalamo–hypophysial nerve tract*. Neurophysin is involved in the transportation of oxytocin to the posterior pituitary gland. Receipt of nerve impulses within the nuclei hastens the transportation of oxytocin/neurophysin granules down the neurons through the pituitary stalk for subsequent storage in the neurosecretory terminals of the posterior pituitary gland. A characteristic of oxytocin neurons is intermittent *bursting activity* prior to release of oxytocin in response to suckling. Each burst of activity is a coordinated event among all oxytocin neurons and is associated with a subsequent coordinated pulse release of oxytocin into blood. Bursting is a phenomenon that allows oxytocin to reach the mammary gland in high concentration thereby ensuring that contractions of the myoepithelial cells occur synchronously in all parts of the mam-

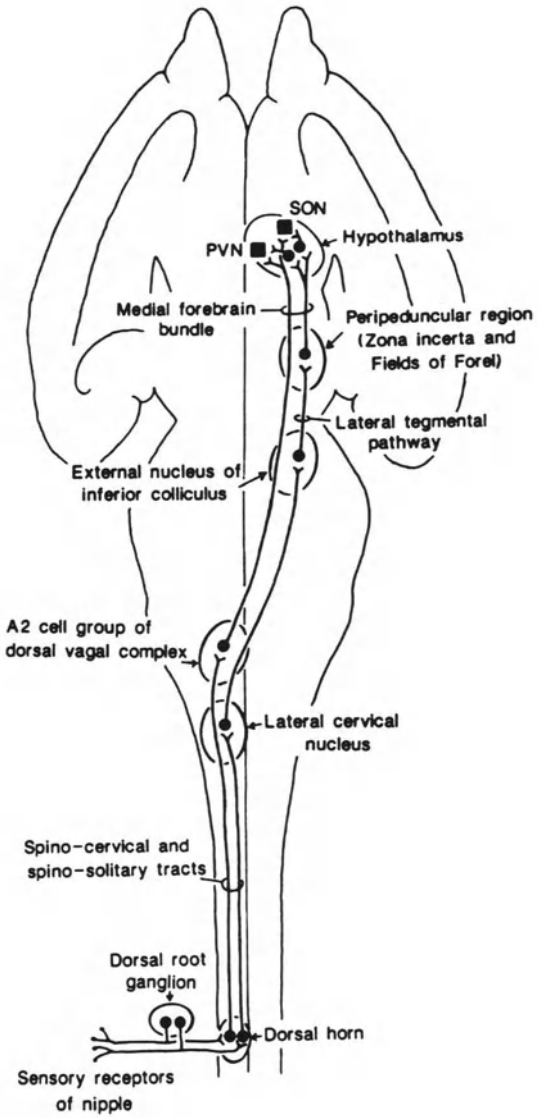

Fig. 13. Proposed route of suckling-induced neural signals from the teat to the paraventricular (PVN) and supraoptic (SON) nuclei. One route ascends ipsilaterally to the lateral cervical nucleus, synapsing in the external nucleus of the inferior colliculus, in the peripeduncular region, and in the hypothalamus. Another route involves the A2 noradrenergic neurons of the dorsal vagal complex, which project directly to the paraventricular and supraoptic nuclei complex. From Wakerley JB, Clarke G, Summerlee AJS. In: Knobil E, Neill JD, ed. *The Physiology of Reproduction*, 2nd ed., New York: Raven, 1994: 1131.

mary glands. Bursting activity of the oxytocin neurons is controlled in the central nervous system (CNS), not at the level of the teat. A central gating mechanism affects the afferent nerve signals, which permits the oxytocin neurons to fire only at discrete intervals.

7.2. Oxytocin Release from the Posterior Pituitary Gland into Blood Causes Milk Ejection

Acetylcholine and cholinomimetics induce release of oxytocin, and presence of cholinergic receptors in the supraoptic nucleus suggests that oxytocin neurons may receive cholinergic input and participate in the mediation of suckling-induced release of oxytocin. The neurotransmitter, *norepinephrine*, acting via α_1-*adrenergic receptors* in the supraoptic and paraventricular nuclei, also induces release of oxytocin in response to suckling. Other neurotransmitters implicated in stimulating release of oxytocin include *glutamate* and dopamine. In addition, oxytocin itself increases bursting activity in the paraventricular and supraoptic nuclei. Oxytocin receptors are present in these nuclei, and this local action of oxytocin on the nuclei is postulated to facilitate release of oxytocin. There is substantial evidence that neurons of the hypothalamo–hypophysial tract transmit nerve impulses in response to suckling, and these impulses are involved in release of oxytocin into blood. Release of oxytocin from terminals of the posterior pituitary gland involves exocytosis of the oxytocin granules in response to a rise in calcium induced by the neural signal initiated with the suckling or milking stimulus. In addition to suckling or milking stimuli, a variety of exteroceptive stimuli such as the cries or sight of a newborn offspring or the sound of a milking machine (in cattle) may induce release of oxytocin. There is also evidence that exteroceptive stimuli may lead to a reduction of the sympathetic tone in the mammary gland, which promotes leakage of milk from the mammary gland before application of the suckling or milking stimulus.

The posterior pituitary gland normally contains a large reserve of oxytocin, but during prolonged lactation these stores become reduced. Indeed, in cattle the quantity of oxytocin released into blood decreases as lactation advances. This may contribute to gradual curtailment of milk synthesis as lactation progresses because frequent removal of milk from the mammary gland is essential for maintenance of milk secretion. If milk is not removed from the ducts and alveoli it is resorbed, and the mammary gland begins to involute, which leads to a marked decline in milk synthesis. This process is especially noticeable after weaning.

Once released from nerve terminals, the secretory granules containing oxytocin/neurophysin fuse with adjacent basement membranes of the endothelial cells

of capillaries of the posterior pituitary gland. In cattle, evidence suggests that neurophysin is released coincidentally with oxytocin into the blood, but the two molecules quickly dissociate.

Oxytocin is then delivered to the capillary beds of the mammary gland where it diffuses into the intercellular space and binds with high affinity to the myoepithelial cells, which are located in close apposition to the mammary capillaries. Upon binding to receptors on the myoepithelial cell, oxytocin causes contraction of the myoepithelial cells, which squeezes the alveoli and shortens (thus widens) the mammary ducts (*see* Fig. 1), thereby forcing the rapid ejection of milk out of the mammary gland for the nourishment of the offspring or in the case of domestic livestock harvesting of milk for human food.

Oxytocin has a half-life in serum of only 1.5–2 min. Thus, to obtain the maximal amount of milk, milking must be closely coordinated with the actual release of oxytocin. During machine milking of cattle, concentrations of oxytocin in blood remain elevated throughout milking, a procedure that normally lasts 4–8 min. Milking also reduces the tone within the mammary gland because of a local inhibition of *epinephrine* secretion from sympathetic nerves in the mammary gland. Reduced activity in the sympathetic neurons is associated with an increased rate of removal of milk. In rats, there is a 10–15 min delay in milk ejection in response to suckling. This delay is associated with release of epinephrine. But as suckling continues, release of epinephrine abates, which facilitates oxytocin action on the myoepithelial cell. Although suckling may be continuous, release of oxytocin from the posterior pituitary gland is intermittent. In pigs, which nurse approximately once every hour, each milk-ejection episode lasts only approximately 20 sec. Thus, for survival it is important that the piglets are actively suckling a teat at the time of milk ejection. Frequency of nursing is wildly variable among species. For example, shortly after birth, joeys attach to the teat of the mother kangaroo and nurse continuously. Dolphins may nurse as frequently as once every half hour, cattle every 4–6 h, rabbits once every 24 h, and northern fur seals once a week.

7.3. Factors that Inhibit Milk Ejection

Various stimuli inhibit milk ejection. The most common cause of inhibition is associated with emotional disturbances, fear, and various nonspecific stresses. Such stimuli activate neural pathways in the CNS that are inhibitory to the release of oxytocin despite application of adequate suckling or milking stimuli to the nipple or teat. Descending pathways from higher centers in the brain to the paraventricular and supraoptic nuclei have been associated with inhibition of oxytocin release and milk ejection. Factors involved in inhibition of milk ejection are described below.

Endogenous opioid peptides normally suppress oxytocin release from axon terminals and dendrites of magnocellular oxytocin neurons, but how these peptides interact with neurotransmitters that cause release of oxytocin is not known. Nonetheless, enkephalin and endorphin nerve fibers innervate the paraventricular and supraoptic nuclei, and these opioids are colocalized in oxytocin neurons. Activation of μ-opioid receptors inhibit firing of oxytocin neurons in the hypothalamus, and κ-receptors mediate opioid blockade of release of oxytocin in the posterior pituitary gland. Blockade of opioids with *naloxone* increases suckling-induced release of oxytocin. Collectively, it appears that opioids mediate stress-induced suppression of oxytocin release. Although binding of norepinephrine to α_1-adrenergic receptors is stimulatory to release of oxytocin, binding of norepinephrine to β-*adrenergic* receptors suppresses oxytocin release. Nitric oxide is another factor that suppresses milk ejection. Nitric oxide disrupts synchronized bursting activity of oxytocin neurons. Finally, *gamma aminobutyric acid* (GABA) neurons innervate oxytocin neurons, and activation of GABA neurons inhibits milk ejection. Surprisingly, inhibition of GABA also inhibits milk ejection.

Administration of oxytocin overcomes central inhibition of oxytocin release. In cattle, central inhibition of milk ejection is common during the first few milkings of primiparous animals. In such cases, exogenous oxytocin is given therapeutically at the time of milking to ensure maximal removal of milk. This is important because leaving large quantities of milk in the mammary glands leads to involution of the mammary gland. Within a few days, as these animals become accustomed to the milking procedure, the use of exogenous oxytocin usually can be eliminated.

Under extremely stressful conditions epinephrine may be released from nerves innervating the arterioles within the mammary gland to such an extent that concentrations of oxytocin at the myoepithelial cells are reduced. In addition, sufficiently high concentrations of epinephrine will bind to the oxytocin receptor directly on the myoepithelial cell and block its ability to contract. In these instances, injection of oxytocin

will not overcome this peripheral inhibition of the milk-ejection reflex.

8. SUMMARY

Secretion of milk from epithelial cells of mammary glands is required for survival of mammalian neonates. Most development of the mammary gland and initiation and maintenance of lactation occurs as a result of changes in hormone secretion during pregnancy, parturition, and suckling (or mechanical milking), respectively. Specifically, increased secretion of estrogen and progesterone coupled with secretion of prolactin and growth hormone induce mammary epithelial cell growth. In the periparturient period, there is a sharp decline in secretion of progesterone, followed by decreased activity of TIDA neurons, thereby decreasing the secretion of the prolactin inhibiting factor, dopamine. In addition, there is increased release of a variety of prolactin releasing factors. The combination of reduced dopamine and increased secretion of prolactin releasing factors induces a major surge in secretion of prolactin. Prolactin, in turn, binds to receptors on the mammary epithelial cells, which initiates lactation. During lactation the suckling (or milking) stimulus is essential to remove milk from the mammary via a neuroendocrine reflex called milk-ejection, which involves neural stimuli inducing acute release of oxytocin from the posterior pituitary gland and subsequent contraction of myoepithelial cells that surround the mammary alveoli. This contraction forces milk from the lumen of the mammary alveoli through the mammary ducts to the exterior of the nipple or teat. The suckling stimulus also activates receptors in the nipple (or teat), which generates nerve impulses that are integrated within the hypothalamus to cause release of prolactin, adrenocorticotropin and in some species, growth hormone. With respect to prolactin, suckling activates the opioidergic system, which suppresses activity of TIDA neurons thereby increasing release of prolactin. Suckling also activates a variety of prolactin releasing factors. Prolactin is essential to maintain lactation in nonruminants, but not ruminants. In species where suckling induces release of growth hormone, opioid and α_2-receptor mechanisms within the arcuate nucleus are activated to increase secretion of GHRH and subsequently growth hormone. In ruminants, the primary galactopoietic hormone is growth hormone. Histaminergic neurons in the hypothalamus probably mediate suckling-induced release of adrenocorticotropin, which in turn increases secretion of glucocorticoids. Glucocorticoids are also required for maintenance of lactation. In addition, during lactation the hypothalamus-pituitary-adrenal axis is hyporesponsive to stress. This hyporesponsiveness is associated with suckling-induced suppression of noradrenergic inputs to corticotropin-releasing hormone neurons in the paraventricular nucleus.

SELECTED READINGS

Flint DJ. Hormone regulation of uptake and metabolism of milk precursors in normal lactating mammary gland. In: Tucker HA, Pettigrew JE, Petitclerc D, eds. Second International Workshop on the Biology of Lactation in Farm Animals, J Anim Sci, Suppl. 2, 1995; 73:61.

Imagawa W, Yang J, Guzman R, Nandi S. Control of mammary gland development. In: Knobil E, Neill JD eds. The Physiology of Reproduction, 2nd ed., vol 2. New York: Raven, 1994:1033.

Moore KE. Interactions between prolactin and dopaminergic neurons. Biol Reprod 1987; 36:47.

Tucker HA. Lactation and its hormonal control. In: Knobil E, Neill JD, eds. The Physiology of Reproduction, 2nd ed., vol 1. New York: Raven, 1994:1833.

Wakerley JB, Clarke G, Summerlee AJS. Milk ejection and its control. In Knobil E, Neill JD, eds. The Physiology of Reproduction, 2nd ed., vol 2. New York: Raven, 1994:1131.

11 Neuroendocrine Regulation of Growth Hormone Secretion

William B. Wehrenberg, PHD and Andrea Giustina, MD

CONTENTS

1. INTRODUCTION

The neuroendocrine regulation of growth hormone (GH) secretion is unique and yet simple to understand. It is unique in that GH secretion is the only pituitary hormone regulated by both a hypothalamic releasing hormone, growth hormone-releasing hormone (GHRH), and a hypothalamic release-inhibiting hormone, somatostatin. Yet in spite of this dual regulatory system, it is easy to understand because GHRH and somatostatin interact in a manner analogous to the accelerator and brake of an automobile, a system with which we are all very familiar (Fig. 1). Thus, when there is a physiological demand for GH, we observe the physiological equivalent of the hypothalamus stepping on the gas (releasing GHRH) and simultaneously releasing the brake (inhibiting somatostatin release). Along these same lines, when GH secretion needs to slow down, we observe the physiological equivalent of the hypothalamus putting on the brake (releasing somatostatin) and taking its foot off the gas (decreasing GHRH release). However, there are questions that should immediately come to mind. For example, does GH secretion ever "coast?" That is to say, if neither GHRH and somatostatin are secreted, what happens to GH secretion? Can the accelerator override the effect of the brake and vice versa? What other factors affect this accelerator/braking system?

To begin, one must recognize that no matter how sophisticated a model is presented, the physiological regulation of pituitary GH secretion follows classic neuroendocrine feedback mechanisms. Hypothalamic neuropeptides (GHRH and somatostatin) are released in response to central nervous system signals (*see* Chapter 7) and then carried to the pituitary gland via the hypothalamic-pituitary portal system (*see* Chapter 2). The somatotrophs (GH secreting cells in the pituitary) in turn respond by increasing or decreasing the secretion of GH. Although GH is recognized as having some direct cellular effects on the growth and metabolism of cells, the predominant effect is on the synthesis and secretion of the insulin-like growth factors (IGFs). The most important one is IGF-I, which is synthesized in the liver. The IGFs, in turn, modulate the hypothalamic and pituitary components of this growth axis in a classic negative feedback manner. The secretion of GH is dynamic, and therefore feedback regulation is also dynamic. In addition, many

From: *Neuroendocrinology in Physiology and Medicine,* (P. M. Conn and M. E. Freeman, eds.),

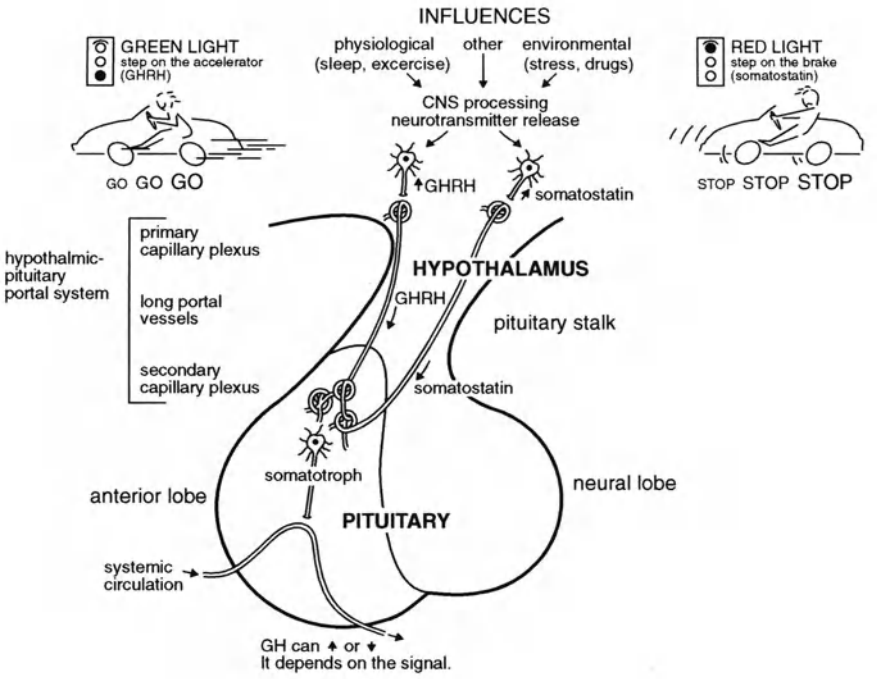

Fig. 1. A stylistic representation of the neuroendocrine regulation of GH secretion. Numerous influences are integrated by the hypothalamus. If the overall demand is for increased GH release, there is an increase in GHRH and a decrease in somatostatin. If decreased GH release is needed, somatostatin is released and GHRH is inhibited.

other parameters also affect overall GH homeostasis. The following chapter attempts to detail our present understanding of the interactions and feedback regulation of GH secretion by the hormones constituting the GH axis. In addition, we will detail the long-loop feedback effects of several other hormones and factors that are involved in the neuroendocrine regulation of GH secretion.

Endocrine feedback loops have long been recognized. Unfortunately, several terms having the same definition have found their way into the neuroendocrine literature, and therefore it is necessary to define the terms that will be used in this chapter. The first one is *ultrashort-loop feedback.* This term is used to define the system in which the secretory products of a cell directly regulate the function of the secretory cell itself; for example, pituitary somatotrophs (GH secreting cells) that respond directly to GH (Fig. 2A). Autoregulation and autocrine regulation have been used as synonyms for this term. The second term is *short-loop feedback.* This system reflects feedback effects, which are localized in their sites of action; for example, the regulation of hypothalamic function by pituitary hormones through local pathways rather than the general circulatory system. An example is the short-loop feedback effects of GH on hypothalamic GHRH and somatostatin (Fig. 2B). This system has also been referred to as paracrine feedback. The final term is *long-loop feedback.* This term has been defined in earlier chapters and involves the IGFs and numerous other hormones and molecules that feed back to interact with the GH axis at the level of the hypothalamus and pituitary through the general circulatory system (Fig. 2C).

2. PRIMARY NEUROENDOCRINE REGULATION OF GH SECRETION

2.1. Somatostatin

Many interesting observations have been reported in the field of neuroendocrinology as it developed. One such example is the discovery of somatostatin. The original neuroendocrine hypothesis focused on hypothalamic releasing factors, not release-inhibiting factors. Indeed, efforts designed to isolate and identify a growth hormone-releasing factor led to the initial reports that specific fractions from hypothalamic extracts had the capacity to inhibit GH secretion. Such reports were received with great scepticism. Yet in 1973, the purification and amino acid characterization of somatostatin was reported (*see* Fig. 3).

Somatostatin is a cyclic peptide consisting of 14 amino acids. It is now recognized as a rather ubiquitous molecule, having numerous significant neuroendocrine and gastrointestinal actions. A second, 28 amino-acid form of somatostatin has also been identi-

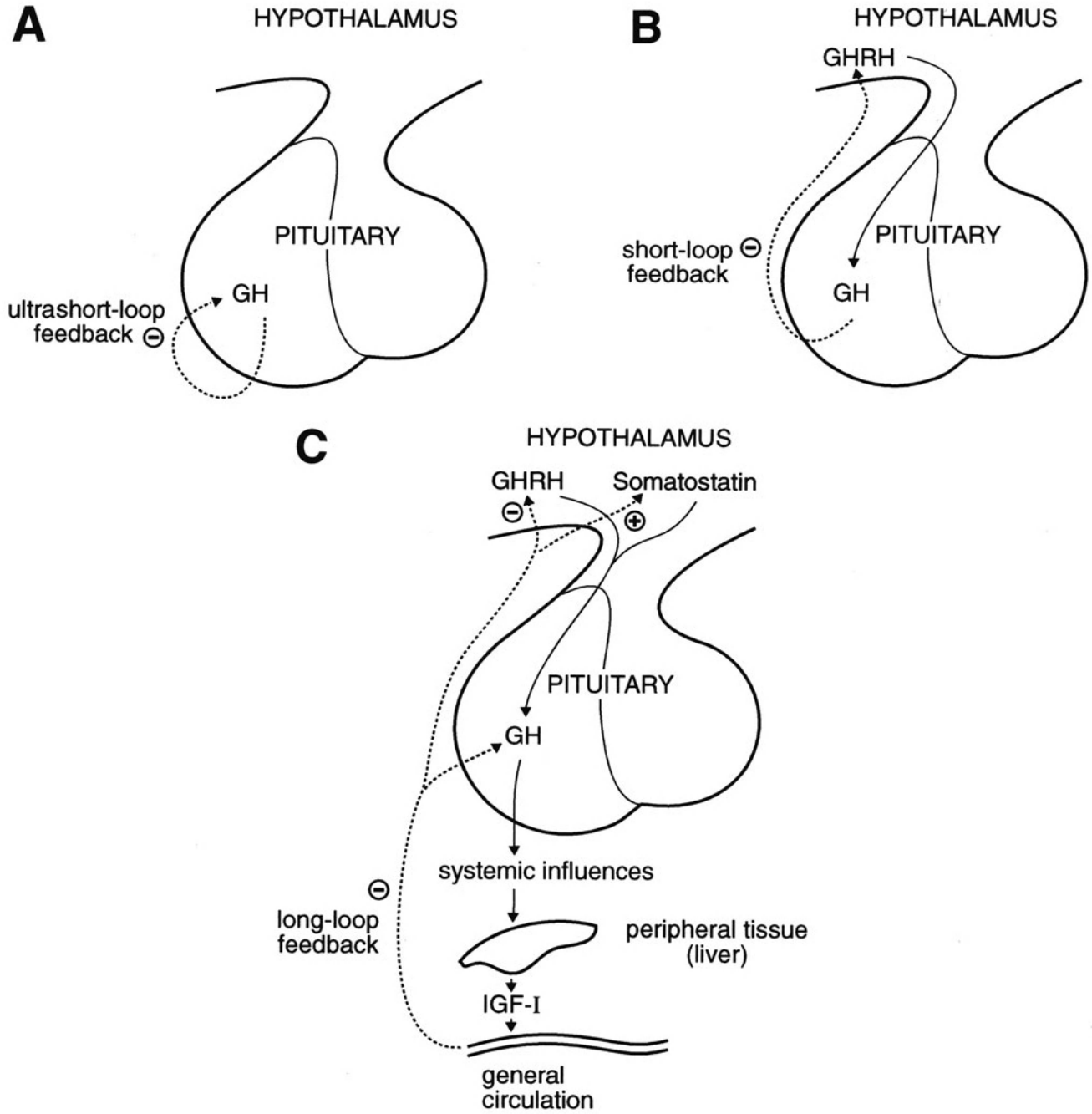

Fig. 2. **(A)** Schematic illustration of an ultrashort-loop feedback system. In this example, GH has a direct negative feedback effect on itself. **(B)** Schematic illustration of a short-loop feedback system. In this example, GH has a localized negative feedback effect on GHRH. **(C)** Schematic illustration of a long-loop feedback system. In this example, IGF-I has a systemic feedback effect on GH, GHRH, and somatostatin.

fied. This molecule results from modified cleavage of the somatostatin prohormone. Somatostatin 14 is the primary regulator of the GH axis whereas the longer version is involved in regulating the gastrointestinal system. One should be aware that somatostatin also inhibits thyrotropin secretion and has numerous other inhibitory actions. Within the hypothalamus, somatostatin is synthesized in the anterior hypothalamic periventricular system.

To understand the neuroendocrine regulation of GH secretion by somatostatin, it is first necessary to recognize that the secretory pattern of GH is pulsatile

SOMATOSTATIN

Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys

SOMATOSTATIN-28

Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-Ala-
Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys

Fig. 3. Molecular structure of somatostatin and somatostatin-28.

(Fig. 4). GH pulses are regulated by the pulsatile release of both somatostatin and GHRH in a reciprocal fashion. As stated earlier, somatostatin acts as a brake or inhibitor of GH secretion. This neuropeptide exerts its action by binding to one of five receptor subtypes that have been identified. As an aside, it appears that the numerous receptor subtypes play a role in tissue specificity and sensitivity to somatostatin. Somatostatin receptors have transmembrane sequences and involve the G proteins to mediate their effects. After somatostatin binds to its receptor the intracellular actions of somatostatin include decreased adenylate cyclase and lowered cellular calcium concentrations. During periods of elevated somatostatin secretion, its inhibitory effects predominate over the stimulatory effects of GHRH (*see* Fig. 5A and 5B). That is to say that in the presence of high somatostatin influences, GHRH can not stimulate GH secretion. The facts that somatostatin can completely interrupt pulsatile GH secretion in vivo and somatostatin analogues are used

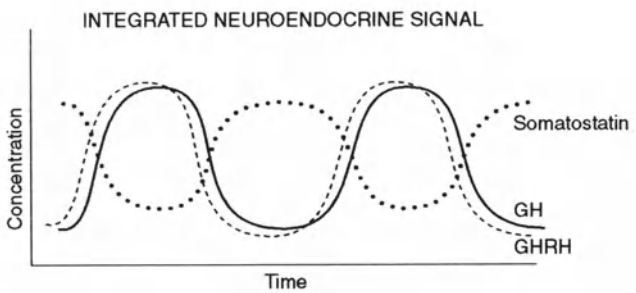

Fig. 4. Pulsatile secretion of GH, GHRH, and somatostatin. The secretion of GH reflects the integrated influences of the two regulatory neuropeptides. There are gender-related differences and the pattern of GH pulses is more frequent and of lower amplitude in females.

to treat acromegaly also argue for the predominant role of somatostatin.

2.2. GHRH

GHRH was finally characterized and sequenced in 1981. Its isolation from hypothalamic extracts proved to be very elusive. Indeed, GHRH was first isolated from a human pancreatic tumor found in a patient with acromegaly. Further research confirmed that tumor and hypothalamic GHRH are identical. Three molecular forms have been characterized: GHRH 1-44-NH_2, GHRH 1-40-OH, and GHRH 1-37-OH (*see* Fig. 6). The 1-44-NH_2 molecule has been reported to be most potent and this has led to the suggestion that the shorter two peptides are catabolic products. GHRH is synthesized primarily in the arcuate nucleus and stored in the medial aspects of the hypothalamus (*see* Fig. 7). GHRH immediately stimulates pituitary GH secretion, but its action is very short-lived, reflective of the short half-life of GHRH. The pituitary shows a remarkable capacity to respond to repeated injections of the releasing peptide. Exogenous administration of GHRH suggests that the pituitary has the capacity to respond as frequently as every hour (*see* Fig. 8). This capacity of the pituitary is consistent with the observed increases in pulsatile GH secretion observed in humans during adolescence.

2.3. Primary Interactions Between GHRH and Somatostatin

As mentioned in the Introduction, GHRH and somatostatin regulate GH secretion though a very simple, yet elegant interaction. Acting like an accelerator and a brake, these two neuropeptides are secreted in a reciprocal fashion into the hypothalamic-pituitary portal vessels. The timing of GHRH and somatostatin release is critical. For example, when somatostatin concentrations are elevated, exogenous GHRH is not able to induce GH release from the pituitary (Fig. 4). The critical role of somatostatin in inhibiting the GH response to GHRH has been demonstrated by neutralizing the effects of somatostatin by passively immunizing animals with antibodies against somatostatin. To summarize this relationship, pulses of GH reflect a positive pituitary response to GHRH under conditions of low somatostatin secretion (Fig. 4). During periods of GH nadirs, GHRH secretion is low, whereas somatostatin secretion is elevated. All of the subsequent discussion relating to factors modulating the neuroendocrine feedback regulation of GH are subservient to this relationship.

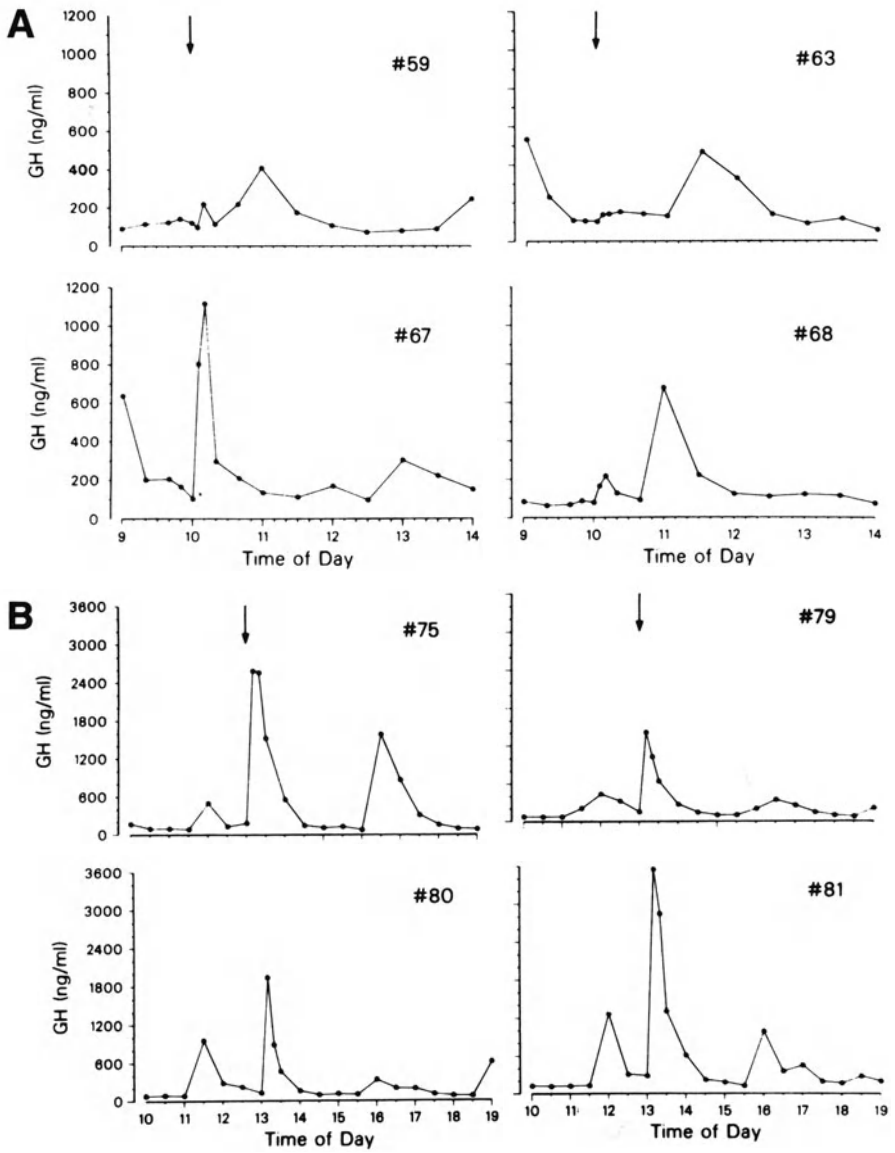

Fig. 5. (A) The effect of a pharmacological dose of GHRH on GH secretion in four individual, conscious, freely moving male rats. Injections (indicated by arrows) were made at a time known to be between spontaneous GH pulses. Note the absence and partial response in most animals. **(B)** The effect of a ten-fold lower dose of GHRH on GH secretion in four individual, conscious, freely moving male rats pretreated with antibodies against somatostatin. Injections (indicated by arrows) were made at a time known to be between spontaneous GH pulses. (From Wehrenberg WB, Ling N, Bohlen P, et al., Physiological roles of somatocrinin and somatostatin in the regulation of growth hormone secretion. *Biochem Biophy Res Commun* 1982; 109:562–567.

Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu- Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-Gln-Gln-Gly-Glu-Ser (plus)

-Asn-Gln-Glu (GHRH 1-37)

-Arg-Gly-Ala (GHRH 1-40)

-Arg-Ala-Arg-Leu-NH_2 (GHRH 1-44NH_2)

Fig. 6. Molecular structure of human GHRH. Three molecular forms have been reported for the human.

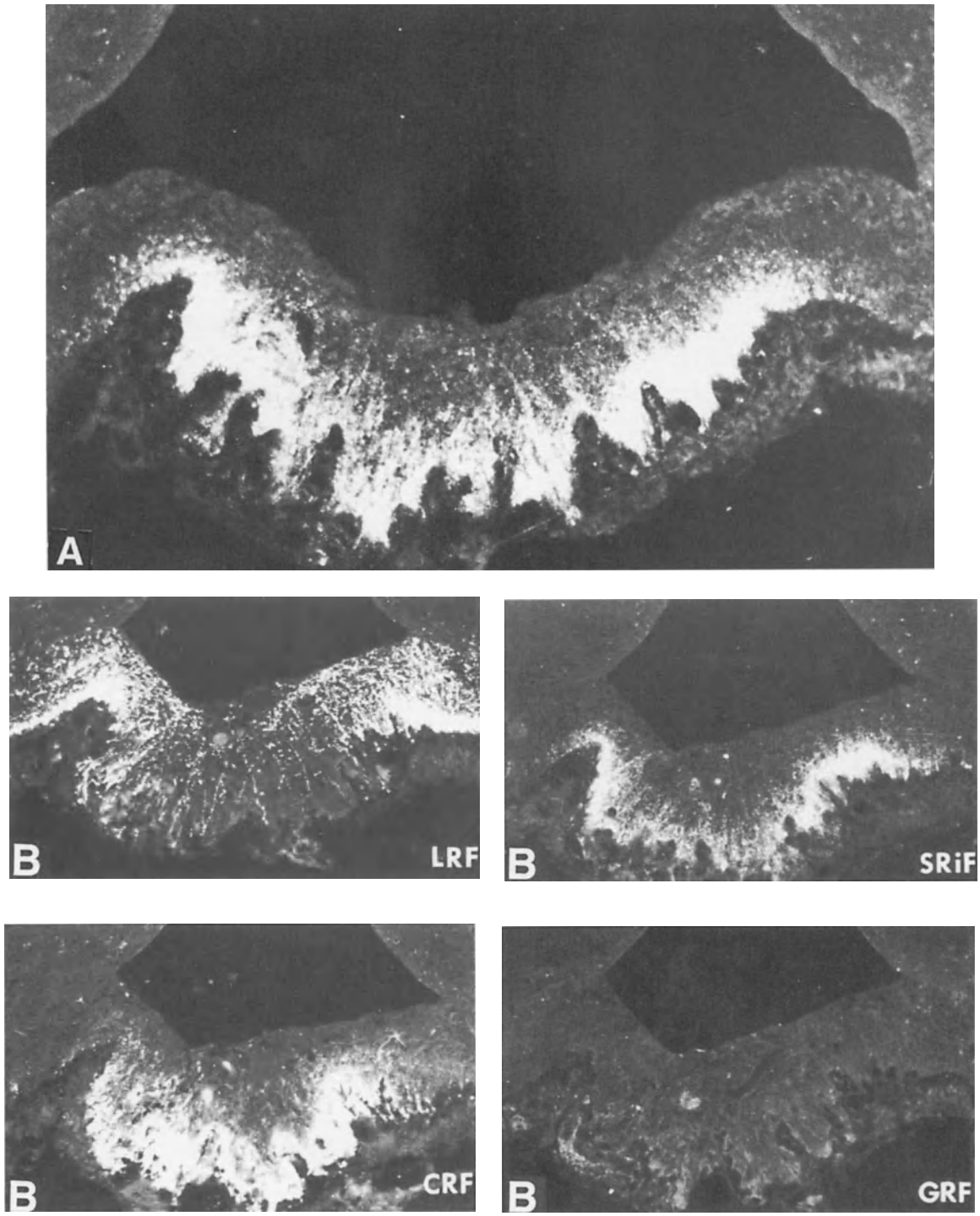

Fig. 7. (A) General view of a frontal section of the median eminence of an adult rat showing GHRH-immunoreactive fibers in contact with the hypotalamic-pituitary portal vessels. **(B)** Immunofluorescent staining of the median eminence of adult rats treated with monosodium glutamate during the first 10 days of life, a procedure known to destroy the cells producing GHRH in the arcuate nucleus. Immunoreactive fibers were observed in sections stained with antiluteinizing hormone releasing hormone (LRF) antiserum, antisomatostatin (SRiF) antiserum, and anticorticotropin releasing hormone (CRF) antiserum, but were conspicuously absent from sections stained with antigrowth hormone releasing hormone (GRF) antiserum. Note the distribution of GHRH terminals within the median eminence. They are medial and inferior. The pattern and location of distribution is different for the other neuropeptides. (From Bloch B, Ling N, Benoit R, et al., Specific depletion of immunoreactive growth hormone-releasing factor by monosodium glutamate in rat median eminence. *Nature* 1984; 307:272–273.)

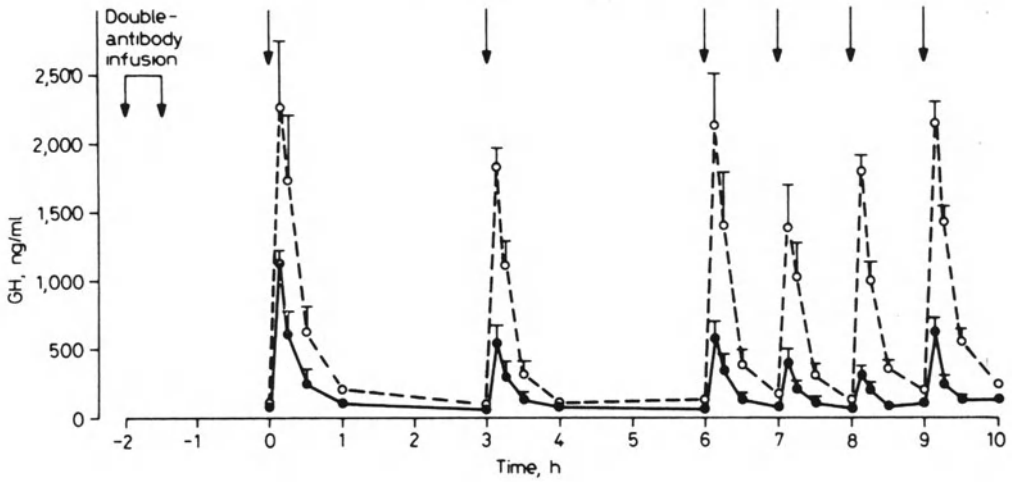

Fig. 8. The capacity of the pituitary in conscious freely moving male rats to secrete GH in response to repeated intravenous injections of a moderate (●) and maximal (○) dose of GHRH. Two hours before the first injection, rats were treated with antiserum against somatostatin and GHRH. Arrows indicate the injection of GHRH. (From Wehrenberg WB, Brazeau P, Luben R, et al., A noninvasive functional lesion of the hypothalamo-pituitary axis for the study of growth-hormone releasing factor. *Neuroendocrinology* 1983; 36:489–491.)

3. FEEDBACK CONTROL OF GROWTH HORMONE SECRETION

3.1. Ultrashort-Loop Feedback (Autocrine Regulation) (Fig. 9)

3.1.1. Ultrashort-loop Feedback Regulation Of GHRH by GHRH and of Somatostatin by Somatostatin

There is limited information on the autoregulation of GHRH by GHRH and of somatostatin by somatostatin because of the difficulty of establishing in vitro hypothalamic cell-culture preparations. However, indirect evidence suggests ultrashort-loop feedback may occur, particularly for somatostatin. This is primarily based on the observation that at low doses somatostatin analogs can suppress the release of somatostatin from cultured hypothalamic cells. Presently, the overall physiological significance of such feedback is not established.

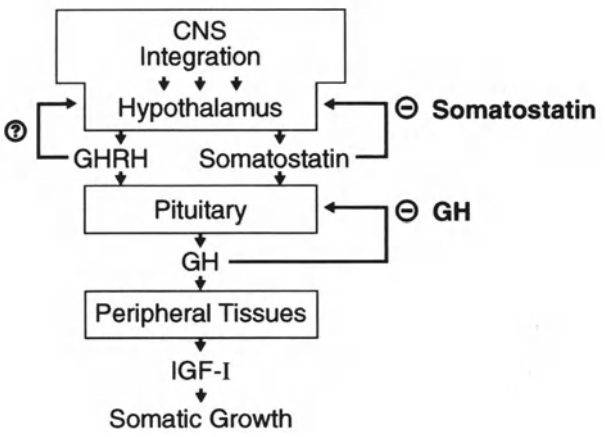

Fig. 9. Neuroendocrine regulation of GH secretion: Ultrashort-Loop Feedback.

3.1.2. Ultrashort-Loop Feedback Regulation of GH by GH

A prerequisite for an ultrashort-loop feedback mechanism is that the secretory cell must itself possess receptors for the product which it secretes. This has been demonstrated for GH in that GH-receptor mRNA, GH-receptor immunostaining, and GH-receptor binding have all been identified in the pituitary gland. Histological and electron microscopical studies of pituitary tissue have also demonstrated the functionality and distribution of the GH receptor in the pituitary. However, in vitro and in situ studies designed to demonstrate GH as an ultrashort-loop feedback regulator of GH secretion are difficult to perform because results are always confounded by the fact that IGF-I serves as a potential autocrine and paracrine regulator in the pituitary. This is further exacerbated in in vivo studies, in which other extrapituitary signals can also feed back on the somatotrophs. Regardless of these weaknesses, there are studies that characterize the autocrine role of GH in the pituitary. For example, exogenous GH decreases basal GH secretion in vitro. There are a large number of in vivo studies that suggest that GH may be able to regulate its own transcription, translation, and

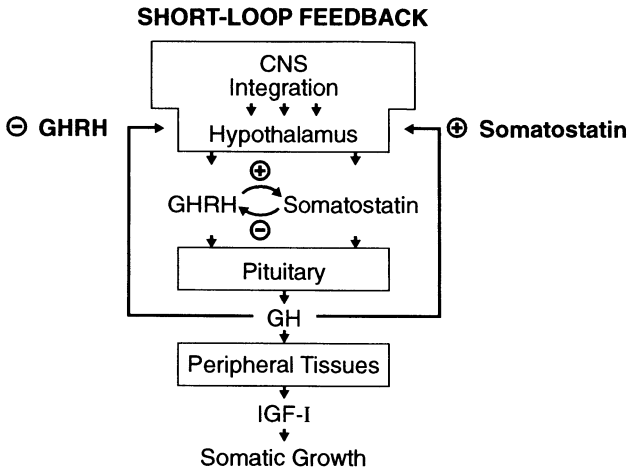

Fig. 10. Neuroendocrine regulation of GH secretion: Short-Loop Feedback.

secretion. For example, somatotrophs from transgenic mice expressing ovine, bovine, or human GH demonstrate a reduction in pituitary GH-mRNA content and the pituitary undergoes atrophy and hypoplasia. In a human model, GH replacement therapy decreases pulsatile GH secretion and the acute GH response to various GH secretagogues. However, as noted earlier in these human studies, the putative autoregulation may actually reflect the negative actions of endogenous IGF-I.

3.2. Short-Loop Feedback (Fig. 10)

3.2.1. Intrahypothalamic Short-Loop Feedback Pathways

The fundamental role of GHRH and somatostatin is to control the secretion of GH from the pituitary gland; yet other control pathways exist. One is the reciprocal modulation of the synthesis and secretion of each neuropeptide by each other. This is directly regulated within the hypothalamus and represents an important link in the short-loop regulation of GH secretion.

3.2.1.1. Short-loop feedback regulation of somatostatin by GHRH. The regulation of somatostatin by GHRH is based upon evidence which suggests that GHRH neurons synapse on somatostatin neurons within the hypothalamus. The preponderance of these connections have been reported to be in the periventricular and preoptic areas of the hypothalamus. Furthermore, hypothalamic somatostatin content, release, and hypothalamic-pituitary portal blood levels of somatostatin are all increased following GHRH exposure. Microinjections of GHRH into the cerebral ventricles of the brain result in a decrease in plasma GH concentrations, a response consistent with increased somatostatin secretion. However, it has been argued that such results suggest an ultrashort-loop feedback system of GHRH on itself. If GHRH increases somatostatin secretion within the hypothalamus, then it is logical to predict that a decrease in hypothalamic GHRH would result in a decrease in somatostatin tone. Indeed, this is the case. For example, lesioning the arcuate nucleus, a procedure that destroys GHRH neurons, decreases the concentration of somatostatin in the median eminence. Immunonuetralization of GHRH with anti-GHRH antibodies has also been shown to decrease somatostatin content and release from hypothalami obtained from fetal rats.

3.2.1.2. Short-loop feedback regulation of GHRH by somatostatin. The interactions between GHRH and somatostatin are bidirectional. Terminals of somatostatinergic cells have been reported to synapse on GHRH neurons. Somatostatin receptors are located on GHRH cell bodies and are up-regulated when somatostatin concentrations are decreased. In addition, the removal of somatostatin increases GHRH release in the rat. Direct measurement of GHRH in hypothalamic-pituitary portal blood in sheep has also indicated that somatostatin decreases physiological GHRH secretion. Direct feedback effects have also been demonstrated in vitro.

3.2.2. Pituitary-Hypothalamic Short-Loop Feedback Pathways

3.2.2.1. Short-loop feedback regulation of GHRH by GH. There is ample evidence to suggest that a short-loop feedback system exists between GH and GHRH. At the cellular level, hypothalamic GHRH-mRNA content increases following hypophysectomy and pituitary stalk transection. Similarly, concentrations of GHRH mRNA in GH-deficient animals are increased. As expected, elevated GH concentrations result in a decrease in GHRH mRNA and GHRH content in the hypothalamus.

3.2.2.2. Short-loop feedback regulation of somatostatin by GH. Short-loop feedback by GH also plays a role in the regulation of hypothalamic somatostatin. Conditions that decrease the circulating concentrations of GH result in a decrease in hypothalamic somatostatin content. For example, hypophysectomy, passive immunization of GH with anti-GH antibodies, and hypothalamic-hypophyseal stalk transection all result in a decrease in hypothalamic somatostatin con-

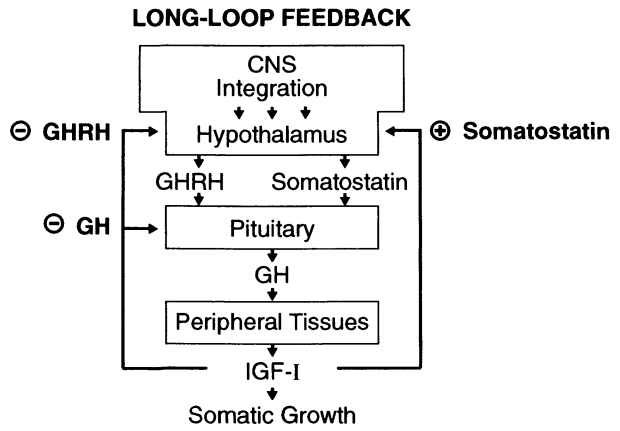

Fig. 11. Neuroendocrine regulation of GH secretion: Long-Loop Feedback. Numerous other factors also feed back on the GH axis. Please refer to Table 1.

tent. These effects are reversed by GH replacement therapy. It appears that these changes are mediated at several levels. These include: (1) somatostatin gene transcription, where a decrease in GH increases the expression of the somatostatin gene and (2) somatostatin release, where somatostatin secretion increases following GH treatment. The feedback effect of GH on hypothalamic somatostatinergic pathways appears to be tissue specific. Thus, whereas feedback effects of GH are seen at the hypothalamus, somatostatin-expressing cells in the brain and gastrointestinal tract do not appear to be affected.

3.3. Long-Loop Feedback (Fig. 11)

3.3.1. Insulin-Like Growth Factors: IGF-I and IGF-II

Several lines of evidence indicate that IGF-I plays a pivotal role in the negative feedback control of GH secretion (Fig. 11). The primary source of IGF-I is the liver. In the hypothalamus, IGF-I increases somatostatin secretion and decreases the pituitary response to GHRH. Interestingly, the pituitary itself has been shown to express IGF-I. Thus, whereas IGF-I behaves as a true long-loop feedback regulator, it is possible that it also has short-loop or paracrine actions in the pituitary. IGF-I decreases pituitary GH gene transcription, GH mRNA content, and GH secretion. IGF-I also suppresses expression of transfected GH genes. IGF-I does not appear to regulate the secretion of other pituitary hormones. The inhibitory IGF-I effects on GH are proportional to the number of IGF-I receptors on the somatotrophs. Overall, it functions through classic negative-feedback mechanisms.

The role of IGF-II in regulating the GH axis is less significant than IGF-I. The primary feedback role of IGF-II occurs during fetal development. Its effect has been shown to be mediated by the pituitary IGF-I receptor.

3.3.2. Adrenal Hormones

The glucocorticoids have very interesting and important roles in modulating the GH axis. The glucocorticoids stimulate GH synthesis in the pituitary and augment the pituitary response to GHRH, yet the steroids' overall physiological effects are to decrease somatic growth. It appears that the inhibition of growth is accomplished by glucocorticoid-induced increases in somatostatin and by direct catabolic influences at the cellular level.

Somatostatin synthesis, storage, and secretion are all stimulated by the glucocorticoids. These increases in hypothalamic somatostatin concentrations, mRNA content, and gene transcription are all easily observed in the hypothalamus. The in vitro incubation of fetal hypothalamic cells with corticosterone increases their content of somatostatin. Somatostatin content in the median eminence of the hypothalamus increases following glucocorticoid treatment (*see* Fig. 12). Glucocorticoid effects on GHRH appear to be quite minor. Thus, within the hypothalamus the overall net effect of the glucocorticoids is inhibitory.

However, in contradistinction to these negative effects of the glucocorticoids at the level of the hypothalamus, the glucocorticoids have a strong stimulatory effect at the pituitary level. Here, glucocorticoids increase pituitary GH-mRNA content. They increase the number of GHRH receptors on the pituitary and increase the rate of GH gene transcription. This is consistent with the observation that there is a glucocorticoid regulatory element on the human GH gene. In vitro studies confirm that glucocorticoids stimulate GH synthesis and secretion. Glucocorticoids also increase the pituitary GH response to GHRH more than fivefold.

The important question is, how are the opposite hypothalamic and pituitary signals interpreted when this information is carried to an in vivo setting? Based on the effects of glucocorticoids in vitro, it is logical to assume that GH secretion is suppressed by hypoadrenalism. Indeed, adrenalectomy without hormone replacement therapy significantly decreases the pituitary GH response to GHRH. Yet, hyperadrenalism does not have the opposite effect. Hyperadrenalism induced by chronic dexamethasone (a synthetic glucocorticoid) treatment suppresses circulating GH concentrations. The reason hyperadrenalism, with its

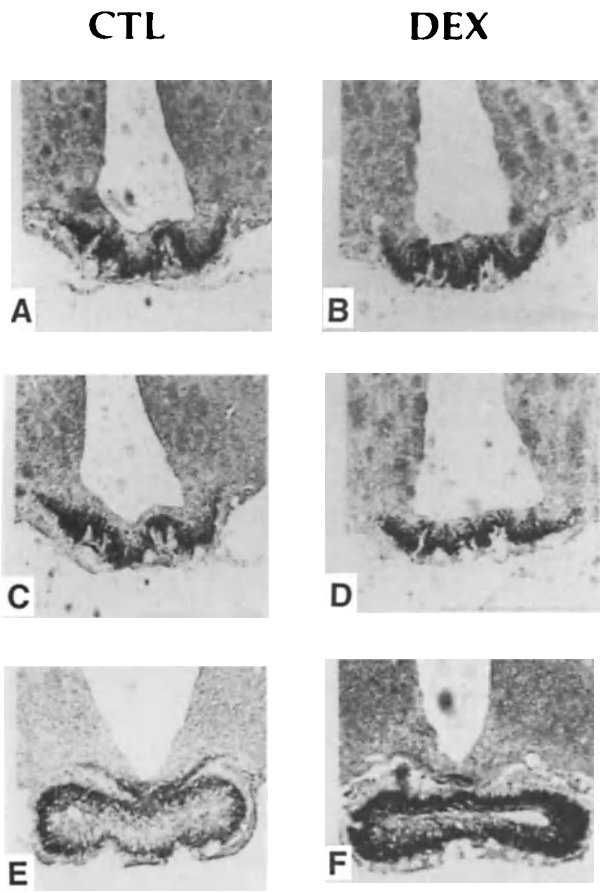

Fig. 12. Immunocytochemical localization and distribution of somatostatin in the rostral **(A,B)**, middle **(C,D)**, and caudal **(E,F)** sections of the median eminence of the hypothalamus of control-treated (CTL, left panels) and glucocorticoid-treated (dexamethasone (dex), right panels) rats. Visible increases in the area and intensity of immunostaining are observed in glucocorticoid-treated vs control animals in all the regions examined. (From Fife SK, Brogan RS, Giustina A, et al., Immunocytochemical and molecular analysis of the effects of glucocorticoid treatment on the hypothalamic-somatotropic axis in the rat. *Neuroendocrinology* 1996; 64:131–138.)

stimulatory effects on pituitary GH, does not increase circulating GH can be explained by recalling that at the hypothalamic level, glucocorticoids also increase somatostatin tone. This has led to the hypothesis that the inhibitory effects of somatostatin predominate during conditions of hyperadrenalism. This hypothesis is supported by the observation that the GH response to GHRH in normal, conscious, freely moving rats is significantly lower in glucocorticoid-treated rats than in saline-treated rats (*see* Fig. 13A). However, when similarly treated animals are passively immunized with somatostatin antiserum, the GH response is completely reversed; that is, glucocorticoid-treated rats have a significantly higher GH response to GHRH than saline-treated rats (*see* Fig. 13B). Overall, hyperadrenalism inhibits GH concentrations by increasing hypothalamic somatostatin tone, which predominates over the stimulatory effects of these steroids on pituitary GH. Studies investigating the feedback effects of mineralocorticoids on the GH axis have thus far been limited, and very little meaningful data are presently available.

3.3.3. Gonadal Hormones

The pattern of pulsatile GH secretion is clearly different in males and females (*see* Fig. 14). This fact suggests that the gonadal steroids modulate GH secretion. Indeed, there is significant evidence to support this hypothesis. Hypothalamic GHRH-mRNA content is approximately two to three times higher in male rats as compared to female rats. This suggests that the higher GH pulses noted in males as compared to females reflect an increased release of GHRH in males. GH feedback inhibition of GHRH-mRNA content has also been shown to be gender-specific. For example, exogenous GH precipitates a much greater decrease in the hypothalamic content of GHRH mRNA in male rats as compared to female rats. Yet, the role of the gonadal steroids in regulating the gender difference is unclear because hypothalamic GHRH-mRNA concentrations are not altered by gonadectomy or pharmacological sex-steroid replacement therapy in either adult male or female rats. In female rats, GHRH-mRNA levels remain constant throughout the estrous cycle and pregnancy. This latter point is particularly interesting because circulating GH concentrations rise during pregnancy. The data concerning the role of the estrogens in modulating GHRH suggest that the role is minor. On the other hand, testosterone may enhance GH secretion through an increase in both GHRH synthesis and secretion. Overall, sex-steroid feedback regulation on GHRH is poorly understood.

At the present time, it is not clear whether there are gender differences in somatostatin-mRNA content of the hypothalamus of male and female rats because differing reports can be found in the literature. However, the effects of exogenous gonadal steroids on hypothalamic somatostatin-mRNA content are well documented. In male rats, gonadectomy reduces hypothalamic somatostatin-mRNA concentrations. Testosterone replacement therapy can reverse this effect of castration. Similar results have been observed in females. Ovariectomy results in a significant decrease in hypothalamic somatostatin-mRNA and estradiol treatment reverses this effect. There is

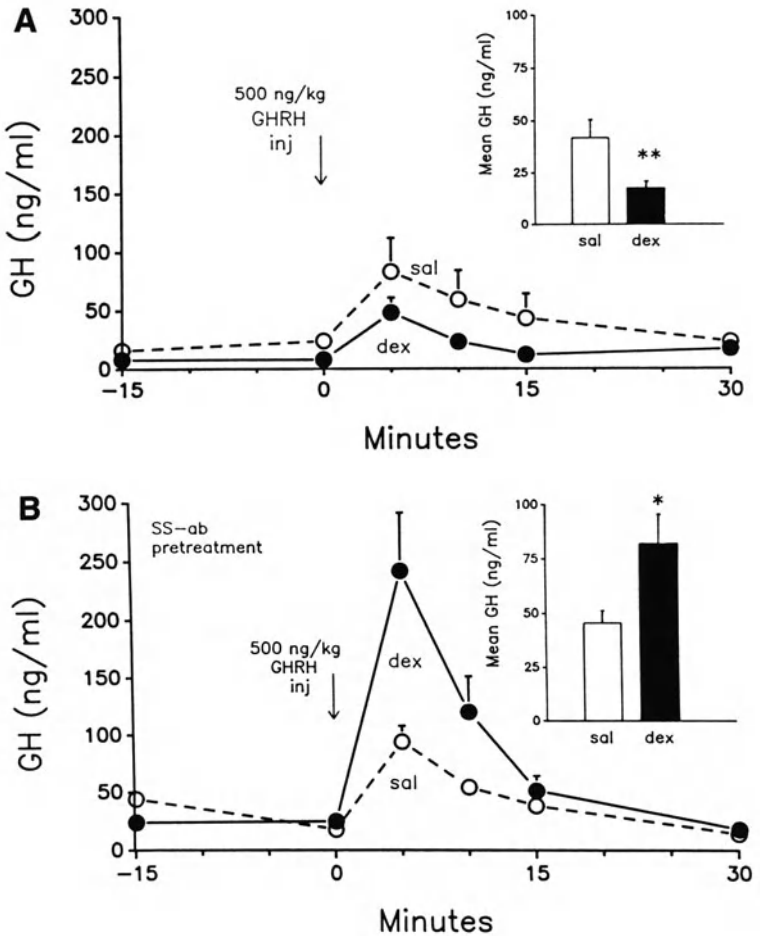

Fig. 13. (**A**) The pituitary GH response to GHRH administered to conscious, freely moving rats treated for four days with saline (sal) or dexamethasone (dex, a synthetic glucocorticoid). Basal and peak GH concentrations were suppressed, leading to a significantly lower overall mean GH response in the dexamethasone-treated animals. (**B**) The GH response to GHRH administered to rats treated for four days with saline (sal) or dexamethasone (dex) and pretreated with 0.25 mL somatostatin antiserum (SS-ab) 30 min before the initiation of blood sampling. Peak GH concentrations were enhanced, leading to a significantly higher overall mean GH response in the dexamethasone-treated animals. (From Wehrenberg WB, Janowski BA, Piering AW, et al., Glucocorticoids: potent inhibitors and stimulators of growth hormone secretion. *Endocrinology* 1990; 126:3200–3203.)

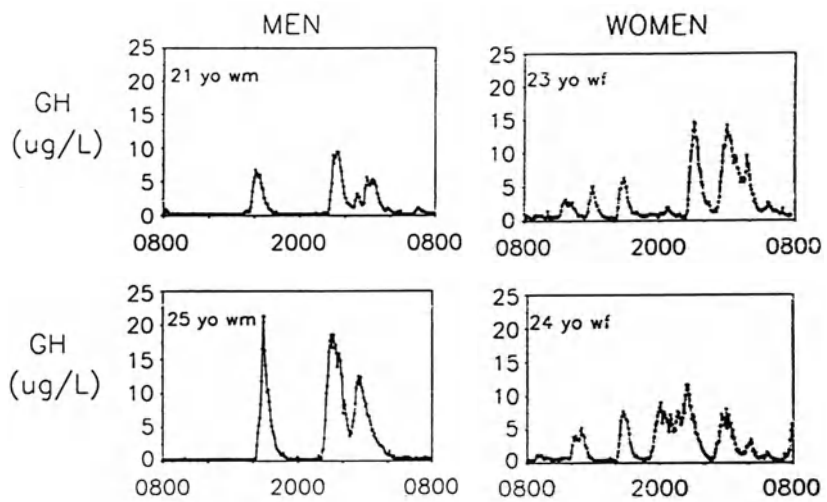

Fig. 14. GH profiles of two normal men and two normal women. Blood sampling was performed every 5 min for 25 h. (From Hartman ML, Veldhuis JD, Vance ML, et al., Somatotropin pulse frequency and basal concentrations are increased in acromegaly and are reduced by successful therapy. *J Clin Endocrinol Metab* 1990; 70:1375–1384.)

no direct evidence demonstrating sexually dimorphic patterns of somatostatin release. However, indirect studies do provide some insight. The time-dependent responses to exogenous GHRH, which are seen in conscious, freely moving male rats (*see* Fig. 5A) are because of endogenous somatostatin secretion (*see* Fig. 5B). This information, coupled with other observations that conscious, freely moving female rats more consistently respond to GHRH administration than male rats, suggests that hypothalamic release of somatostatin is different in male and female rats. Indeed, the consensus is that in males, somatostatin is released in higher amplitude, but lower frequency pulses than in females.

A sex difference in the pituitary content of GH mRNA has been observed, with male rats exhibiting approximately two to six times greater content than females. This is consistent with GH pulses being greater in males. It is not clear if this difference reflects an action of gonadal steroids on GH gene expression directly at the pituitary level or indirectly via the hypothalamic neuropeptides. However, it has been recognized that pituitary GH content is greater in male rats than in female rats. This, coupled with the fact that GH secretory pulses are of greater amplitude in males than in females, suggests that many of the observed sexually dimorphic differences are mediated at the pituitary level. The pituitary GH responses to exogenous administration of GHRH in laboratory rats exhibit sexually dimorphic trends. In vitro, pituitaries obtained from female rats released less GH in response to GHRH treatment than pituitaries obtained from male rats. In all, the effects of the sex steroids on the responsiveness of the pituitary to GHRH are consistent. Androgens appear to have an important stimulatory effect, whereas estrogens play a more minor role.

By nature, studies in humans are much more restrictive, yet the evidence obtained by indirect approaches are consistent with laboratory results obtained in other models. Correlation analysis has suggested that serum testosterone concentrations are positively correlated with the daily GH secretory rate in pubertal boys. Studies of GH secretion in untreated and testosterone-treated hypogonadal subjects demonstrate that the amount of GH released is significantly higher after testosterone treatment. This suggests that in males, increases in testosterone occurring during the early stages of puberty are able to increase both the number and frequency of spontaneous GH peaks. In adults, a positive correlation between mean serum GH concentrations and serum testosterone concentrations has been reported in healthy men. Similarly, serum testosterone concentrations are also positively correlated with GH pulse amplitude.

During puberty in females, serum GH concentrations increase and are correlated with changes in serum estradiol levels. GH release, including GH pulse amplitude, increases two- to threefold from the onset of prepuberty to menarche and this increase correlates with serum estradiol concentrations. In addition, there is a two-fold change in serum GH concentrations across the menstrual cycle. Estradiol taken orally increases serum GH concentrations and decreases IGF-I levels. Moreover, treatment of females with fertility hormones increases serum estradiol and concurrently GH concentrations several fold. In short, there is a basis for inferring a positive effect of estrogen on the release of GH in the human. Accordingly, it is possible to conclude that in humans the neuroendocrine feedback regulation of GH secretion is affected by the stage of the menstrual cycle and the estrogen environment. Studies in the female rat suggest that estrogens do not play a major role in that species.

3.3.4. Thyroid Hormones

GHRH synthesis is affected by circulating concentrations of thyroid hormone. It is clear that hypothyroidism increases hypothalamic GHRH-mRNA content whereas hyperthyroidism decreases it. The hypothalamic content of GHRH is also affected by hypothyroidism and hyperthyroidism. Hypothyroidism results in a decrease in GHRH content. This is consistent with the hypothesis that there is increased GHRH release in an attempt to compensate for the low plasma GH concentrations present under conditions of hypothyroidism. Hyperthyroidism has also been reported to decrease the hypothalamic content of GHRH, as well as that of GHRH mRNA. The explanation for why both hypo- and hyperthyroidism decrease GHRH and its mRNA is not clear at the present time.

The hypothalamic content of somatostatin has also been investigated under hypo- and hyperthyroid conditions. The content of somatostatin in the median eminence of male rats subjected to surgical thyroidectomy is significantly lower than that observed in normal rats. The effect appears reversible as somatostatin values return to normal following replacement therapy. Hyperthyroidism induced in the rat by a subcutaneous implant of T_4 does not seem to alter the level of hypothalamic somatostatin. Although the secretion

of somatostatin from the hypothalamus of conscious animals is obviously difficult to measure, direct in vivo quantification of somatostatin secretion has been reported in pentobarbital-anesthetized rats. In this model, neither hypothyroidism nor hyperthyroidism affect hypothalamic-pituitary portal blood concentrations of somatostatin. Although these results would suggest that the thyroid environment does not affect somatostatin secretion, this conclusion must be tempered by the fact that anesthesia has inhibitory effects on somatostatin secretion.

T_3 stimulates pituitary GH-mRNA accumulation and increases the rate of GH gene transcription. Thyroid hormones also have important effects on pituitary GH content; that is, hypothyroidism decreases pituitary GH content. Thyroidectomy has a profound effect on pituitary GH content, with values dropping to less than 1% of those characteristically found in normal animals. Thyroxine replacement therapy can partially restore GH pituitary content.

In humans, as in other species, hypothyroidism severely impairs postnatal growth. Moreover, the measurement of spontaneous nocturnal GH secretion, which is considered to be a good indicator of physiological GH secretion, is consistently reduced in the hypothyroid state. Consistent with the data derived from studies on spontaneous GH secretion are reports that the GH response to common physiological and pharmacological stimuli, such as GHRH, insulin-induced hypoglycemia, and arginine is blunted in hypothyroid patients. However, the GH response to GHRH is usually normalized two weeks after starting T_4 replacement therapy.

Thyroid-hormone excess consistently impairs the GH response to several secretagogues in man. Thyrotoxicosis is associated with a blunted and delayed GH response to GHRH. Several other clinical studies have been performed to identify the mechanisms for the reduced GH secretion characteristic of hyperthyroidism. These studies suggest that the reduced GH response following GHRH administration observed during hyperthyroidism may be explained by an increase in hypothalamic somatostatin tone. Whether this effect is caused directly by thyroid hormone or by an elevation in the circulating concentrations of IGF-I remains to be established. Alternatively, it can be hypothesized that the defective GH response to acute pharmacological challenges may be because of either chronic GHRH deficiency leading to somatotroph atrophy or to direct toxic effects of elevated thyroid hormone levels on the somatotroph.

3.3.5. Other Hormones

3.3.5.1. Galanin. The hypothalamus is particularly rich in cell bodies and fibers containing galanin-like immunoreactivity, with the highest concentration of galanin-like immunoreactivity being located in the median eminence. Specific binding sites for galanin have been demonstrated in the mediobasal hypothalamus. Galanin increases plasma GH levels when administered via numerous routes. The mechanism underlying the action of galanin is unknown; evidence seems to suggest that galanin may act at the level of the hypothalamus to increase GHRH. The physiological role of galanin is still being clarified.

3.3.5.2. Vasoactive Intestinal Polypeptide (VIP) and Glucagon. Administration of VIP increases plasma GH levels in patients with acromegaly, but not in normal subjects. It also stimulates GH release in vitro from pituitary tumors obtained from these patients. This stimulatory effect may be mediated via GHRH receptors, because VIP is closely related to GHRH structurally (Table 1). Similarly, the peripheral administration of glucagon weakly stimulates GH release in normal subjects. This has led to glucagon's use as a diagnostic test for pituitary function. Whereas these hormones can stimulate GH secretion, they do not play a physiological role in the neuroendocrine regulation of GH secretion.

3.3.6. Metabolic Factors

3.3.6.1. Carbohydrates

3.3.6.1.1. Hypoglycemia. An increase in blood glucose levels in normal subjects reduces basal and stimulated GH concentrations, whereas hypoglycemia stimulates GH release. The mechanism by which hypoglycemia causes GH release is unclear, although the pathway is probably largely independent of GHRH. The evidence supporting this view is as follows: GHRH and hypoglycemia have additive effects on GH release and selective neurotransmitter blockade of the GH response to GHRH only slightly reduces the GH response to hypoglycemia. It is hypothesized that hypoglycemia increases GH secretion by reducing somatostatin release from the hypothalamus.

3.3.6.1.2. Hyperglycemia. Diabetes mellitus is routinely classified as insulin-dependent (IDDM; Type I) or noninsulin-dependent (NIDDM; Type II). The former condition is usually characterized by patients who are young, have normal body mass indexes, and are insulin-deficient. The latter condition

Table 1
Sequence homologies between GHRH and various intestinal peptides

GHRH-44	YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGESNQERGARARL-NH_2
PHI-27	HADGVFTSDFSRLLGQLSAKKYLESLI-NH_2
VIP	HSDAVFTDNYTRLRKQMAVKKYLNSILN-NH_2
Glucagon	HSQGTFTSDYSKYLDSRRAQDFVQWLMNT-OH
Secretin	HSDGTFTSELSRLRDSARLQRLLQGLV-NH_2
GIP	YAEGTFISDYSIAMDKIRQQDFVNWLLAQKGKKSDWKHNITQ-OH
Motilin	FVPIFTYGELQRMQEKERNKGQ-OH

is characterized by patients who are older and who have a predisposition toward obesity and hyperinsulinemia. GH secretion is also different in these two types of diabetes, being elevated in IDDM and suppressed in NIDDM.

Studies have shown elevated 24-h GH secretion in untreated and insulin-treated IDDM patients. GH pulse frequency and interpulse GH concentrations also are elevated. Exaggerated GH secretory responses to the exogenous administration of GHRH have been consistently reported. Overall, decreased somatostatinergic tone appears to account for the elevated GH secretion and exaggerated response to pharmacological stimuli observed in IDDM patients. There is a consensus in the literature that obese, NIDDM patients, as well as nondiabetic obese subjects, have an impaired ability to respond to GHRH.

3.3.6.2. Free fatty acids. Administration of free fatty acids (FFAs) to normal subjects reduces the GH response to a variety of different stimuli including hypoglycemia, physical exercise, L-dopa, clonidine, arginine, sleep, and GHRH. This reduced GH response is also seen in patients with spontaneously elevated FFAs and/or triglycerides. This effect is probably caused by increased somatostatin release because FFAs reduce the GH response to GHRH in normal rats, but not in rats treated with antisomatostatin antibodies. However, this does not exclude an additional direct pituitary action of FFAs.

3.3.7. Growth Hormone-Releasing Peptides

Studies have shown that opioids have GH releasing activity. Further investigations led to the identification of several peptide analogs of met-enkephalin that stimulate GH secretion. These peptides are biologically active via oral and nasal routes in addition to intravenous and subcutaneous administration and therefore hold clinical interest. However, to date investigators have not found endogenous molecules that parallel the actions of the growth hormone-releasing peptides. Therefore, these molecules are presently considered to be more drug-like than endogenous hormone-like.

4. SUMMARY

The neuroendocrine feedback regulation of GH secretion is unique for the pituitary hormones. GH is the only pituitary hormone regulated by both a releasing (GHRH) and inhibiting (somatostatin) factor. The actual feedback mechanisms are well established. There is limited evidence to support the concept that there is an ultrashort-loop feedback system for GHRH within the hypothalamus. More compelling evidence for an ultrashort-loop system is present for hypothalamic somatostatin and pituitary GH. Evidence strongly supports the existence of reciprocal short-loop feedback regulation of GHRH by somatostatin and vice versa. GH also plays an important feedback role on GHRH and somatostatin via a short-loop system. In the presence of low GH concentrations, the GHRH system is activated and the somatostatin system is suppressed. The opposite occurs in the presence of high GH concentrations. Long-loop feedback systems are numerous and complex. Although clearly a simplification of the overall picture, Table 2 provides an overview of the most significant peripheral hormones feeding back on the hypothalamic-pituitary somatotrophic axis.

Table 2
The feedback effect on numerous hormones and glucose on hypothalamic somatostatin and GHRH and pituitary GH

HORMONE	*condition*	*Somatostatin*	*GHRH*	*Pituitary GH*
Glucocorticoids	hypo	?	?	⇓
	hyper	⇑⇑⇑	minor	⇑⇑⇑
IGF-I	hyper	⇑	⇓	⇓
Thyroid Hormones	hypo	minor to ⇓	⇑	⇓⇓
	hyper	⇑	⇓	minor to ⇓
Androgens	hyper	⇑	⇑	⇑⇑
	hypo	⇓	⇓⇓	⇓
Estrogens	hypo	⇑	minor to ⇑	⇑⇑
Glucose	hypo	⇓	minor	⇑
	hyper	⇑	minor	⇓

SELECTED READINGS

Arimura A. Regulation of growth hormone secretion. In Imura H, ed. The Pituitary Gland, 2nd ed. New York: Raven, 1994.

Bercu BB, Walker RF, eds. Growth Hormone II: Basic and Clinical Aspects. New York: Springer-Verlag, 1994.

Frisch H, Thorner MO, eds. Hormonal Regulation of Growth. New York: Raven, 1989.

Harvey S. Growth Hormone. Boca Raton, FL: CRC, 1994.

Kostyo JL, ed. Handbook of Physiology, Section 7: The Endocrine System. Volume V: Hormonal Control of Growth. New York: Oxford University Press, 1999, pp. 299–328.

Wilson JD, Foster DW, Kronenberg HM, Larson PR, eds. Williams Textbook of Endocrinology 9th Edition. Philadelphia, PA: W.B. Saunders, 1998.

12 Neuroendocrine Regulation of Puberty

Sergio R. Ojeda, DVM and Marie Bilger, MD

CONTENTS

1. INTRODUCTION

Puberty is a developmental phase during which profound hormonal, physical, behavioral, and psychological changes occur. The individual becomes capable of reproduction, that is, viable gametes are produced and transferred through a mature reproductive system.

The onset of mammalian puberty depends on an orderly and complete sequence of maturational changes that are initiated within the brain and occur at all levels of the hypothalamic-pituitary-gonadal axis. The qualitative and quantitative characteristics of these changes, and importantly, their physiological linkage are now being more clearly comprehended thanks to the development of powerful methodologies to unravel the molecular mechanisms underlying the process. Studies in experimental animals, including nonhuman primates, have led to the identification of several of the basic mechanisms regulating the hypothalamic-pituitary-gonadal axis during development. The importance of these findings for the understanding of human puberty is underscored by the fact that many of these mechanisms have also been shown to operate in humans.

From: *Neuroendocrinology in Physiology and Medicine,* (P. M. Conn and M. E. Freeman, eds.), Totowa, NJ.

In mammals, no obvious relationship can be discerned between gestation, onset of puberty, and length of estrous or menstrual cycles (*see* Fig. 1). Nevertheless, the age of the onset of puberty is characteristic for each species. Humans require the longest time in this maturational process; this is probably a phylogenetic advantage for our species, as it assures a complete psychosexual maturation before the individual becomes involved in adult social responsibilities.

We can distinguish mechanisms that control the activation of the hypothalamic-pituitary-gonadal axis at puberty and factors that influence when these changes take place. We will discuss in detail only the former. Among these latter factors are inheritance and a variety of environmental factors acting via the central nervous system (CNS), such as geography, altitude, light perception, socioeconomic conditions, especially as these conditions influence the level of nutrition and general well being of each individual.

2. THE CENTRAL ROLE OF LHRH IN SEXUAL DEVELOPMENT

Neither the pituitary gland nor the gonads play a major role in initiating the onset of puberty. In contrast, the CNS has been implicated in causing true precocious puberty. Hence, clinical and animal

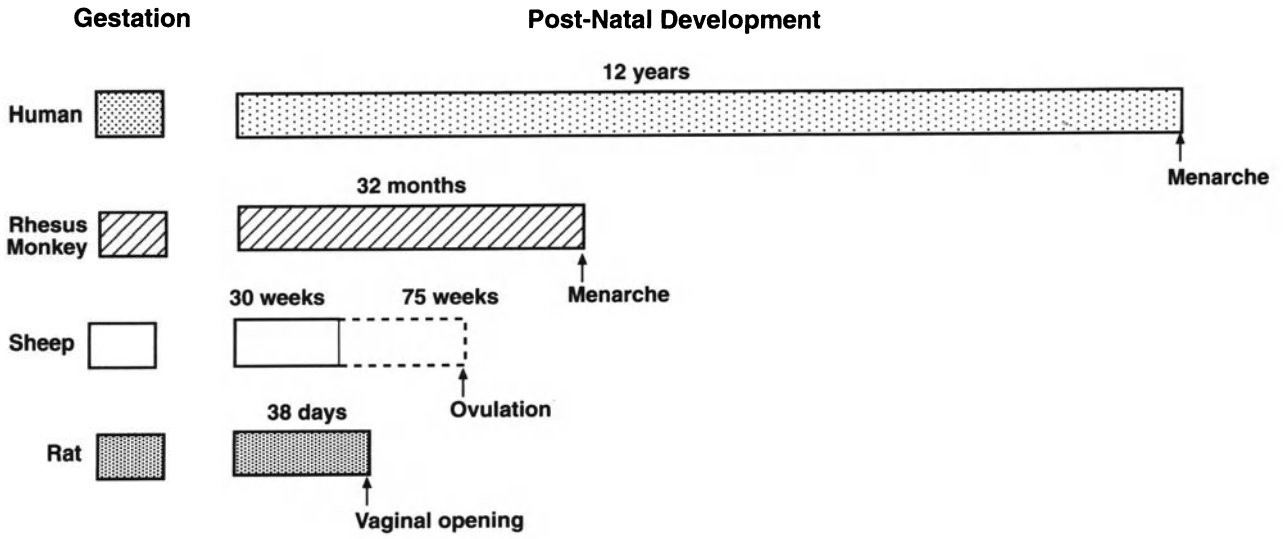

Fig. 1. Lack of correlation between the length of pregnancy and the interval between birth and puberty in female individuals from four representative mammalian species. In each case, the interval between birth and puberty is represented as a relative value in relation to the duration of gestation, which is given a unitary value. The dotted box in the case of sheep represents the seasonal delay in puberty observed when the animals are born during the "off" season, and thus are too young to reach puberty during winter, the breeding season.

researchers have focused a great deal of their attention on gaining insight into the role that the CNS may play in determining the onset of puberty. It is now clear that both the initiation and the tempo of the pubertal process are controlled by a handful of specialized, interconnected neuroendocrine neurons located in the hypothalamus. These neurons, which form a network of about 1000 cells, secrete, in a pulsatile fashion, a decapeptide known as luteinizing hormone-releasing hormone (LHRH) or gonadotropin hormone-releasing hormone (GnRH) (*see* Chapter 3). The name LHRH was originally given to this neurohormone because of its potency in inducing luteinizing hormone (LH) release from the anterior pituitary gland. The subsequent finding that LHRH also stimulates the release of another pituitary gonadotropin, follicle-stimulating hormone (FSH), led to the now-widespread use of the name GnRH.

We prefer the term LHRH because of the possibility that other hypothalamic peptides may have a preferential FSH-releasing activity. As in the case of the gonads and the pituitary gland, LHRH neurons do not appear to be a limiting factor for puberty to occur. Activation of excitatory neurotransmission (such as that provided by glutamate, the major excitatory amino acid in the brain), hastens the initiation of puberty via stimulation of LHRH release. Conversely, a decrease in inhibitory transsynaptic influences [such as those provided by gamma amino butyric acid, (GABA), the major inhibitory neurotransmitter in the brain], leads to a premature activation of LHRH release. It is thus clear that the central mechanisms responsible for the initiation of puberty reside in cellular circuitries functionally linked to the LHRH neuronal network, but not within LHRH neurons themselves. LHRH neurons are, however, essential for sexual development. Their absence (usually because of genetic defects) or their dysfunction (reduced secretory output and/or blunted pulsatile release of the decapeptide), results in impaired maturation of the reproductive system and inevitably leads to reproductive failure, unless therapeutical intervention occurs.

3. DEVELOPMENTAL PATTERNS OF HORMONE SECRETION

3.1. LHRH and Pituitary Gonadotropins

3.1.1. LHRH

LHRH neurons originate outside the brain, in the epithelium of the olfactory placode from which they migrate along the pathway of the olfactory nerves, before diverging toward their final destination in the forebrain. Although no data exist concerning the secretory capacity of migrating neurons, the unambiguous detection of the decapeptide in the perikarya of migrating cells makes it likely that they are not only accumulating, but also releasing LHRH as they

approach and enter the brain. Once they reach the hypothalamus (in the human, at about 9 wk of fetal life), LHRH neurons send axons to the median eminence where they presumably begin to release their secretory products into the developing portal vasculature. Experiments in sheep have shown that LHRH secretion is already episodic in fetal life. The human pituitary gland begins to respond to LHRH as early as the tenth week of fetal life, i.e., shortly before development of the vascular connections between the median eminence of the hypothalamus and the pituitary gland (which occurs by 11.5 wk of gestation). After birth, and throughout puberty, the hypothalamic content of LHRH changes little, indicating that the peripubertal activation of LHRH neuronal function is mostly a function of an increase in LHRH release instead of a change in biosynthetic capacity.

3.1.2. Gonadotropins

By 10 wk of fetal life, the pituitary gland synthesizes FSH and LH. The content of both gonadotropins rises from this gestational age, reaching maximum levels at 25 to 29 wk and then declining as pregnancy progresses. The pituitary of a newborn infant has a 2- to 5-times smaller concentration of gonadotropins than that of fetuses at 25 to 29 wk of gestation.

Circulating levels of LH and FSH are detected by 12 wk. (The earliest fetus studied.) They increase thereafter to reach very high values (in the castrate range) by 21 wk of gestation, i.e., before the peak in pituitary gonadotropin content (Fig. 2). From then on, there is a fall in serum levels to term. However, during the first 2 to 4 yr of life, serum gonadotropins are higher than during the midchildhood period (*see* Figs. 3 and 4). Interestingly enough, girls have higher levels of FSH than do boys in fetal life, infancy, and early childhood.

This particular pattern of gonadotropin release, in which FSH (and possibly LH) are secreted in increasing amounts during the second third of gestation in such a way that peak serum values are reached between 14 and 24 wk, is thought to be related, at least in part, to the maturation of the negative-feedback mechanism exerted by gonadal steroids. In the absence of this inhibitory feedback, FSH and LH are secreted autonomously or at least are relatively unrestrained. Later in fetal development, the inhibitory feedback mechanism matures and becomes operative (Fig. 2). This increasing sensitivity of the hypothalamic-pituitary unit to circulating sex steroids leads to suppression of the synthesis and release of FSH and LH.

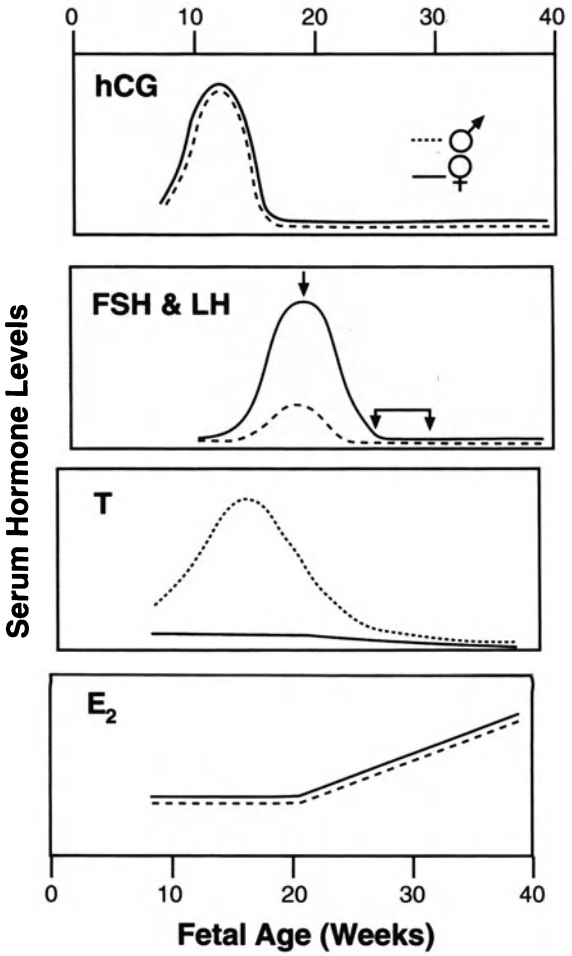

Fig. 2. Changes in serum gonadotropin and steroid levels in human fetuses from both sexes during gestation. hCG = human chorionic gonadotropin, T = testosterone; E_2 = estradiol. The double arrows indicate the peak in pituitary gonadotropin content. The single arrow points to the approximate time when steroid negative feedback begin to develop. Redrawn with permission from Faiman et al., *Clinics Obstet Gynaecol.* 1976; 3:467–483.

The lower gonadotropin levels in males compared with females can be attributed to the existence in the male of a negative-feedback mechanism that begins to operate at an earlier age during fetal life than in the female. Although no studies examining this hypothesis have been conducted in humans because of the obvious experimental difficulties involved, it has been fully proven in other species.

Studies carried out in the rat (an animal born at an age equivalent to 14 wk of human gestation) have demonstrated that the different patterns of circulating FSH in the male and the female are because of the different ages at which the inhibitory gonadal feedback mechanism becomes operative. Estrogen, which

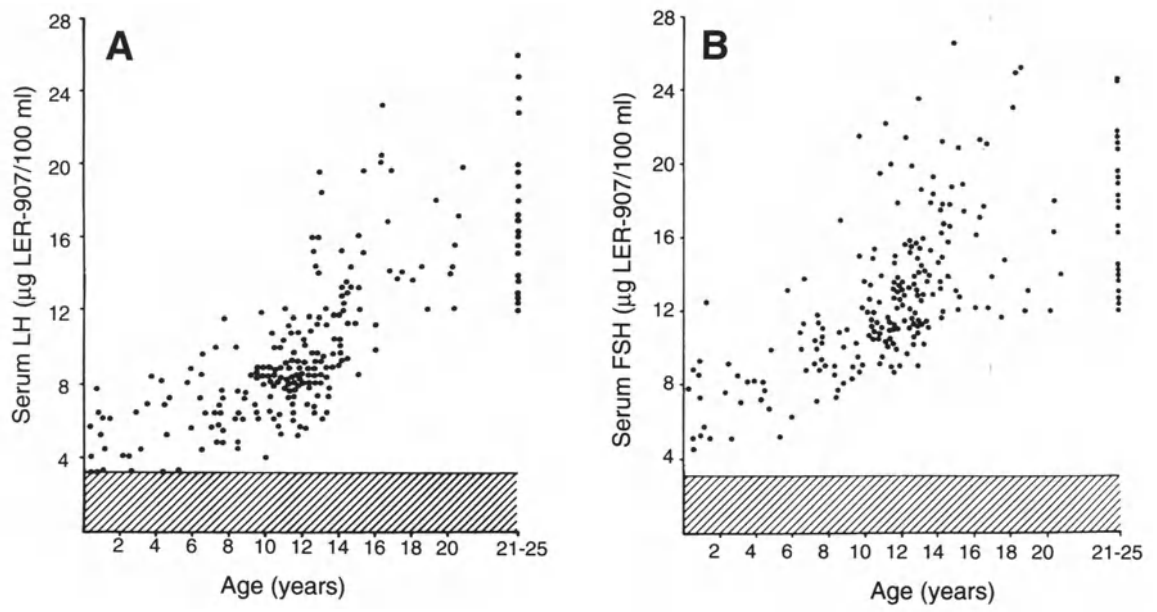

Fig. 3. Changes in serum LH **(A)** and FSH **(B)** levels at different postnatal ages in human males. The shaded area represent the limit of sensitivity of the assay. Notice that serum FSH levels begin to increase at least two years earlier that LH. Reproduced with permission from Winter and Faiman, *Pediat Res* 1972; 6:126–135.

is one of the main steroid hormones produced by the adult ovary, is ineffective in reducing the high plasma FSH levels found in the rat during the first 2 wk of postnatal life (= 14–21 wk of gestation in the human). By contrast, neonatal castration of male rats induces a prompt increase in circulating FSH and LH levels, which is readily prevented by treatment with testosterone, the main steroid produced by the adult testis. Studies with sheep fetuses, in which the changes (as in humans) occur during fetal life, identified testicular inhibin as an additional factor responsible for the earlier gonadal feedback inhibition of gonadotropin secretion in males than females.

Cross-sectional and longitudinal surveys of blood gonadotropin levels during postnatal sexual maturation have shown that the levels of both LH and FSH increase two- to fourfold from the prepubertal period to adulthood (Figs. 3 and 4). Daily urinary FSH and LH excretion per day increases 4 to 8 and 8 to 30 times, respectively. The general pattern of change in blood gonadotropin during puberty is similar in boys and girls. FSH levels increase in the early pubertal years, with the attainment of adult levels while LH values are still rising. In boys, this elevated FSH level correlates well with the period of most rapid testicular growth (*see* Fig. 6), whereas in girls, it correlates with the early rise in plasma estradiol levels (*see* Fig. 8).

LH levels increase throughout puberty. In males, the initial increase in LH occurs before plasma testosterone values rise (*see* Figs. 3 and 6), indicating that LH is, in fact, responsible for the activation of the Leydig cells around the time of puberty. Mean-serum FSH and LH levels cease to rise after about ages 15 to 17 yr. In girls, LH levels may not increase until secondary sexual development is quite advanced. In later pubertal females, there is a wide range of serum LH and FSH values, reflecting a superimposed menstrual cyclicity (Fig. 4). Episodic increases in LH levels (ovulatory spikes) have occasionally been recorded in girls within several months of menarche. A short rise in progesterone levels following these LH peaks has also been found in these girls, suggesting corpus luteum formation and ovulation. The luteal phase in these girls appears to be shortened. Accordingly, the infertility of the young adolescent girl may be related to either anovulatory cycles or to inadequate corpus luteum function.

The first endocrinological manifestation of puberty onset is an amplification of a diurnal pattern of LH release. In prepubertal children, LH is secreted in a pulsatile manner, but the magnitude of the pulses is small. In addition, greater LH levels are seen during sleep than during waking hours. At the end of childhood, and before mean gonadotropin levels become more elevated, the diurnal difference in LH secretion becomes more pronounced and the magnitude of the LH pulses increases (*see* Fig. 5). These changes not

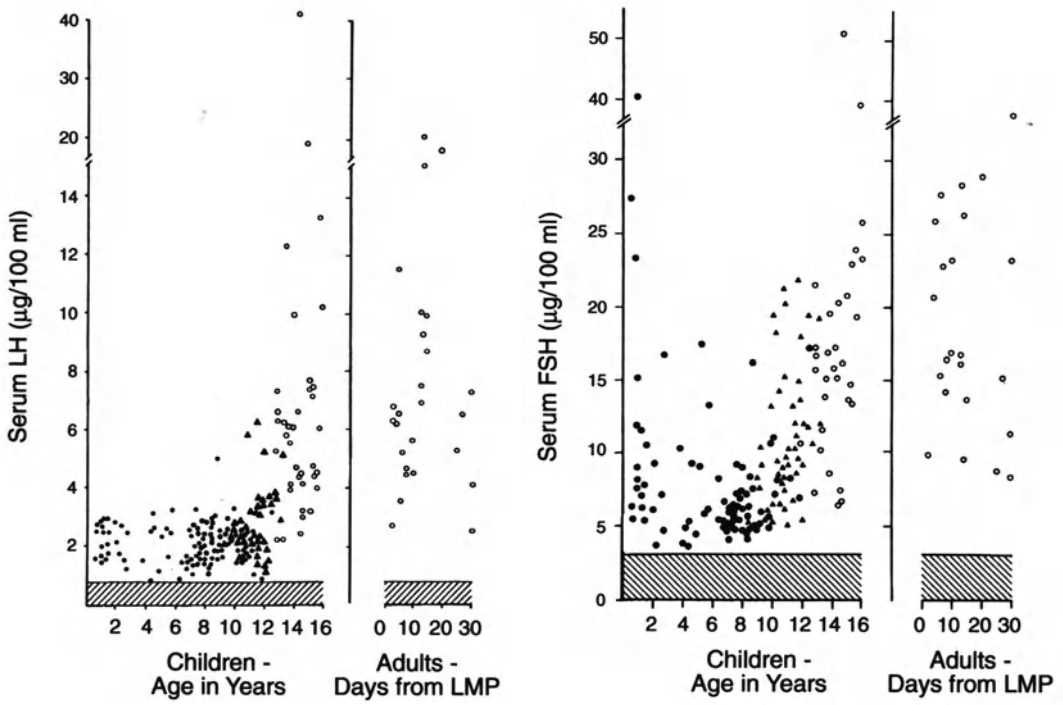

Fig. 4. Changes in serum LH (**A**) and FSH (**B**) concentrations in human females at different postnatal ages during prepubertal development, and during the adult menstrual cycle. The shaded area represents the limit of sensitivity of the assay. ● = prepubertal; ▲ = premenarcheal with breast development; ○ = postmenarcheal. Values for the 16- to 25-yr-old subjects are plotted on the right as days from the last menstrual period (LMP). Notice the elevated FSH values during the first year after birth. Redrawn with permission from Faiman and Winter, In: Grumbach MM, Grave GD, & Mayer FE, eds, *The Control of the Onset of Puberty,* New York: Wiley, 1974; 32.

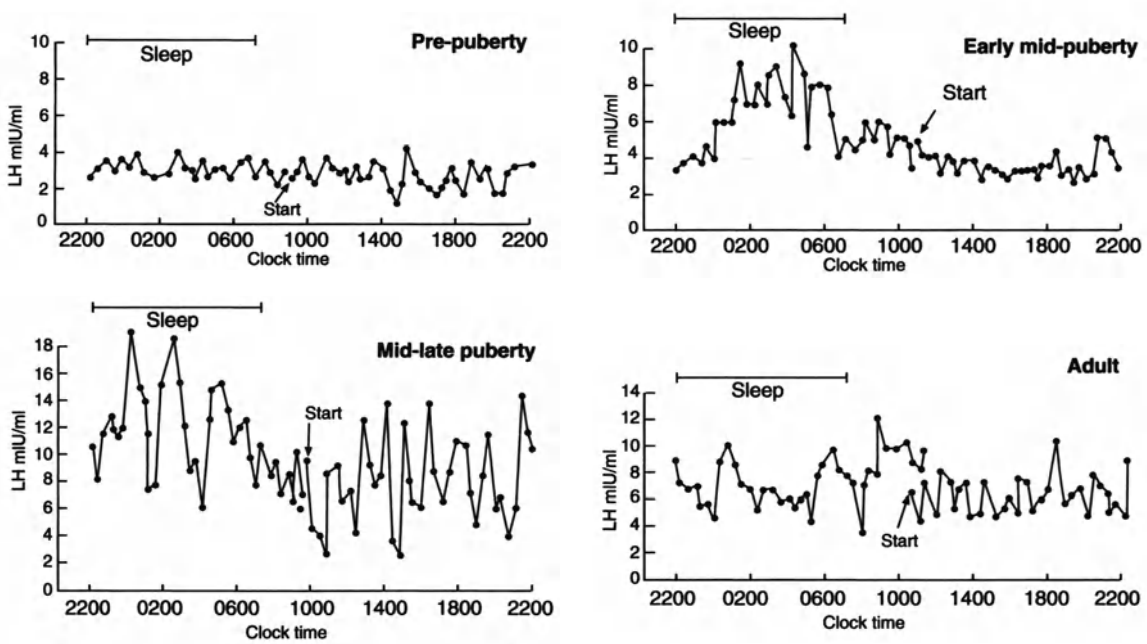

Fig. 5. The changes in pulsatile LH release during human puberty. Notice the transient increase in nocturnal episodes of LHRH release between early and midpuberty. Redrawn with permission from Boyar et al., *N Engl J Med* 1972; 287:582.

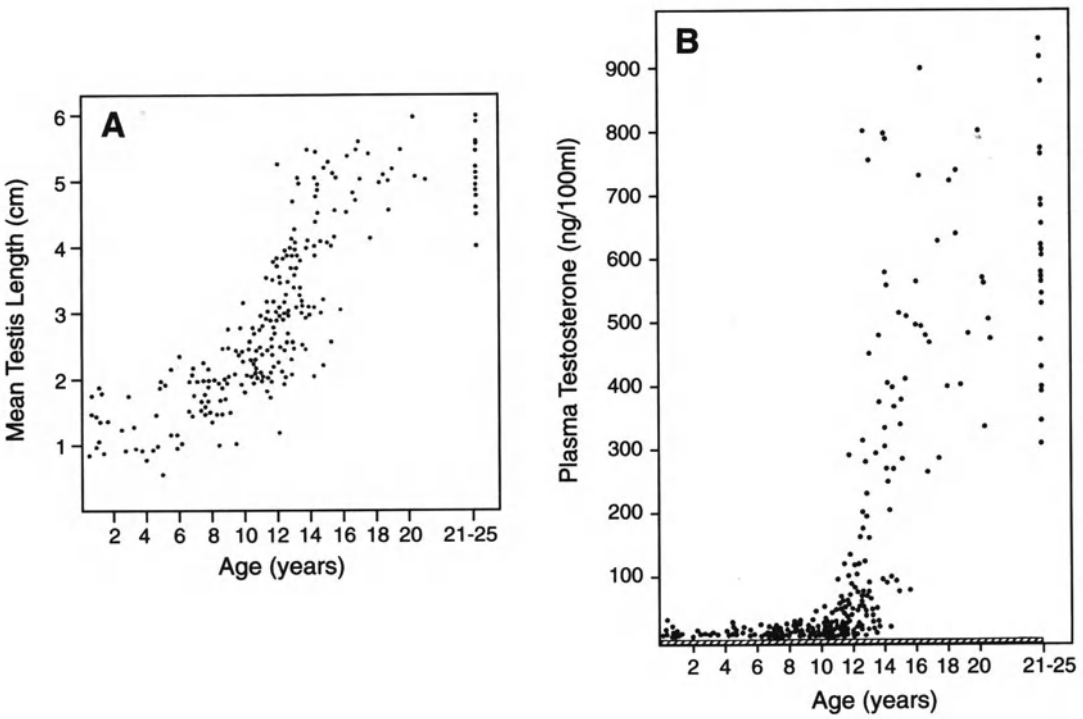

Fig. 6. Correlation between testis size **(A)** and serum testosterone **(B)** levels during postnatal development of human males. Values for testicular size correspond to the mean of the longest diameters of both testes. The shaded area in **B** represents the limit of sensitivity of the assay. Redrawn with permission from Winter and Faiman, *Pediatr Res* 1972; 6:126–135.

only reflect the initial activation of the central mechanism governing LHRH secretion, but are also believed to be the initial endocrine event leading to the attainment of puberty.

3.2. Gonadal Steroids and Peptides

3.2.1. The Testis and its Hormones

During prepubertal years, the seminiferous tubules, consisting of Sertoli cell precursors and germ cells, are dormant. The testes grow slowly between 6 and 10 yr of age and then more rapidly after intense gonadotropin stimulation begins (*see* Figs. 3 and 6A). The age of onset of increased testis volume in North American boys ranges from 9–14 yr and represents the earliest sign of male puberty, occurring before systemic androgenic manifestations. Histologically, at puberty there is active proliferation of interstitial fibroblasts, the Leydig cell precursors, and enlargement of the seminiferous tubules with maturation of the germinal epithelium.

In the fetus, the number of Leydig cells and their activity are maximal during the 11th to 17th wk of gestation, a time when their main stimulus is hCG rather than LH (*see* Fig. 2), and then decline until term. At birth, the Leydig cells are sparse, but active. After the first 6 mo of extrauterine life, this activity declines, remaining low until the onset of puberty. Plasma testosterone levels reflect these changes in biosynthetic activity. They are relatively elevated during the first months of life, declining by 7 mo and remaining at low values until the initiation of prepubertal gonadal growth (at about 11 yr of age).

Testosterone is the main androgen secreted by the adult testis; other steroids such as dihydrotestosterone, androstenedione, and estradiol are also secreted. During prepubertal years, androstenedione is an important secretory product. Between 10 and 17 yr of age there is a 20- to 30-fold increase in plasma testosterone (Fig. 6B) accompanied by more rapid phallic growth and the appearance of axillary and pubic hair. Plasma testosterone levels increase in response to the pubertal, sleep-associated increase in LH secretion (Fig. 7). testosterone has considerable influence on the growth and maturation of seminiferous tubules, acting synergistically with FSH and, as such, is involved in the earliest physical changes in male pubescence. As in the case of FSH and LH, after age 17 no further change occurs in mean testis size, secondary sexual characteristics, or plasma testosterone concentrations.

Whereas androgens are produced by the Leydig cells of the testis, Sertoli cells in the seminiferous

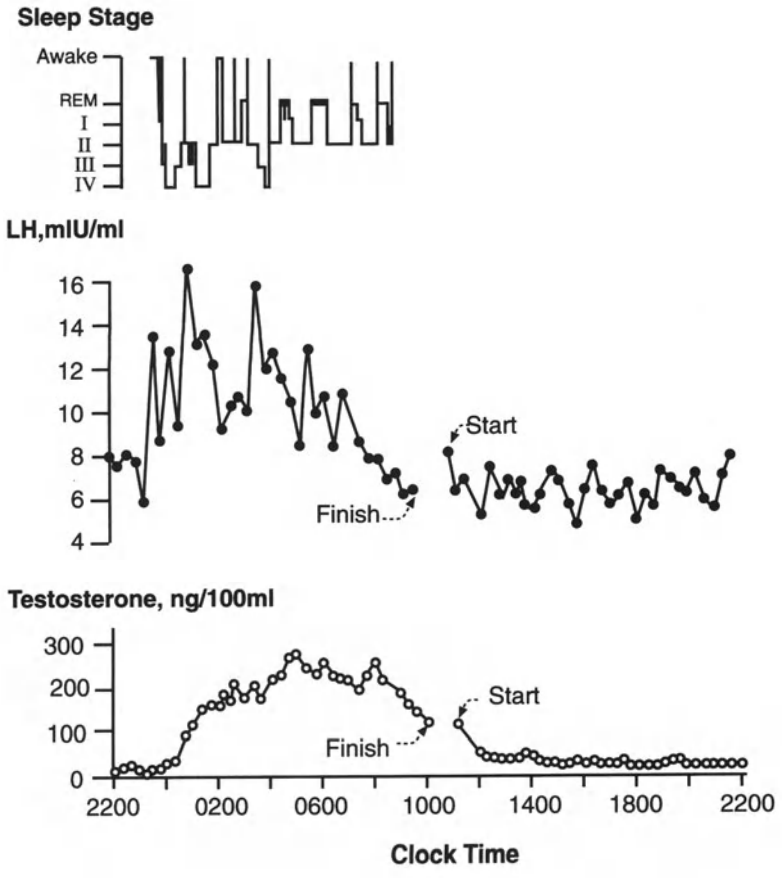

Fig. 7. Diurnal changes in plasma LH and testosterone during early human puberty. The subject studied was a 14 yr-old boy in pubertal stage 2. Blood samples were drawn every 20 min. The histogram above the period of nocturnal increase in LH levels depicts the different sleep stages observed during the period of study. Each sleep stage is identified with a roman numeral and the stage of REM (rapid eye movement) by a thicker horizontal line. Notice that rise in testosterone begins about 30 min after the initial increase in LH levels. Reproduced with permission from Boyar et al., *J Clin Invest* 1974; 54:609–618.

tubules produce inhibin, a heterodimeric glycoprotein that selectively inhibits FSH secretion from the anterior pituitary. Inhibin is a member of the transforming growth factor β superfamily that include proteins such as activin and Mullerian-inhibiting substance; it is composed of an alpha subunit and one of two beta subunits. Synthesis of the resulting molecules, known as inhibin A and inhibin B, is stimulated by FSH. Both alpha and beta subunits are already expressed by 16 wk gestation, but the level of expression declines after the first four mo of birth. Serum inhibin B levels increase significantly between juvenile development and the initiation of puberty, likely as a consequence of FSH stimulation. The increase in inhibin B coincides with the pubertal changes in serum LH and testosterone that occur at this time.

3.2.2. The Ovaries

The prepubertal ovary demonstrates morphologic signs of activity, such as follicular maturation to the stage of antral follicle and subsequent follicular atresia. The cellular residue of atretic follicles contributes substantially to the mass of the human ovary, which increases rectilinearly from birth to puberty. Steroidogenic capability is present from birth. Estradiol is the major ovarian secretory product although estrone, androstenedione, testosterone, and other steroids are also produced. Estradiol is responsible for the development of secondary sexual characteristics in the female, that is, growth and development of breasts and reproductive organs, fat redistribution, and bone maturation. Circulating estradiol levels are very low in prepubertal girls and begin to rise around 10 yr of age, reaching a plateau by age 14 (Fig. 8). Interestingly, umbilical artery levels of estradiol (that reflect fetal production of the steroid) are more than 100 times higher than in adult females despite considerable metabolism of estrogen by the fetus. Estrogen levels decline rapidly in the first postnatal week and then remain at less than 10 pg/mL until the onset of puberty.

Ovarian production of inhibin is negligible during fetal life, and remains low after birth, until early puberty, at which time serum levels of both inhibin A and B increase significantly.

Recent studies have provided evidence for the concept that ovarian development is also regulated by direct neural influences that arrive at the ovary via

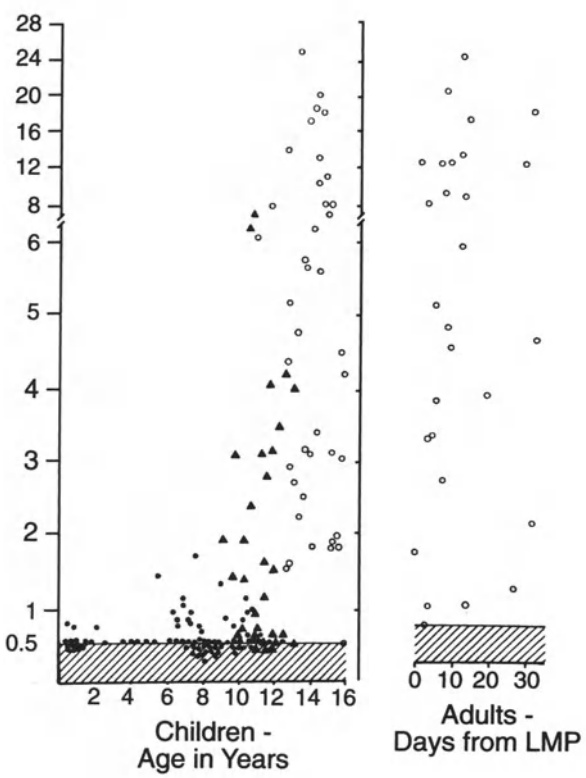

Fig. 8. Serum estradiol levels in human females at different postnatal ages during prepubertal development and during the adult menstrual cycle. The shaded area represents the limit of sensitivity of the assay; ● = prepubertal; ▲ = premenarcheal with breast development; ○ = postmenarcheal. Values for the 16- to 25-yr-old subjects are plotted on the right as days from the last menstrual period (LMP). From Faiman and Winter, In: Grumbach MM, Grave GD, Mayer FE, eds, *The Control of the Onset of Puberty.* New York: Wiley, 1974: 32–35.

the extrinsic innervation. Ovarian follicles are innervated by sympathetic and sensory nerve fibers, which also innervate the interstitial and vascular components of the gland. That neurotransmitters contained in ovarian nerves can affect ovarian steroidogenesis has been shown by studies in which norepinephrine (NE) and vasoactive intestinal peptide (VIP) were found to stimulate steroid secretion, including progesterone and androgens in the case of NE, and also estradiol in the case of VIP. Other experiments in rodents have demonstrated that elimination of the sympathetic innervation of the ovary during early development leads to stunted follicular development, reduced steroidal responsiveness to gonadotropins, delayed puberty, and inability of the ovary to maintain a normal pattern of cyclicity. Thus, it appears that the ovary acquires reproductive competence under the concerted influence of hormonal and neural inputs.

3.3. Metabolic Hormones: Growth Hormone, Insulin-like Growth Factor-I, Leptin

3.3.1. Growth Hormone (GH)

Circulating GH levels increase during puberty in both girls and boys. This increase is to a significant extent caused by the rising gonadal steroid levels that accompany the pubertal process. The steroid responsible for the stimulation of GH secretion in both boys and girls is estradiol. Androgens stimulate GH secretion after conversion to estrogen via peripheral aromatization. GH is secreted episodically throughout life. The amplitude, but not the frequency, of these secretory episodes increase at puberty or following androgen administration. The pubertal increase in GH secretion occurs earlier in girls than in boys, at the time of breast development in girls and during phase 4 of genital development (see below) in boys. That the pubertal increase in GH secretion may contribute to facilitating the progression of the pubertal process is suggested by the delayed puberty observed in individuals with isolated GH deficiency, which often resolves after treatment with GH. Studies with rodents have, in fact, demonstrated that experimental induction of GH deficiency results in delayed puberty.

3.3.2. Insulin-Like Growth Factor (IGF-I)

Serum IGF-I levels increase at puberty in all mammalian species thus far examined. In humans, serum IGF-I values begin to rise during late juvenile development, and in both sexes reach maximal values after the peak of pubertal growth spurt (Fig. 9). The pubertal increase in serum IGF-I levels is, to a significant extent, because of an indirect effect of gonadal steroids exerted via stimulation of GH release. However, data from experimental animals indicate that part of the increase occurs via a gonadal-independent mechanism involving the hypothalamus. IGF-I not only stimulates LHRH release in vitro, but also accelerates the initiation of puberty upon intracerebral administration to laboratory animals in vivo. These actions have led to the suggestion that IGF-I is one of the metabolic signals long suspected to play a role in facilitating the onset of puberty in mammals.

3.3.3. Leptin

Leptin is a cytokine-like protein produced by adipocytes that acts on the hypothalamus to induce satiety. Leptin also acts centrally to promote sympathetic activity and energy expenditure, and importantly, it affects the neuroendocrine reproductive system by

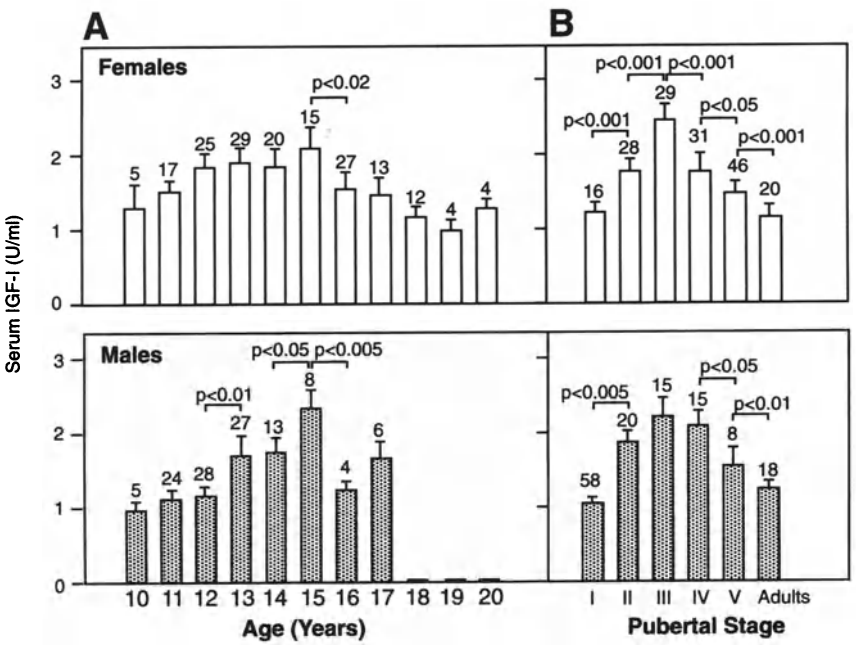

Fig. 9. Changes in plasma IGF-I levels in human males and females at different chronological peripubertal ages **(A)** and in relation to their pubertal stage **(B)**. In both sexes, peak levels are attained at midpuberty, i.e., Tanner stage 3. Notice that the first significant increase occurs between the late juvenile phase (Stage 1) and the initiation of puberty (Stage 2). Reproduced with permission from Grumbach MM and Styne DM, In: Williams, Foster, Kronenberg, Larsen, Zorab, eds. *Williams Textbook of Endocrinology,* Philadelphia, PA: WB Saunders, 1998:1509–1625.

stimulating LHRH and LH release. Because of these actions and the finding that administration of leptin to leptin-deficient mice reversed the infertility of these animals it was postulated that leptin is a major metabolic trigger of puberty. This hypothesis, however, has not been confirmed by detailed studies in monkeys and humans, which failed to show a correlation between circulating leptin levels and early pubertal maturation. Although there is an increase in serum leptin levels during early puberty, the changes detected at this time and throughout both male and female puberty are moderate (Fig.10), and better correlated with changes in body fat and age than with pubertal progression. It is now believed that leptin plays a permissive role in the process by providing to the hypothalamus information concerning the status of energy stores in the body.

4. SOMATIC CHANGES AT PUBERTY

The maturation of the hypothalamic-pituitary-gonadal axis during puberty leads to an increase in the production of sex steroids and results in secondary sexual characteristics, a pubertal growth spurt, and fertility. In girls, puberty begins when ovarian estrogens lead to breast development, the onset of which is termed thelarche. The first sign of puberty in a boy is testicular enlargement; testicular androgens then lead to further genital growth. During this maturation of the gonadal axis, there is also a maturation of the adrenal axis. The zona reticularis of the adrenal gland grows and there is an increase in adrenal 17,20-lyase activity, which results in enhanced androgen (dehydroepiandrosterone and androstenedione) production in response to the pituitary hormone, adrenocortico-

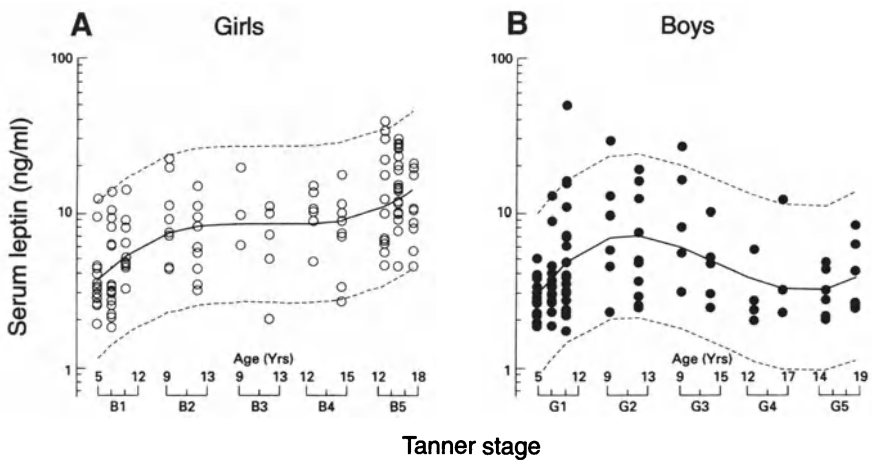

Fig. 10. Changes in plasma leptin levels in human males and females at different pubertal stages. Tanner stages B1-B5 in females refer to breast development, and G1-G5 in males refer to genital development. Notice the minor changes in leptin concentration at the initiation of puberty, i.e., between the pubertal stages 1 and 2. Redrawn with permission from Clayton et al., *Clin Endocrinol* 1997; 46:727–733.

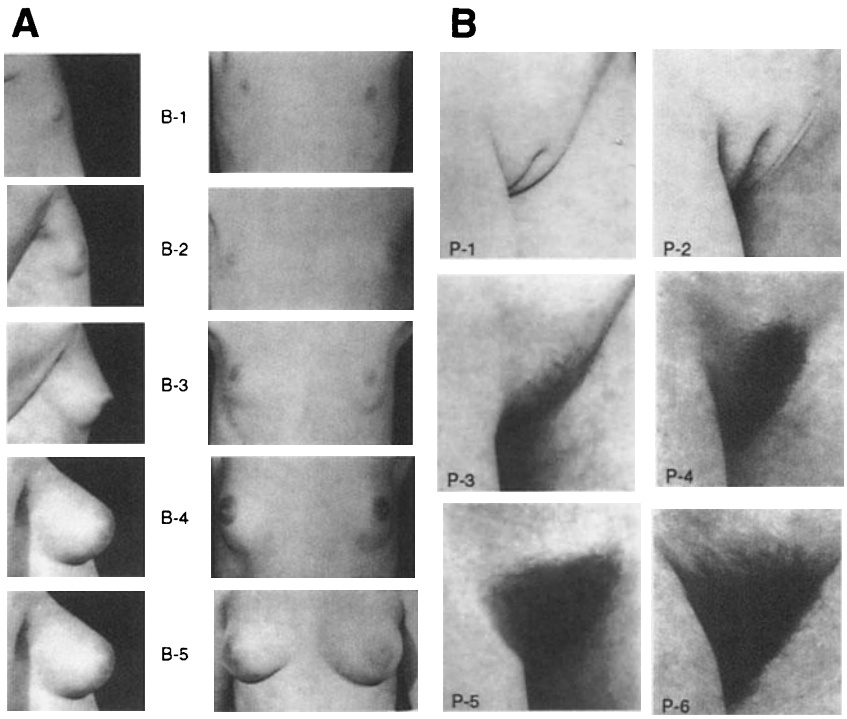

Fig. 11. Physical changes in breast development **(A)** and pubic hair **(B)** currently used to assess the stage of sexual maturation in girls. The changes occur in five stages, termed Tanner stages B1 to B5 (B = breast) and P1 to P5 (P = pubic hair). Some authors add a P6 stage for women showing a further upward extension of hair growth. The specific changes in breast development and pubic hair that occur at each stage are described in detail in the text. Reproduced with permission from Van Wieringen JC, et al. *Growth Diagrams,* 1965 Netherlands: Second National Survey on 0–24 year olds. Netherlands Instit Prevent Med TNO. Groningen:Wolters-Nardhoff, 1971.

tropin hormone (ACTH). Adrenal androgens are responsible for the development of pubic hair or pubarche. The increase in adrenal androgen production is called adrenarche.

4.1. Tanner Stages

This commonly used classification scheme was first proposed in 1962 by J.M. Tanner. He used specific details of the physical examination to divide the progression of secondary sex characteristics into separate independent stages. Figs. 11 and 12 illustrate these stages. The sequence of somatic events that occur at puberty in both males and females is depicted in Fig. 13.

4.1.1. Females

Tanner's classification proposes five stages of breast and pubic hair development. They are as follows:

Breast development (*see* Fig. 11A):

B1: prepubertal; no breast tissue.

B2: onset of puberty; breast buds form as small mounds with enlargement of areolar diameter, which may be unilateral and/or tender at first. This stage is also known as thelarche.

B3: further enlargement of breast and areola, but with no separation of their contours. Breast tissue extends beyond the areola.

B4: further enlargement of breast with protrusion of areola above the breast to form a secondary mound. Areola and nipple pigmentation appear, and nipple papilla diameter increases to greater than 3–4 mm. Some breasts skip this stage, or it is very transient.

B5: mature breast; areola and breast again in the same plane. Nipple papilla have a final dimension of about 9 mm.

Note: The final size and shape of the breasts are determined by genetic and nutritional factors.

Pubic hair development (*see* Fig. 11B):

P1: prepubertal; no pubic hair.

P2: onset of adrenarche; few straight, long, pigmented hairs appear, chiefly along the labia. This stage is also known as pubarche.

P3: darker, coarser, curlier hair appears sparsely over the mons.

P4: thick, adult-type hair appears, covering the mons only.

P5: mature pattern; thick, adult-type hair in inverse triangular distribution with extension to internal sides of upper thighs. Some women have hair continuing up the linea alba.

Girls also undergo subtle changes in the appearance of their genitalia: the thin, shiny, reddish vaginal mucosa becomes duller, thicker, and a lighter pink.

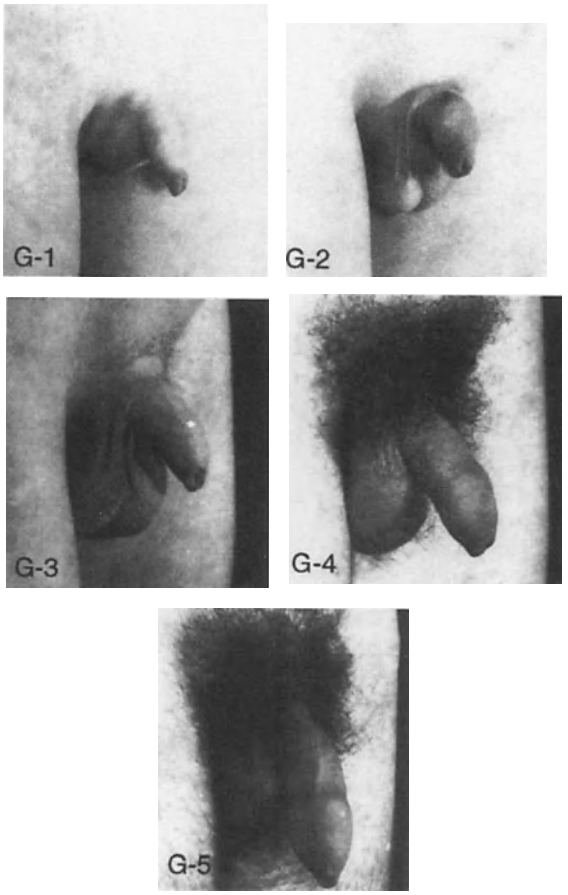

Fig. 12. Physical changes in male genital and pubic hair development currently used to assess the stage of sexual maturation of developing boys. The changes occur in five stages, termed Tanner stages G1 to G5 (G = genital) and Pl to P5 (P = public hair). The specific changes in genital development that occur at each stage are described in detail in the text. Reproduced with permission from Van Wieringen JC, et al. *Growth Diagrams,* 1965 Netherlands: Second National Survey on 0–24 year olds. Netherlands Instit Prevent Med TNO. Groningen:Wolters-Nardhoff, 1971.

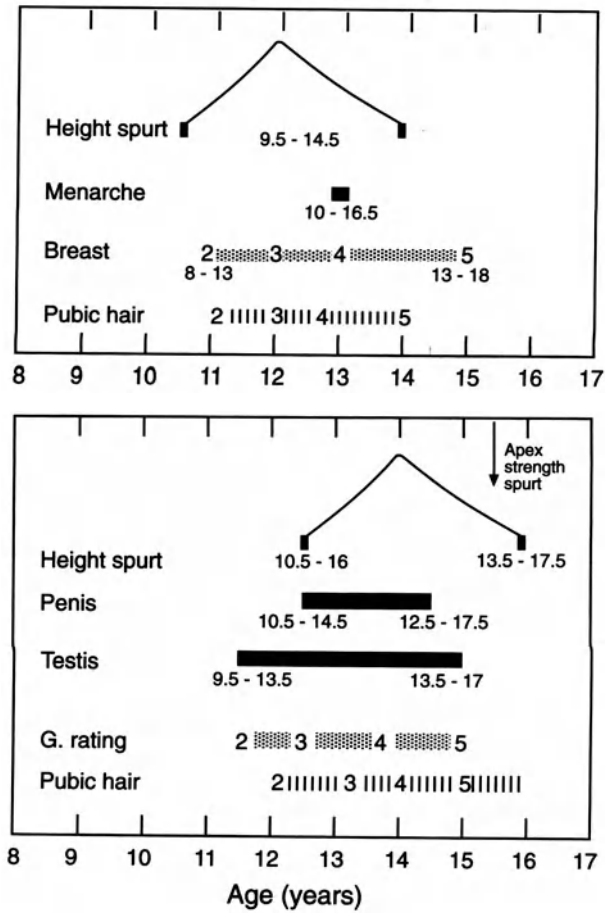

Fig. 13. Schematic sequence of somatic maturational changes at puberty. The range of ages within which each event may begin and end is given by the figures placed directly below it, indicating both the start and finish of the event. Reproduced with permission from Tanner, In: Grumbach, Grave, Meyer, eds. *The Control of the Onset of Puberty,* New York: Wiley, 1974:448–470.

The labia majora and minora become thicker and more rugated, the clitoris enlarges slightly, and there is fat deposition under the mons pubis. The urethral opening becomes more prominent, and the vagina increases in length from about 8 cm prepubertally to about 11 cm at menarche. The pelvis enlarges and tips forward such that the position of the vulva changes from being vertical to more progressively horizontal. Menarche generally occurs between Tanner stages 4 and 5 (Fig. 13), at an average age of 12.8 yr in Caucasian-American girls and 12.2 yr in African-American girls. The first cycles are anovulatory, with about 5 yr passing until regular ovulation occurs during each cycle.

4.1.2. Males

As in females, Tanner's classification proposes five stages of pubertal development, which in this case refer to both the genitals and pubic hair.

Genital development (*see* Fig. 12):

G1: prepubertal; testes less than 2.5 mL, scrotum, and penis prepubertal.

G2: onset of puberty; enlargement of testes to greater than 2.5 mL; scrotum enlarges with the skin thinning, reddening, and more rugated in texture. Penis does not change.

G3: scrotum and testes continue to grow (8–10 mL), penile growth occurs mainly in length. Spermarche (presence of sperm in the urine) occurs between this stage and the next.

G4: testes continue to grow (10–12 mL), scrotum is larger with further darkening of the skin, penis is further enlarged in length and breadth with development of the glans.

G5: mature genitalia; testes size greater than 15 mL. Right testis is usually larger than left and located higher in the scrotum.

Pubic hair development (*see* Fig. 12):

P1: prepubertal; no pubic hair.

P2: few straight, long, pigmented hairs appear, chiefly at the root of the penis or only on the scrotum.

P3: darker, coarser, curlier hair appears sparsely over the root of the penis.

P4: thick, adult-type hair appears, covering the base of the penis.

P5: mature pattern; thick, adult-type hair in inverse triangular distribution with extension to internal sides of upper thighs. Most men will have further spread up the linea alba and down the medial thighs.

Up to 60–70% of normal boys will have a small degree of breast development known as gynecomastia. It is usually less than 3 cm in diameter and disappears spontaneously within 2 yr.

4.2. Other Somatic Changes

4.2.1. Skeletal Development/Bone Age

Skeletal maturation is assessed by the morphological appearance of the bones on radiographs. Bone ossification centers appear at different ages in childhood. Also, the size and shape of the bones change as they grow, and the epiphyseal growth plates mature and eventually fuse. This epiphyseal fusion is dependent on estrogen. There are published standard radiographs for the appearance of ossification centers in the left hand and wrist at different ages; the radiograph of an individual child is compared to these standards to estimate his/her bone age. Bone age can be different from the chronological age, either advanced or delayed, but bone ages within two standard deviations of chronological age are considered to be within normal limits. Bone mineral density also increases during puberty and peaks at age 17.5 in boys and age 15.8 in girls, i.e., after the peak height velocity has occurred (*see* Fig. 13).

4.2.2. Growth Spurt

Boys and girls undergo a pubertal growth spurt after a prepubertal growth slowdown (Fig. 14). Boys have a late peak height velocity during Tanner stage 3 to 4. Girls have this peak velocity at an average of two years earlier, between Tanner stages 2 and 3 (Fig. 13); some girls will even have this height spurt before thelarche. Growth slows down after the spurt, and girls generally only grow 5 to 7.5 cm more after menarche. Boys are taller than girls when they begin the pubertal spurt, and they have a greater gain in height (about 28 cm versus about 25 cm) during the spurt. This contributes to the greater final height of males than females. Adult men are an average of 12.5 cm taller than adult women.

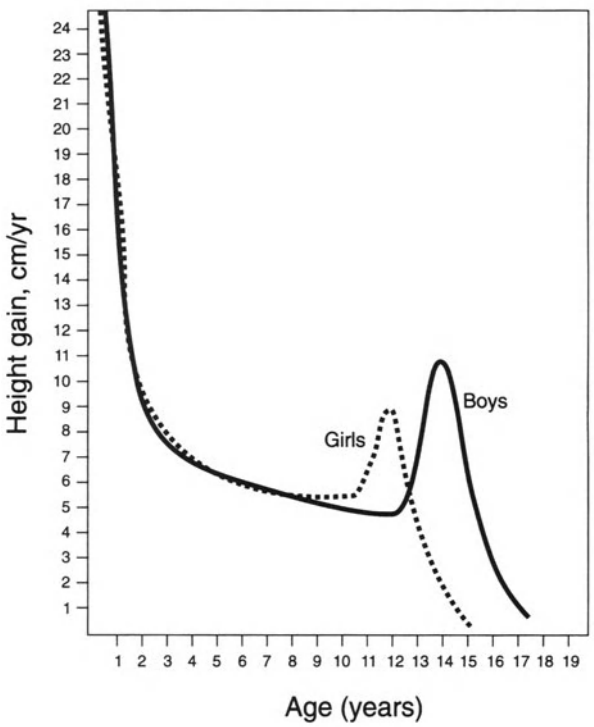

Fig. 14. The adolescent growth spurt in boys and girls. Notice the marked deceleration of height gain that follows the initial postnatal phase of rapid growth, and the reacceleration of height gain ("growth spurt") at the time of puberty. Also notice that the growth spurt occurs two years earlier in girls than in boys. Reproduced with permission from Tanner, In: Grumbach, Grave, Meyer, eds. *The Control of the Onset of Puberty,* New York: Wiley, 1974:448–470.

Among the gonadal sex steroids, estrogen seems to be the most important in regulating GH secretion and bone maturation. Estrogen augments GH secretion from the pituitary and acts locally on the bone and cartilage to stimulate IGF-1 production and maturation of the epiphyseal plates. Testosterone stimulates GH secretion and facilitates growth velocity via aromatization to estrogen. Nonaromatizable androgens are able to stimulate some increase in growth velocity, but do not affect GH secretion. Both GH and

the sex steroids are necessary for a normal pubertal growth spurt. It appears that GH is required at a threshold level, but that the extent of the pubertal growth spurt is proportional to the level of sex steroids. An important clinical point is that a young child with GH deficiency and coexisting precocious puberty, a not infrequent occurrence in children who have undergone brain irradiation, may grow at a "normal" rate, precluding identification of the GH defect unless they are carefully examined for pubertal changes. Children with just a sex-steroid deficiency will not have a growth spurt, but will eventually grow to a normal adult height because of the delay in the closure of their epiphyseal growth plates. The importance of estrogen on epiphyseal growth plate maturation is demonstrated in patients who either do not respond to estrogens or cannot make estrogen from androgens. They exhibit osteopenia and very delayed bone ages; for example, a 24-yr-old man with one of these defects had a bone age of 14.5 yr and was still growing. Growth in the absence of epiphyseal fusion leads to abnormal "eunuchoid" body proportions with long arms and legs and an upper to lower body ratio of less than 0.9.

4.2.3. Body Composition

Before puberty, boys and girls have a similar body composition. Lean body mass, primarily because of muscle, begins to increase in early puberty in both boys and girls, but this process progresses more gradually and diminishes earlier in girls. Boys also have a greater increase in skeletal mass with longer, thicker bones. In the late stages of puberty, girls tend to have an increase in fat mass whereas boys tend to show a loss of fat. After puberty, men have about 1.5 times more skeletal and muscle mass and about half as much body fat as women.

4.2.4. Axillary and Facial Hair

Axillary hair becomes evident about 1 yr after pubic hair appears and develops through similar stages. As the axillary hair appears, the sweat glands begin to function, and an apocrine body odor becomes apparent. This occurs at about 13 yr in girls and 14 yr in boys. Facial hair usually appears in boys during pubic hair stage 3 and is only completed in development after pubic hair stage 5.

4.2.5. Behavior

Parents describe more emotional and oppositional behavior. Adolescent development normally involves separating from the family and concentrating instead on peers and personal independence. Brain function alters as concrete thinking patterns change to abstract thought processes. Sleep patterns also change with older adolescents naturally tending toward being "night owls," staying up later and sleeping in longer than their schedules generally allow.

4.2.6. Other Changes

A sexually dimorphic change associated with puberty is laryngeal growth. It consists of a dramatic increase in the length of the vocal cords in boys, but not in girls. The voice of most boys begins to break at about 13 yr, and they achieve an adult timbre by 15 yr. A pubertal change common to both boys and girls is the development of acne and seborrhea of the scalp. The condition results from increased adrenal androgen secretion.

4.3. Metabolic Signals and the Onset of Puberty

Clinical evidence has shown that the mean weight of girls at the time of initiation of the adolescent growth spurt (around 30 Kg), at the time of peak velocity of weight gain (around 39 Kg), and at menarche (around 47 Kg) does not differ for early and late maturing girls. This finding resulted in the advancement of the concept that attainment of a critical weight causes a change in metabolic rate per unit mass (or per unit of surface area) which, in turn, affects the hypothalamic ovarian feedback by decreasing the sensitivity of the hypothalamus to gonadal steroids. The resulting increase in gonadotropin release would then trigger the pubertal process. Though attractive, this hypothesis has not been supported by several reports showing no obvious correlation between body weight and the initiation of puberty. It is also clear that a change in hypothalamic sensitivity to ovarian steroid negative feedback is not a major factor involved in determining the onset of puberty (see below).

The hypothesis of a critical weight was later modified to state that a particular ratio of fat-to-lean mass is normally necessary for puberty and the maintenance of female reproductive ability. Further refinement of the hypothesis states that there is an alteration in body energy metabolism required for puberty to occur. As indicated above, there is evidence that metabolic factors may, indeed, affect hypothalamic activity and play active or permissive roles in the initiation and/or progression of puberty. Although not all (or the most critical) of these factors have been identified, there at least two peptidergic factors produced by peripheral tissues that appear to facilitate the pubertal process: IGF-I and leptin (see above). Serum levels

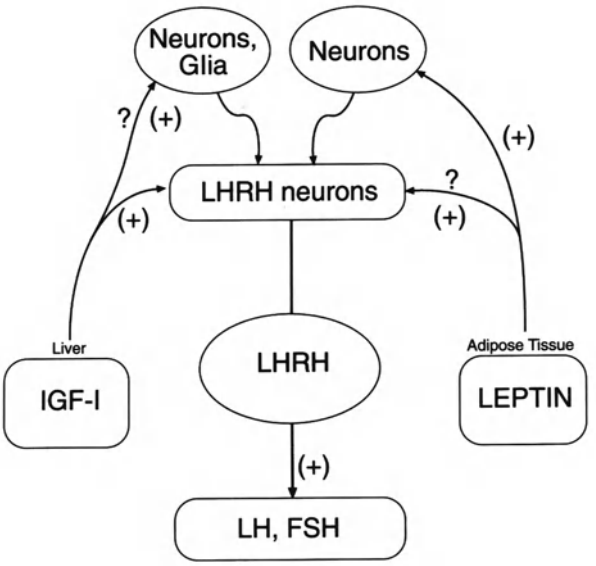

Fig. 15. Effects of IGF-I and leptin on the reproductive hypothalamus. Both peptide hormones are postulated to serve as metabolic signals affecting the onset and/or progression of puberty. Circulating levels of both peptides increase at the onset of puberty, and thus may primarily contribute to the progression of puberty. There is evidence that IGF-I can act directly on LHRH neurons to stimulate LHRH secretion. Such an action has not been reported for leptin, which appears to stimulate LHRH indirectly via neuronal networks connected to LHRH neurons. (+) = stimulation.

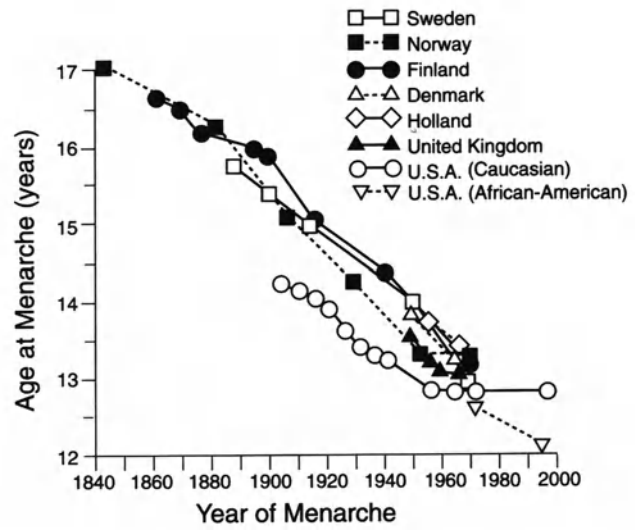

Fig. 16. The age of menarche in the United States and Western Europe has decreased since 1840, although this trend has slowed or ceased over the last 40 yr in most populations, with the exception of African-American girls. The most recent data gives an age of 12.88 yr for Caucasian-American girls (○-○) and 12.16 yr for African-American girls (▽-▽). Modified from Tanner JM, Eveleth PB, In: Berenberg SR, ed. *Puberty, Biologic and Psychosocial Components.* Stenfert Kroese-Leiden, 1975:256–273 (with permission from Kluwer Academic, Dordrecht, The Netherlands).

of both proteins increase during early puberty (Figs. 9 and 10), and both IGF-I and leptin can act on the hypothalamus to increase LHRH release (Fig.15). Despite their ability to advance puberty when administered to experimental animals, no evidence that they play a major role in triggering human puberty has been provided as yet.

5. THE TIMING OF PUBERTY

Puberty seems to be occurring at an earlier age than it did just 150 years ago. The average age of menarche has been decreasing by 2–3 mo every 10 yr for the last 100 yr in Europe and the United States until around 1960 when it stabilized at an average menarchal age of 12.8 yr, (Fig. 16). This trend is thought to be because of improvements in nutrition and general health. The timing of the pubertal growth spurt is more related to a child's bone age than to his or her chronological age.

5.1. Girls

Most American girls will begin puberty (i.e., begin breast development) at age 11, with a normal range currently described as being between 8 and 13 yr. However, a 1997 cross-sectional study of more than 17,000 healthy girls examined in private office settings suggests that puberty is occurring earlier than previously described. Tanner stage 2 breast development was noted at a mean age of 9.96 ± 1.82 yr in Caucasian girls and at a mean age of 8.87 ± 1.93 yr in African-American girls. This is about 1 yr earlier than previously described for Caucasian girls and about 2 yr earlier than previously described for African-American girls. Also earlier is the development of Tanner Stage 2 pubic hair, at a mean age of 10.51 ± 1.67 yr for Caucasian girls and 8.78 ± 2.0 yr for African-American girls. Importantly, at 7 yr of age, a time previously defined as prepubertal, 15.4% of African-American girls and 5.0% of Caucasian girls had Tanner Stage 2 breast development. Also at this age, 17.7% of African-American girls and 2.8% of Caucasian girls had at least Tanner Stage 2 pubic hair. The average age of menses in Caucasian girls (12.88 yr) was not significantly different from values reported in previous studies. However, in the case of African-American girls, there was a further advancement in the age at menarche (from 12.5 yr in 1970 to 12.16 yr in 1997).

Girls progress from Tanner stage 2 to 5 in about 4.2 yr and from Tanner stage 2 breast development

to menarche in 2.3 ± 1.0 yr. Girls who enter puberty at a younger age tend to have a longer period of time before menarche, 2.77 yr for girls who start puberty at 9 yr of age, and 1.44 yr for girls who start puberty at 12 yr of age.

5.2. Boys

Boys show the first signs of puberty (i.e., testicular enlargement) later than girls, at an average age of 11.5 yr. The normal range is from 9 to 14 yr of age. They progress from Tanner stage 2 to 5 in about 3.5 yr.

6. THE NEUROENDOCRINE CONTROL OF THE ONSET OF PUBERTY

A hormonal change required for the attainment of puberty is an increase in the pulsatile secretion of gonadotropins. This increase, which is initiated during sleep at the end of childhood, is not an independent phenomenon intrinsic to the pituitary gland. The pubertal changes in gonadotropin secretion are, instead, caused by corresponding changes in episodic LHRH release from the hypothalamus. Studies in rhesus monkeys have shown that during early puberty, the amplitude of episodic LHRH secretion increases, stimulating the pituitary gland to secrete more gonadotropins (Fig.17). In turn, gonadotropins stimulate the gonads to produce steroids, which are responsible for the development of the secondary sex characteristics in both males and females. Gonadotropins also stimulate the release of gonadal peptides, such as inhibin. Both steroids and peptides feedback on the hypothalamic-pituitary unit to maintain gonadotropin secretion in check. Gonadal steroids can inhibit gonadotropin secretion by acting directly on the pituitary gland, or by inhibiting LHRH release via an effect exerted on neuronal circuits controlling LHRH neurons. Estrogen, on the other hand, induces the preovulatory surge of gonadotropins by acting both on the pituitary and on the neuronal and glial networks controlling LHRH release. Figure 18 depicts the hierarchical arrangement of the different components of the neuroendocrine reproductive axis at puberty.

LHRH neurons appear to have an intrinsic capacity to release LHRH in bursts of secretion. Examination of transformed mouse LHRH neurons in vitro revealed that they can generate pulses of LHRH secretion at a frequency very similar to that observed in intact animals, in the absence of any input from extrinsic neuronal or astroglial cell systems. It is also clear that, in vivo, the LHRH neuronal network does not remain quiescent before puberty. On the contrary,

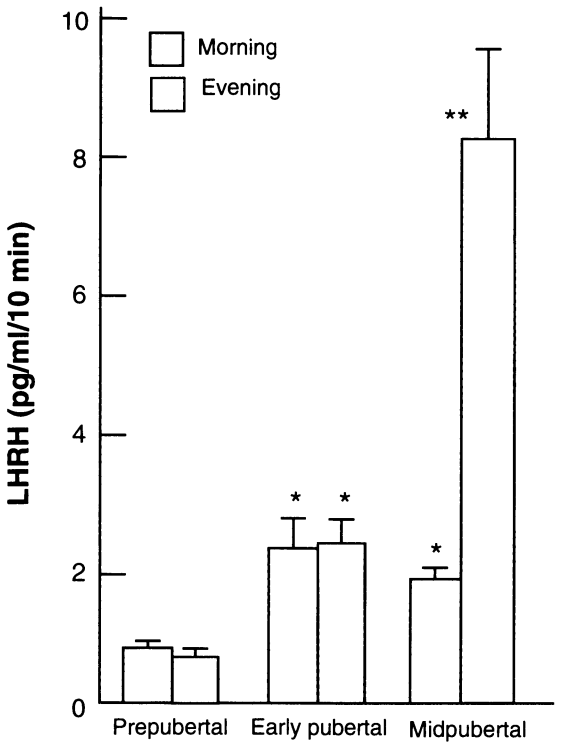

Fig. 17. LHRH release from the hypothalamus increases at puberty. The decapeptide was detected in the median eminence of conscious rhesus monkeys subjected to a microperfusion technique. The values depicted derive from six prepubertal, seven early pubertal and six midpubertal animals. Notice that LHRH release increases significantly at the initiation of puberty. * = significantly greater than values in prepubertal monkeys; ** = significantly greater than morning values. Drawn with permission from Watanabe and Teresawa, *Endocrinology* 1989; 125:92–99.

once the neurons arrive at their final hypothalamic destination during fetal life, they begin stimulating the pituitary secretion of gonadotropins. In humans, gonadotropin secretion, and presumably LHRH output, become maximal by 150 days of gestation. Upon the activation of gonadal negative feedback (see above), gonadotropin secretion decreases toward the end of gestation, to increase again during the first few months of postnatal life upon removal of placental steroids. Examination of the pattern of LH release during this phase of life in gonadectomized rhesus monkeys demonstrated that in the absence of gonadal negative feedback, gonadotropin secretion is not only pulsatile, but also exhibits a periodicity very similar to that of adult animals (Fig. 19).

During juvenile development, the activity of the LHRH "pulse generator" decreases markedly, to increase again at the time of puberty. In the human, episodic LHRH release (and consequently, pulsatile

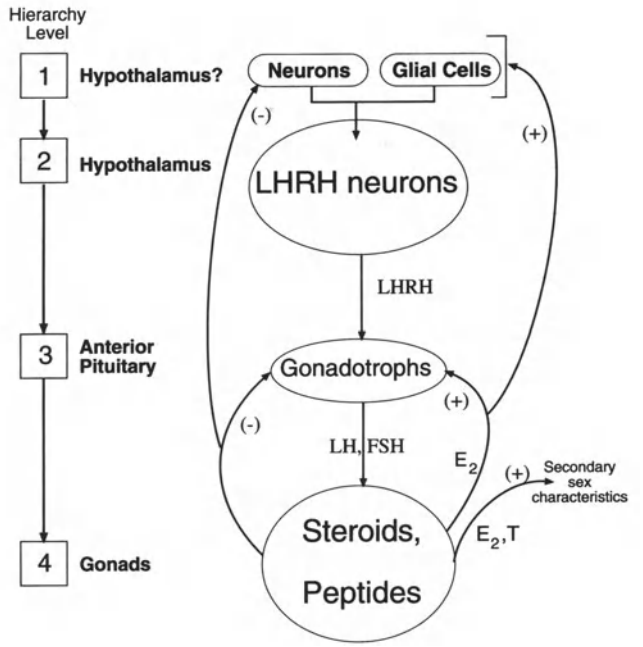

Fig. 18. The hierarchical arrangement within the hypothalamic-pituitary-gonadal axis at the time of puberty. Puberty is initiated by events that take place within the central nervous system **[1]**. These events result in an increased pulsatility of LHRH **[2]**, which leads to an increased secretion of pituitary gonadotropins (LH and FSH) **[3]**. Gonadotropins, in turn, stimulate gonadal development and production of gonadal steroids **[4]**, which are responsible for the development of secondary sex characteristics, and control gonadotropin secretion via negative-feedback loops-acting on both the neurons controlling LHRH secretion and pituitary gonadotropins. Estrogen triggers the first preovulatory surge of gonadotropins (+) by acting on both the neuronal and glial networks controlling LHRH secretion and directly on pituitary gonadotropins.

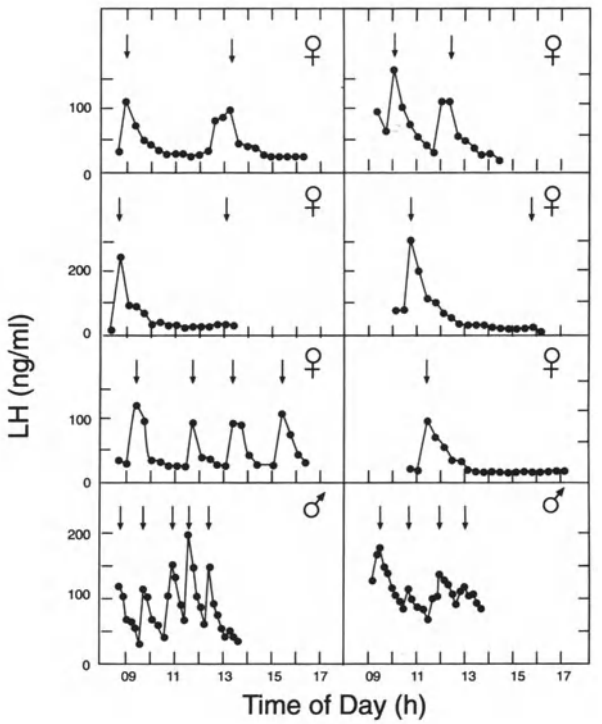

Fig. 19. The release of LH from the pituitary gland is pulsatile long before the initiation of puberty. The results illustrating this concept derive from six infantile female rhesus monkeys (top three panels) and two infantile males (lower panel) (4–7 wk of age) that were bilaterally gonadectomized at 1 wk of age. Notice that the frequency of LH pulses (denoted by arrows), and presumably therefore of LHRH discharges, is markedly slower in females than in males. Drawn with permission from Plant T, *Endocrinology* 1986; 119:539–545.

gonadotropin release) remain low for almost a decade, before being reactivated at puberty. This hiatus in LHRH/gonadotropin secretion is, to a significant extent, a gonadal-independent event as it also occurs in individuals born without gonads (Fig. 20), and in monkeys gonadectomized at birth to simulate the human pathology of gonadal dysgenesis. Thus, LHRH secretion decreases during childhood and is reactivated at puberty in the absence of gonadal inputs, indicating that the factors responsible for these changes must reside within the CNS.

Because it is now evident that LHRH neurons are not a limiting factor for puberty to occur, investigators have turned their attention to the cellular systems that, operating within the CNS, control the secretory activity of the LHRH neuronal network. Two main hypotheses have been put forward to explain the initiation of puberty. One of them, no longer accepted, postulates that the pubertal reactivation of LHRH secretion is determined by a decrease in hypothalamic sensitivity to gonadal steroid negative feedback. The other, currently accepted, indicates that LHRH secretion augments at puberty as a consequence of an increase in a gonadal-independent "central drive." Two concepts have been advanced to explain the intrinsic mechanisms responsible for the manifestation of this central event. One of them states that the LHRH neuronal network is actively inhibited during childhood, and that the pubertal activation in LHRH secretion results from a loss of this "central restraint." The other hypothesis indicates that the juvenile hiatus in LHRH secretion is because of a reduction in excitatory inputs to LHRH neurons, and that the pubertal activation of LHRH release results from an increase in such excitatory influences (Fig. 21). Each of these hypothesis is explained in detail below.

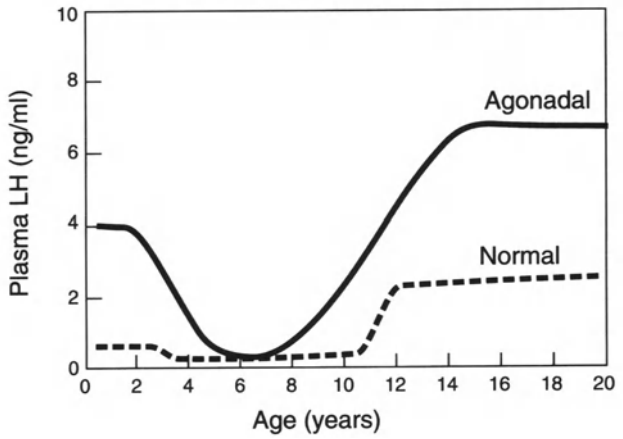

Fig. 20. Changes in circulating gonadotropin levels during postnatal development in normal and agonadal human females. Notice that gonadotropin levels in the agonadal subjects decrease during childhood and increase at puberty similar to normal subjects, indicating that these changes occur independently from the gonads. Redrawn with permission from Grumbach, In: Krieger DT, Hughes JC, eds. *Neuroendocrinology,* Sunderland, MA: Sinauer, 1980:249–258.

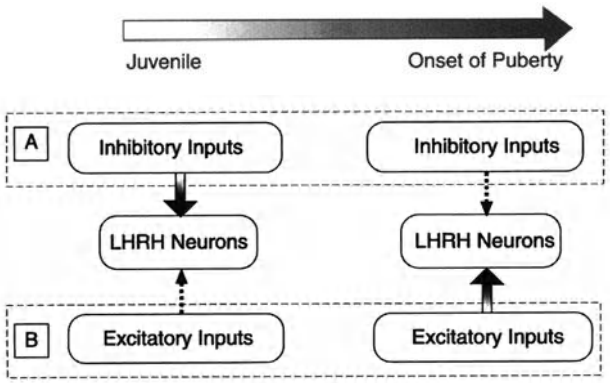

Fig. 21. Current hypotheses concerning the central mechanism(s) responsible for setting puberty in motion. **(A)** Episodic LHRH secretion is postulated to be tonically restrained during childhood by inhibitory transsynaptic inputs; puberty is thereby initiated because of the loss of this central restraint. **(B)** LHRH secretion is postulated to be low during childhood because of the lack of excitatory inputs (direct and/or indirect) to LHRH neurons; puberty is initiated because there is an increase in these stimulatory influences.

6.1. Change in Setpoint of Negative Feedback Mechanism

As already mentioned, the negative feedback of gonadal steroids is poorly developed during fetal life and becomes fully operative during childhood. Several years ago, studies in rats showed that the hypothalamus of prepubertal animals was much more sensitive to the negative feedback effect of circulating androgens and estrogens than that of the adult. The concept was advanced that a pubertal decrease in hypothalamic sensitivity to circulating sex steroids results in increased gonadotropin secretion. The hypothesis states that the low levels of sex steroids in the prepubertal individual suppress the release of FSH and LH. With the approach of puberty, there would be a progressive decrease in sensitivity of the hypothalamus to sex steroids that would result in increased secretion of pituitary gonadotropins, stimulation of sex steroid output and the development of secondary sex characteristics. There is, indeed, clear evidence in both laboratory animals and humans that prepubertal individuals are much more sensitive than adults to the inhibitory effect of gonadal steroids in gonadotropin secretion. Administration of gonadal steroids to humans revealed that juvenile subjects are 6 to 15 times more sensitive than adult individuals.

This different sensitivity notwithstanding, data gathered in rats, nonhuman primates, and humans themselves have shown that the "resetting of the gonadostat" does not occur before the occurrence of the first endocrine manifestations of puberty, and that, consequently, it cannot be considered as the cause of puberty. Studies in humans and rhesus macaques have clearly demonstrated that the change in hypothalamic set point to steroid negative feedback is, in fact, a late phenomenon in puberty, indicating that the "resetting" mechanism is a consequence of the pubertal process rather than its cause.

6.2. Activation of the Hypothalamic "Pulse Generator"

As mentioned earlier, pulsatile release of LH becomes more prominent at the end of childhood. If juvenile monkeys are treated with LHRH, delivered in a pulsatile manner for several weeks, they will show repetitive, normal ovulatory cycles, indicating that an increase in episodic LHRH release suffices to set in motion the pubertal process (Fig. 22). These cycles cease upon removal of the LHRH pulsatile stimulation. Also, if either rats or monkeys are subjected to the pulsatile administration of NMDA, an excitatory amino acid analog that activates a class of excitatory amino acid receptors involved in facilitating LHRH release, the onset of puberty is accelerated (Fig. 23). Hypothalamic lesions can also accelerate the pubertal process. Thus, regardless of the presence of any restraining influence, activation of CNS synaptic circuitries responsive to excitatory influences

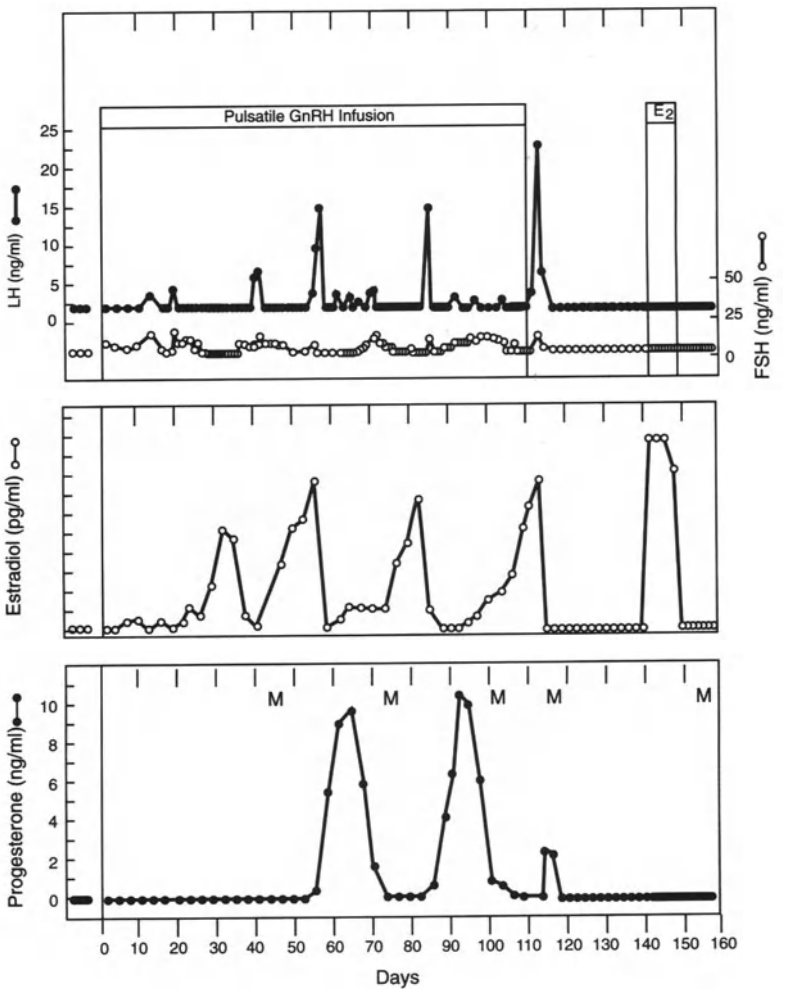

Fig. 22. Induction of puberty by pulsatile LHRH administration. Ovulatory cycles were induced in a premenarcheal rhesus monkey via chronic intermittent intravenous infusion of LHRH (one pulse per hour) initiated on day 0. Notice that the pituitary-ovarian axis reverted to a prepubertal state following termination of the LHRH treatment on day 111, and that subsequent administration of estradiol (indicated by the unshaded area labeled E_2) failed to induce a gonadotropin surge in the absence of LHRH. Menstruations are indicated by M. Reproduced with permission from Wildt et al., *Science* 1980; 207:1373–1375.

brings about the initiation of puberty. Other experiments in nonhuman primates have shown that LHRH secretion is normally inhibited by neurons that utilize gamma aminobutyric acid (GABA) as a neurotransmitter, and that the intensity of this inhibitory influence decreases at puberty (*see* Fig. 24). That GABA is a major component of an intrinsic inhibitory tone restraining LHRH release before puberty was demonstrated by experiments in rhesus monkeys in which GABA production was blocked via intrahypothalamic administration of antisense oligodeoxynucleotides against the enzymes responsible for GABA synthesis. LHRH secretion increased promptly after initiation of the oligodeoxynucleotide infusion (*see* Fig. 25), suggesting that a reduction of GABA availability leads to LHRH release, because the strength of the GABAergic inhibitory control over the LHRH neuronal network decreases. Because glutamate, the major excitatory amino acid controlling hypothalamic functions (and LHRH secretion), is the natural GABA precursor, a decrease in GABA formation would be expected to affect glutamate levels. This is indeed the case, as subsequent experiments by the same authors showed that blockade of GABA synthesis is followed by a prompt increase in hypothalamic glutamate release. It is then clear that a pure loss of inhibitory transsynaptic inputs to LHRH neurons cannot be the only (or most important) event responsible for the reactivation of the LHRH pulse generator at puberty.

Instead, this "awakening" of the pulse generator appears to depend on the occurrence of several related events within the CNS. It now appears that there are three major contributors to the pubertal activation of LHRH release, two of transsynaptic nature, and a third one involving a glial-neuronal communication pathway (*see* Fig. 26). As indicated above, one of the transneuronal events involves a decrease in the inhibitory GABAergic input to LHRH neurons. The other is an increase in transsynaptic stimulation to LHRH neurons. Glutamate, the major excitatory neurotransmitter in the hypothalamus, seems to play a predominant role in this stimulation. The recent demonstration that activation of a subclass of glutamate receptors (kianate receptors) in another brain region

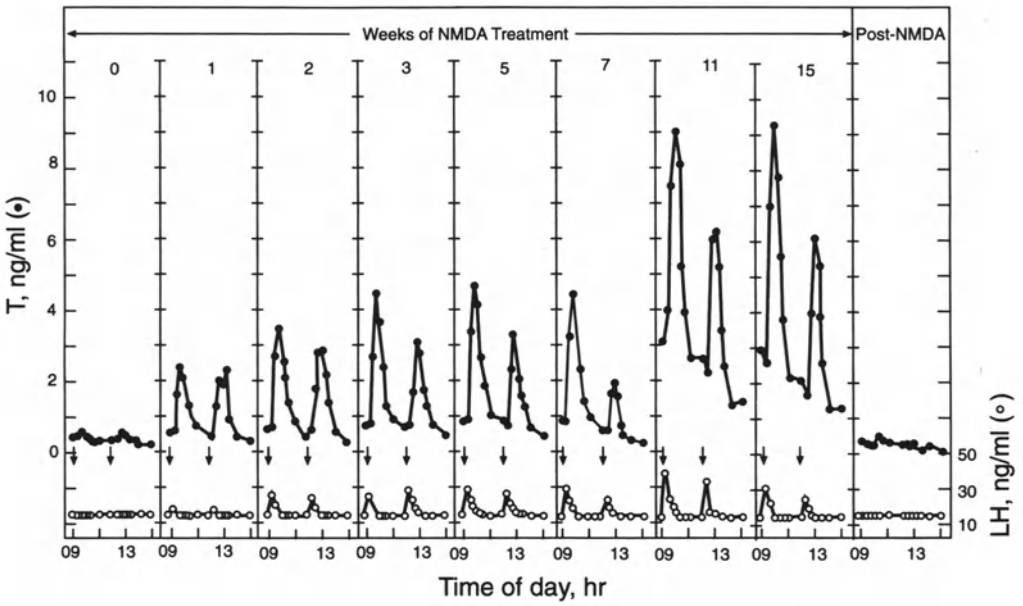

Fig. 23. Premature activation of the hypothalamic-pituitary-testicular axis in prepubertal rhesus monkeys induced by repetitive stimulation of the hypothalamus with NMDA, an excitatory amino acid receptor agonist, administered once every 3 h for 15 wk. The treatment was initiated 1.5–2 yr before the normal age of puberty in this species. The arrows indicate the time of injection. Reproduced with permission from Plant et al., *Proc Natl Acad Sci (USA)* 1989; 86:2506–2510.

results in inhibition of GABA release suggests that the pubertal decrease in GABA transmission may result, at least in part, from an increase in excitatory amino acid inputs to the hypothalamus. Additional neurotransmitters, particularly norepinephrine (NE) and neuropeptide Y (NPY), have also been implicated in the central control of the pubertal process. Studies in nonhuman primates revealed that prepubertal LHRH neurons respond briskly to NE stimulation, but become sensitive to NPY stimulation only after the initiation of puberty. Because endogenous NE release in the median eminence does not increase until midpuberty, it does not appear that activation of noradrenergic transmission is an early, primary event determining the initiation of the pubertal cascade. On the other hand, the failure of LHRH neurons to respond to NPY in early puberty suggests that like NE, an NPY-dependent activation of LHRH secretion is not an early event in puberty, but instead plays a role in facilitating the progression of pubertal maturation.

The nontranssynaptic event underlying the activation of LHRH neurons at puberty is provided by the activation of signals of glial origin, which appear to predominantly affect the release of LHRH from nerve terminals. For many years, astrocytes were considered to play a passive, supporting role in the CNS. Astrocytes were thought to provide nourishment and overall metabolic support to neurons, but not to function as true interactive partners. It is now clear that they can not only generate, but also process information within the brain. They are endowed with a variety of neurotransmitter receptors, and can release several neuroactive substances, including calcium, glutamate, and growth factors. In fact, they respond to neuronal activity with calcium waves that propagate from astrocyte to astrocyte, and that leads to the release of glutamate. In turn, both calcium (reaching neighboring neurons via gap junctional communication) and glutamate (released to the extracellular space) can act on neurons to facilitate their synaptic activity.

In contrast to their sparse innervation, which amounts to less than 2% of their cell membrane, LHRH neurons are profusely apposed by astrocytic processes. This apposition is particularly evident in the median eminence, where LHRH nerve terminals converge to release the decapeptide into the portal vasculature. During periods of reduced gonadotropin secretion, glial processes in the median eminence have been found to prevent the contact of LHRH nerve endings with the endothelial wall of the portal vessels, thereby providing a morphological mechanism for the regulation of LHRH output. These and other studies have shown that the median eminence of the hypothalamus is a major site of glial-neuronal interaction. Despite the potential importance of these morphological features, recent studies have emphasized the

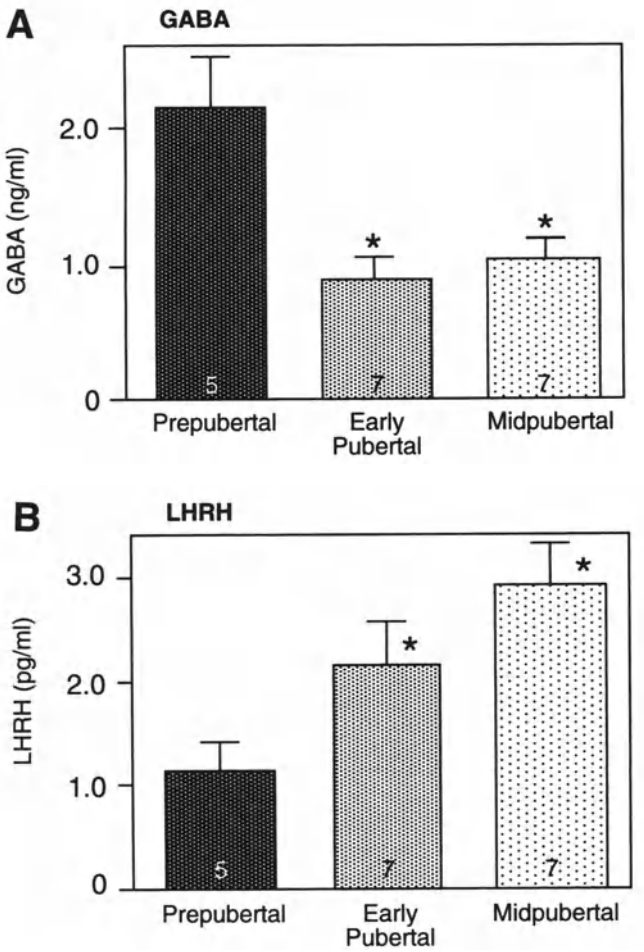

Fig. 24. Decrease in GABA release **(A)** from the rhesus monkey hypothalamus at the time of puberty. Notice that the decrease coincides with the pubertal increase in LHRH secretion **(B)**. Both GABA and LHRH were measured in perfusates collected from the median eminence of conscious animals via a device that allows the continuous perfusion and collection of fluid from the area of interest (push-pull cannula). Numbers inside each bar represent number of animals per group. * significantly different from prepubertal value. Reproduced with permission from Mitsushima et al., *Proc Natl Acad Sci (USA)* 1994; 91:395–399.

importance of a biochemical glial-to-neuron signaling pathway. Some of the signals involved in this communication have been identified as members of the epidermal growth factor (EGF) family of trophic factors, which are known to play complementary roles in the development of the CNS. In particular, transforming growth factor alpha (TGFα), and more recently, the neuroregulins (NRGs) have been shown to facilitate the pubertal process via a cell–cell communication process involving astroglial cells and specific receptors located, not on LHRH neurons, but on astroglial cells. These studies demonstrated that TGFα and NRGs activate specific sets of glial receptors, which leads to the release of prostaglandin E_2. The prostaglandin, in turn, causes LHRH release by acting directly on LHRH neurons. The importance of this glial regulatory system for the initiation of puberty was suggested by the finding, in both rats and nonhuman primates, that expression of the gene encoding TGFα in the hypothalamus increases at the time of puberty. A causal relationship was established by the finding that inhibition of TGFα or NRG actions targeted to the hypothalamus delayed puberty, whereas an increase in TGFα production near LHRH neurons advanced the process.

Thus, puberty appears to result from a concomitant increase in stimulatory transsynaptic inputs and a decrease in inhibitory influences to LHRH neurons, coupled to the increased formation of glial growth factors that stimulate LHRH release indirectly via neuroactive glial-derived substances (*see* Fig. 26). The molecular control of this neuronal/glial activation of LHRH neuronal secretory output remains to be elucidated.

Another factor that operates at the time of puberty is an enhanced pituitary responsiveness to LHRH. The release of LH following the administration of LHRH is minimal in prepubertal children, increases markedly at puberty, and is even greater in adults. There is a sex difference in the rise of serum FSH: prepubertal and pubertal females release more FSH than males at all stages of sexual maturation.

6.3. Maturation of the Positive-Feedback Mechanism

The first demonstration that estrogen can induce ovulation was done in the rat several years ago. Recently, it has become clear that in the adult human female, estrogen is also capable of stimulating gonadotropin release and through it induce ovulation. This positive feedback of estrogen is not demonstrated in prepubertal and early pubertal children, but becomes patent around midpuberty. Studies in monkeys led to the concept that the positive-feedback effect of estradiol is exerted exclusively at a pituitary level. Animals in which the hypothalamus was surgically disconnected from the pituitary responded to estradiol with a preovulatory surge of gonadotropins, provided that the gland was primed by an unvarying regime of pulsatile LHRH administration. Because a LH surge occurred in the absence of increased LHRH pulsatility, the conclusion was reached that LHRH only plays a permissive role in the genesis of the preovulatory LH surge. Inconsistent with this view is the fact that

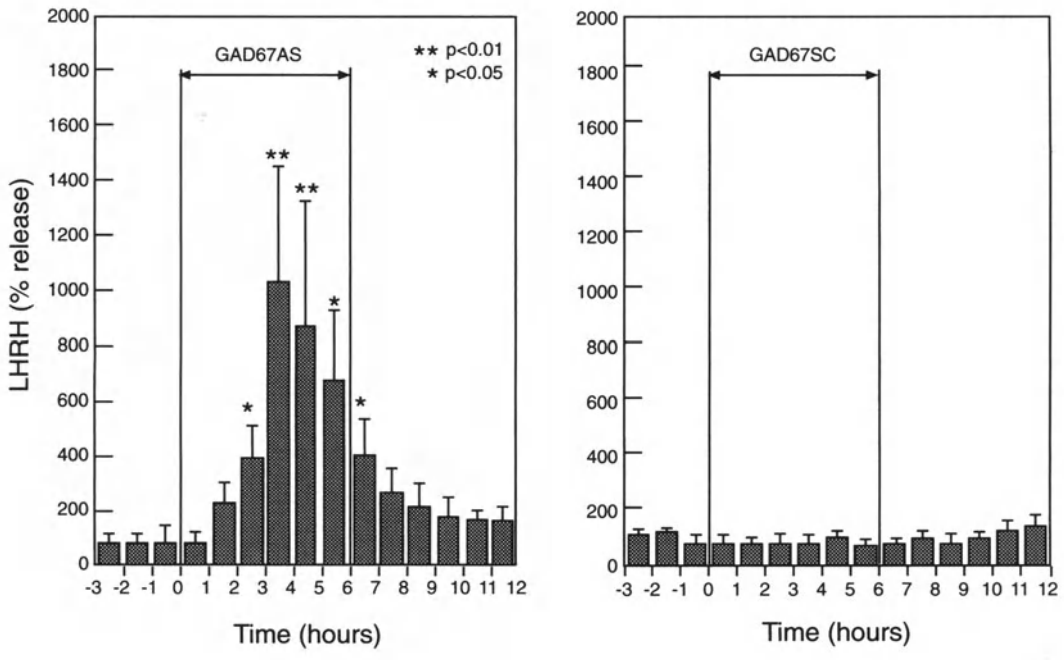

Fig. 25. Increase in LHRH release from the prepubertal monkey hypothalamus caused by blockade of GAD–67 synthesis via intrahypothalamic administration of an antisense oligonucleotide directed against GAD–67 messenger RNA . GAD–67 is one of the two key enzymes involved in GABA synthesis. GAD67AS = antisense to GAD–67 mRNA (six animals); GAD67SC = control oligonucleotide having a scrambled DNA sequence (four animals). Reproduced with permission from Mitsushima et al., *J Neurosci* 1996; 16:2562–2573.

maturation of estrogen positive feedback is a late event in puberty, occurring long after the pituitary begins to respond to LHRH in an adult-like fashion. This observation, and the findings that LHRH secretion does increase during the midcycle preovulatory surge of gonadotropins (*see* Fig. 27), and that a preovulatory dose of estrogen does indeed stimulate LHRH release from the hypothalamus of rhesus monkeys (*see* Fig. 28), indicate that an increase in LHRH release is a component of the mechanism by which estrogen triggers a preovulatory surge of gonadotropins. The late appearance of preovulatory surges of gonadotropin secretion during normal human puberty (Fig. 29) in the face of substantial pituitary responsiveness to LHRH, and the inability of estrogen to evoke an LH surge in early puberty in nonhuman primates suggest that the late manifestation of estrogen positive feedback is related to the inability of LHRH neurons to respond to this activating input until the later phases of puberty. Experiments in laboratory animals indicate that, as in the case of the initiation of puberty, manifestation of the positive feedback effect of estrogen involves the activation of neuronal circuitries stimulatory to LHRH neurons and increased formation of EGF-related growth factors in hypothalamic astroglia.

7. NEUROENDOCRINE DISORDERS AFFECTING THE ONSET OF PUBERTY

7.1. Delay of Puberty

The onset of puberty after 14 yr of age in a boy and 13 yr of age in a girl or failure to complete sexual maturation by 5 yr after the initiation of the pubertal process is considered to be abnormal. Delay of puberty can occur because of abnormalities in the hypothalamus and/or pituitary or in the gonads themselves. A defect in the hypothalamus or pituitary with abnormally low levels of gonadotropins is known as hypogonadotropic hypogonadism. If there is an abnormality in the gonads, the serum levels of gonadotropins will rise as a result of the lack of sex-steroid feedback; this is termed hypergonadotropic hypogonadism.

7.1.1. Constitutional Delay of Growth and Puberty

The most common cause for a delay in puberty, termed constitutional delay of growth and puberty or late bloomer syndrome (*see* Fig. 30), is actually considered to be a variation of the normal growth pattern. It tends to run in families, where it is often accompanied by a parental history of "late blooming."

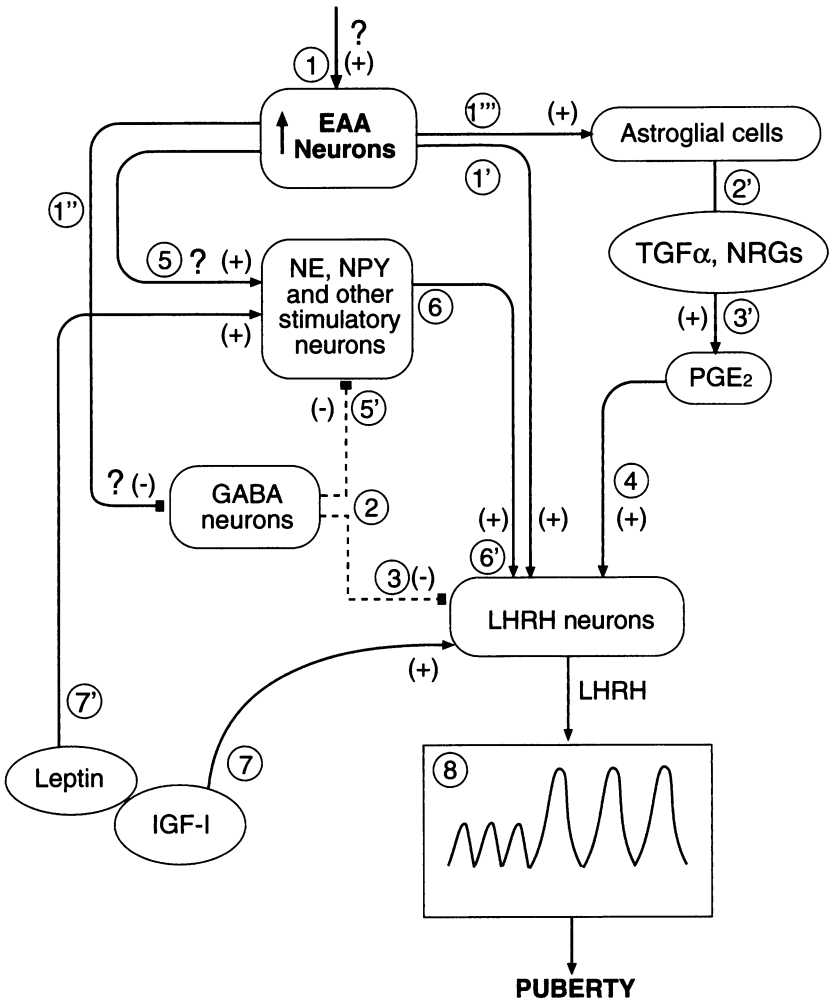

Fig. 26. The central events underlying the initiation of puberty. An increase in episodic LHRH secretion is postulated to be caused by the activation of an integrative mechanism involving both neuron-to-neuron and glia-to-neuron communication processes. The neuron-to-neuron (transsynaptic) component involves the simultaneous activation of excitatory transsynaptic inputs and a reduction in inhibitory inputs to LHRH neurons. The main excitatory transsynaptic input affecting LHRH release is postulated to be provided by neurons that use excitatory amino acids (EAA) as neurotransmitters. Noradrenergic (NE) and Neuropeptide Y (NPY)-containing neurons provide a complementary facilitatory input to the LHRH neuronal network. The chief inhibitory neuronal input restraining LHRH release is provided by GABAergic neurons (GABA). The glia-to-neuron component of the process involves the production of the epidermal growth factor (EGF)-related peptides, TGFα and neuregulins (NRGs), by astroglial cells, and the stimulatory effect of these peptides on the glial production of neuroactive substances, such as prostaglandin E_2, which then act on LHRH neurons to stimulate LHRH secretion. It is postulated that an increase in EAA release **[1]** occurs early in the sequence of events. Excitatory amino acids can act directly on LHRH neurons **[1′]** to stimulate LHRH release. The change in EAA transmission is followed very closely by a decrease in GABA release **[2]** and the activation of the glial production of TGFα/NRGs **[2′]**. The decrease in GABA release may be brought about by the increase in EAA transmission **[1″]**. Initial evidence suggests that the glial activation of growth factor production is also caused, at least in part, by EAA **[1‴]**, acting via a specific set of receptors located on hypothalamic astrocytes. Whereas the decrease in GABA transmission begins to relieve LHRH neurons from GABAergic inhibitory control **[3]**, the increased production of EGF-related glial peptides stimulates the formation of prostaglandin E_2 **[3′]**, which acts directly on LHRH neurons to induce LHRH release **[4]**, amplifying the stimulatory effect of EAA on the secretion of the neuropeptide **[1′]** and magnifying the loss of GABAergic inhibitory control **[3]**. Upon the initiation of these changes, a further increase in EAA stimulation **[5]** and further decrease in GABAergic inhibitory tone **[5′]** lead to activation of NE and NPY neuronal systems **[6]**. Both NE and NPY would then contribute to the progression of the pubertal process by stimulating LHRH secretion **[6′]**. The metabolic signals, leptin and IGF-I further the process along by stimulating LHRH secretion either directly **[7]**, or via functionally connected neuronal networks **[7′]**. The resulting increase in pulsatile LHRH secretion **[8]** then results in the initiation of puberty. —| = inhibition; (+) = stimulation.

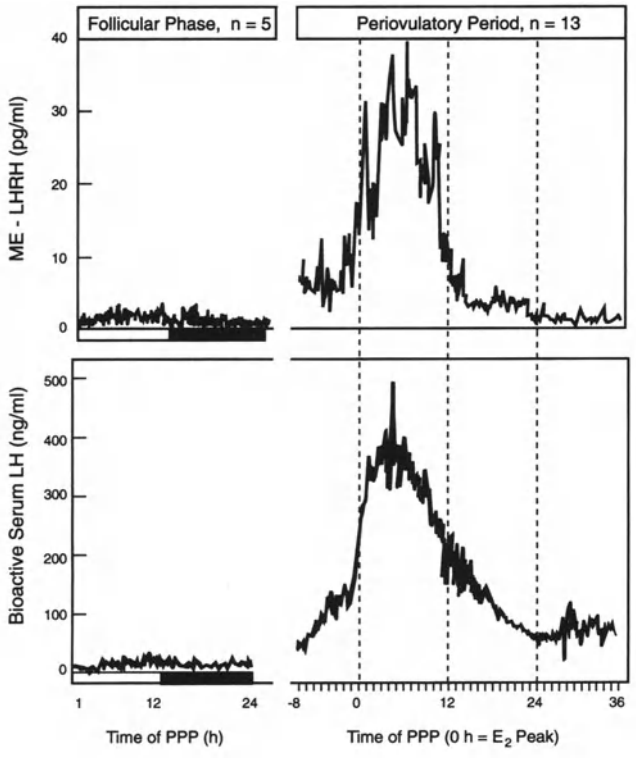

Fig. 27. The preovulatory surge of gonadotropins in primates is accompanied by a substantial increase in hypothalamic release of LHRH. LHRH was measured in samples collected from the median eminence of conscious monkeys via push-pull perfusion (PPP). The profile depicted corresponds to the average of five PPPs performed during the follicular phase of the estrous cycle and 13 PPPs collected during the preovulatory period. The open and solid bar under the follicular phase profiles represent the light/dark cycle. The dotted vertical lines during the preovulatory period simply emphasize the correlation between LHRH and LH secretion before, during and after their respective surges. Modified with permission from Pau et al., *Endocrinology* 1993; 133:1650–1656.

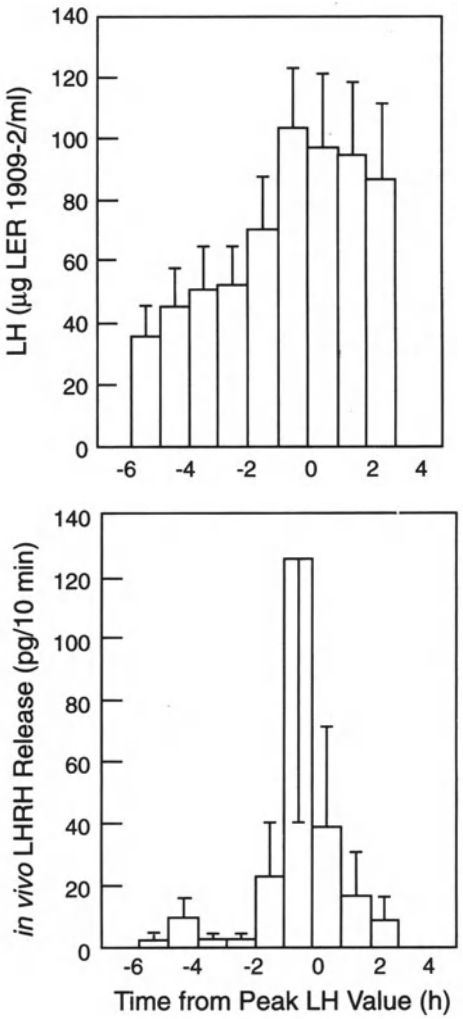

Fig. 28. Estrogen not only induces an discharge of LH from the pituitary gland, but also elicits a surge of LHRH secretion upon its administration to ovariectomized monkeys. Bars represent the mean values ± standard error of hormone levels detected in four monkeys. Reproduced with permission from Levine et al., *Endocrinology* 1985; 117:711–721.

These children are of normal size at birth, have a slow down in growth as toddlers, and then grow at a normal (but not catch-up) rate. They have a delay in their bone age, but they do enter puberty normally when their bone age finally reaches about 12 to 14 yr in boys and 11 to 13 yr in girls. Adrenarche is also usually delayed. They are shorter than their peers as a result of the early slow down in growth rate, but their height is appropriate for their bone age. Early on, it can be difficult to distinguish these children from those with true hypogonadotropic hypogonadism. The mechanism to explain this delay in growth and puberty is unknown, although it is likely to involve the CNS regulation of GH and LHRH release.

7.1.2. Hypogonadotropic Hypogonadism

The hypothalamus, the pituitary, or both may be affected by a variety of disorders that can lead to low or abnormal secretion of gonadotropins. These disorders can be divided into three main categories: anatomical, molecular, and functional.

7.1.2.1. CNS Anatomic Abnormalities. Anatomical abnormalities in the region of the hypothalamus or pituitary, such as developmental defects in brain formation or destructive lesions like tumors, can lead to abnormal hypothalamic and/or pituitary function. Such lesions are generally associated with multiple pituitary hormone deficiencies including the gonado-

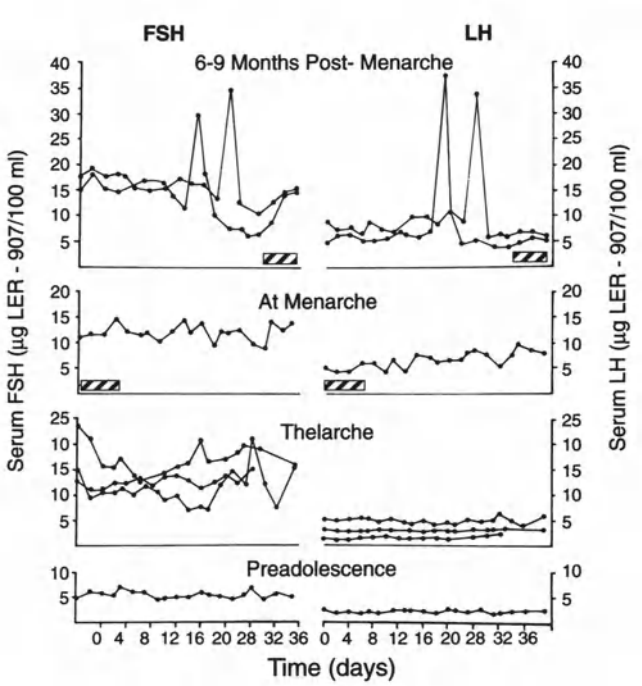

Fig. 29. Maturation of the estrogen positive feedback is a late event in human puberty. The hatched bars denote menses. Reproduced with permission from Faiman and Winter, In: Grumbach, Grave, Meyer, eds. *The Control of the Onset of Puberty,* New York: Wiley, 1974:32.

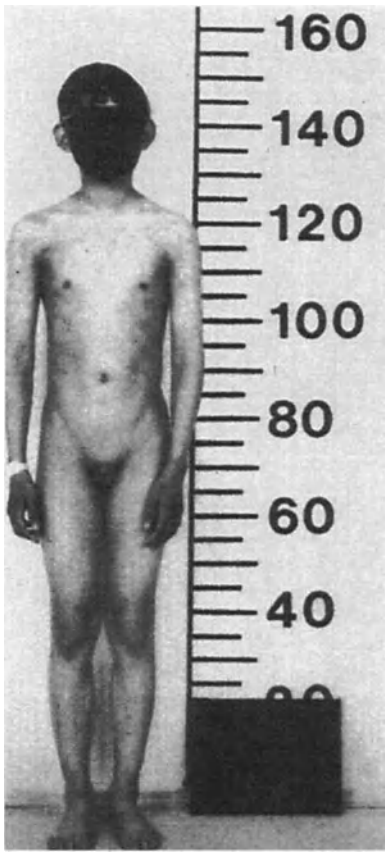

Fig. 30. A boy 16 yr 2 mo of age with constitutional delay of growth and puberty. His bone age was delayed by 4 yr. He eventually progressed through a normal puberty without intervention. Reproduced with permission from Styne DM, Grumbach MM, In: Yen SSC, Jaffe RB, eds., *Reproductive Endocrinology,* Philadelphia, WB Saunders, 1986:345.

tropins, growth hormone, thyroid-stimulating hormone, adrenocorticotropin-stimulating hormone, and antidiuretic hormone. The syndrome of septo-optic dysplasia, owing to abnormal development of the prosencephalon, is an example of a brain defect associated with overall pituitary dysfunction. The most common CNS tumor associated with delayed puberty is the craniopharyngioma, a tumor comprised of cells derived from the Rathke's pouch in the pituitary region. Other CNS tumors that can develop in this area include germinomas, astrocytomas, gliomas, and adenomas. Additional causes of local cellular destruction to the hypothalamus and/or the pituitary include hydrocephalus, trauma, irradiation, vascular lesions, postinfectious inflammation, and granulomas because of sarcoid or tuberculosis.

7.1.2.2. Molecular Defects. Isolated hypogonadotropic hypogonadism can be idiopathic or associated with a known molecular defect. The pattern of LH secretion has been measured in some adults with known isolated LHRH deficiency and found to show either no LH pulses at all, LH pulses only during the night, or decreased LH pulse amplitude or frequency.

Kallman's syndrome is the most common form of isolated hypogonadotropic hypogonadism; it occurs in about 1 in 7500 males and about 1 in 50,000 females. It is caused by a failure of the LHRH neurons to migrate from the olfactory placode to the medial basal hypothalamus and is commonly associated with hyposmia or anosmia, although this is often not noticed by the affected individual. There is a well-described X-linked form, which has been mapped to mutations in the KAL1 gene at Xp22.3, and that encodes a protein with characteristics of a neural adhesion molecule, perhaps important in guiding the migration of LHRH neurons. In the kindreds with X-linked inheritance, there is also a high incidence of unilateral renal aplasia. Other physical features occasionally associated with Kallman's syndrome are cleft lip and palate, seizure disorders, cerebellar ataxia, hearing loss, abnormal eye movements, and short metacarpals. Interestingly, there can be a difference

in phenotype between relatives with the same KAL1 mutation, with some members having the full-blown syndrome with hypogonadotropic hypogonadism and severe hyposmia, and others with only mild olfactory defects and normal sexual development. There have also been families with presumed Kallman's syndrome in which the disorder appears to be inherited as an autosomal dominant and autosomal recessive fashion, as well as families with likely X-linked inheritance of the disorder, but with a normal KAL1 gene.

It is suspected that there are other autosomal genes responsible for LHRH deficiency, and that these genes may actually account for the majority of familial cases. Candidates for this role include the LHRH gene itself located at 8p21–8p11.2. This gene is partially deleted in the hypogonadal (hpg) mouse model where hypogonadism is inherited in an autosomal recessive fashion. No human counterpart has yet been described. Other possible sites for mutations causing hypogonadotropic hypogonadism are being studied by examining chromosomal rearrangements that have been noted in individual patients with this problem.

Partial forms of hypogonadotropic hypogonadism have also been described. A disorder termed "fertile eunuch syndrome" appears to be the result of an incomplete form of LHRH deficiency, perhaps related to Kallman's syndrome. There is enough LHRH to stimulate spermatogenesis and testicular growth, but there is not enough to stimulate Leydig cells to produce enough testosterone for full virilization. They have small genitalia and a eunuchoid body habitus, but normal sized testes and preservation of spermatogenesis.

Hypogonadotropic hypogonadism associated with congenital adrenal hypoplasia is another X-linked disorder. It is because of a mutation in the DAX1 (*D*osage-sensitive sex reversal-*A*drenal hypoplasia congenita on the *X* chromosome gene *1*) gene at Xp21.2–21.3. The disorder affects the hypothalamus and/or the pituitary, although pituitary defects seem to dominate. These patients have severe adrenal insufficiency at birth owing to abnormally formed adrenal glands. A deletion in this area of the X chromosome can also encompass the genes for glycerol kinase and Duchenne's muscular dystrophy, leading to a so-called contiguous gene syndrome. In the mouse, DAX1 is expressed in the adrenal cortex throughout development. It is also found in the developing hypothalamus and anterior pituitary. The developing gonads have a sexually dimorphic expression, with DAX1 expression occurring in both ovaries and testes during early development, but decreased markedly in the male gonad by midgestation. The functions of DAX1 are still being elucidated, although there are some interesting early findings. DAX1 encodes an orphan nuclear hormone receptor that binds to hairpin DNA secondary structures and results in transcriptional repression. An example of its transcriptional silencing effect has been found in the promoter of the gene encoding steroidogenic acute regulatory protein (StAR), a protein required for adrenal steroidogenesis. DAX1 binds to this promoter and inhibits steroidogenesis in adrenal cells. This action may not, however, fully explain the defect in adrenal development seen in DAX1-deficient individuals. Because DAX1 gene expression is regulated by another orphan nuclear hormone receptor termed steroidogenic factor 1 (SF1), and adrenal development is impaired in SF1 knockout mice, it is plausible that at least part of the morphogenic activity of SF1 is mediated by DAX1.

Isolated LH or FSH deficiencies with LH-β and FSH-β mutations have also been described. The FSH mutations appear to be more important for female than male fertility as males with the mutation can be phenotypically normal, in contrast to females, which are infertile. Defects in the LHRH receptor gene have also been reported as a cause of hypogonadotropic hypogonadism.

In addition to these LHRH- and gonadotropin-specific disorders, there are defined genetic syndromes that can include hypogonadotropism as an associated abnormality. Examples of this category are the Prader-Willi and Laurence Moon Biedel syndromes. The cause for the hypogonadotropism in these patients is currently unclear, although it may be because of abnormal regulation of the LHRH neurons.

Functional gonadotropin deficiencies: A multitude of chronic systemic disorders can cause functional gonadotropin deficiencies, perhaps because of poor nutrition and low IGF-I/leptin levels. Examples include heart disease, asthma, inflammatory bowel disease, diabetes, sickle cell anemia, renal failure, and severe weight loss to less than 80% of ideal body weight as in anorexia nervosa. Intense physical exercise like that of ballet dancers or long distance runners can also delay puberty independent of weight. Exercise may influence LHRH release via activation of inhibitory opiatergic pathways. Hypothyroidism, Cushing's syndrome, and hyperprolactinemia are endocrinological abnormalities that can also result in decreased gonadotropins and delayed puberty.

7.1.3. Hypergonadotropic Hypogonadism. Primary gonadal disorders in which the testes or ovaries cannot produce adequate amounts of sex steroids, despite a normal hypothalamic and pituitary function, result in delayed puberty. The most common primary gonadal disorder is Klinefelter's syndrome. The syndrome affects 1 in 1,000 males and is genetically characterized by a 47,XXY karyotype. The affected individuals have seminiferous tubule dysgenesis and abnormal Leydig cells that cannot produce enough testosterone to normally complete puberty. In girls, a 45 XO karyotype leads to Turner's syndrome, which includes primary ovarian failure among a constellation of other physical findings such as short stature, left-sided cardiovascular abnormalities, and lymphedema.

Ovaries can be a target of an autoimmune process (oophoritis) that can lead to tissue destruction and hypogonadism. Metabolic disorders such as galactosemia or carbohydrate-deficient glycoprotein syndrome type 1 can also destroy the gonads, especially the ovaries. Testicular failure can also arise as a result of mumps orchitis or a Coxsackie B viral infection. In addition, both boys and girls can suffer gonadal failure because of local tumors such as gonadoblastomas and dysgerminomas, or metastases from leukemia, as well as from the irradiation or chemotherapy used to treat these processes.

7.2. Precocious Puberty

The definition of precocious puberty has classically been the onset of puberty at an age less than 8 yr in girls and less than nine yr in boys. This definition should probably change to the onset of breast development in Caucasian girls before age seven yr and in African-American girls before six yr, given the new data on the earlier pubertal patterns seen in American girls. Children with precocious puberty have an advanced bone age and an early growth spurt, but then have premature epiphyseal fusion with cessation of any further growth. Because they stop growing so early, they usually have a shorter final height than their genetic potential would indicate. Treatment for precocious puberty is necessary if final height is severely compromised or if there are psychosocial problems because of the early appearance of secondary sex characteristics. LHRH-dependent precocious puberty, is also known as true, complete, or central precocious puberty with high, pubertal levels of gonadotropins stimulating the gonads. When the gonads operate independently of the LHRH pulse generator, precocious puberty is considered LHRH-independent, and is classified as pseudo, peripheral, or incomplete precocious puberty. In addition, there are benign patterns of early development that are not associated with an advanced bone age or loss of final height and may be on one side of a continuum with true precocious puberty.

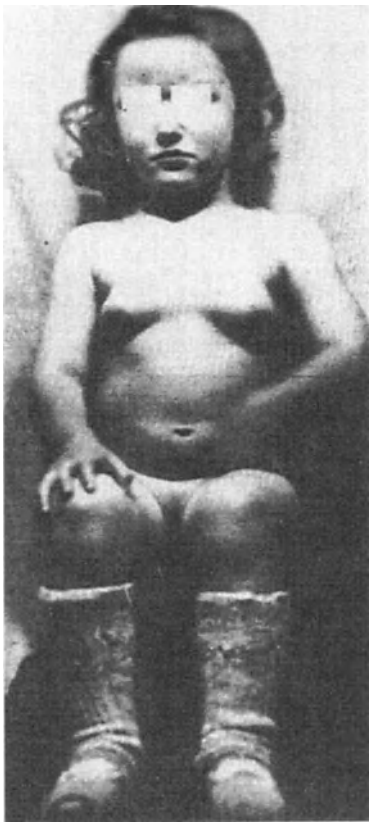

Fig. 31. Central precocious puberty of idiopathic origin in a 3.5 yr old girl. The patient developed pubic hair and started to menstruate at the age of 17 mo. Reproduced with permission from Jolly: *Sexual Precocity. . .*

7.2.1. LHRH-Dependent Precocious Puberty

LHRH-dependent precocious puberty may be a benign, idiopathic process in which there is early maturation of the normal hypothalamic-pituitary-gonadal axis for unknown reasons (Fig. 31). It may also be because of a CNS lesion, such as a tumor, or other CNS disruption. In girls, the majority of LHRH-dependent cases of precocious puberty are idiopathic, but in boys, about 60% are owing to CNS lesions.

Certain congenital conditions such as cerebral palsy or hydrocephalus are risk factors for LHRH-dependent precocious puberty. Babies who are born

prematurely are also at risk for precocious puberty later in life, perhaps because of early mild damage leading to abnormal brain function. An interesting association with precocious puberty has been noted in children adopted from developing countries into developed nations. These children have a history of being malnourished prior to three yr of age, and upon refeeding may show an excess gonadotropin secretion.

The most common CNS tumor that causes precocious puberty is the hypothalamic hamartoma. These nonneoplastic tumors can cause precocious puberty usually before three yr of age and are often associated with gelastic (laughing) seizures and developmental delay. They contain nonmalignant clusters of neurons and astroglial cells that usually develop from the floor of the third ventricle in the hypothalamus. Some of these tumors have been found to contain LHRH neurons. Others have been shown to contain an extensive network of astroglial cells containing TGFα. It is thus possible that hamartomas advance puberty via their ability to produce LHRH and/or glial growth factors able to activate the normal LHRH neuronal network of the patient's hypothalamus. Other tumors that may be associated with precocious puberty include astrocytomas, gliomas, ependymomas, pineal tumors, and craniopharyngiomas. Genetic disorders causing the development of optic gliomas and neurofibromas, such as neurofibromatosis type I, can also lead to precocious puberty.

Other CNS disturbances that can induce excess gonadotropin secretion are trauma, infections, or irradiation. Also, if bone age has been advanced by any other hormonal abnormality, such as a poorly treated congenital adrenal hyperplasia, the normal hypothalamic-pituitary-gonadal axis can be activated and true precocious puberty may result.

7.2.2. LHRH-Independent Precocious Puberty

Excess production of androgens in boys and estrogens in girls, independent of a normal hypothalamic-pituitary unit leads to the development of secondary sexual characteristics and growth, which appear clinically similar to true precocious puberty. One of these such conditions is the McCune-Albright syndrome. It consists of the triad of café-au-lait spots, fibrous dysplasia of the long bones, and constitutive activation of many endocrine glands (ovary, thyroid, adrenal, pituitary, and parathyroid) because of an activating mutation of the α-subunit of the stimulatory guanine nucleotide binding protein. This constitutive activation causes precocious puberty in girls, but rarely in boys. In boys, there is an autosomal dominant disorder termed familial testotoxicosis in which the testes produce testosterone independent of LH; it is because of point mutations in exon 11 of the LH receptor that causes constitutive activation. Boys and girls with congenital adrenal hyperplasia caused by deficiency of 21- or 11-hydroxylase have overproduction of androgens unless they are adequately treated. Other independent sources of sex steroids are tumors that can produce human chorionic gonadotropin (hCG), androgens, or estrogens. These can lead to isosexual (virilization of males, feminization of females) or contrasexual (virilization of females or feminization of males) precocious puberty, depending on the hormone produced. Severe hypothyroidism can lead to overproduction of TRH, which is postulated to increase prolactin secretion and increase synthesis of the common TSH/gonadotropin α subunits, thus leading to incomplete precocious puberty and galactorrhea.

7.2.3. Benign Patterns

There are benign patterns to precocious puberty that are well described. Premature thelarche is a nonprogressive increase in breast tissue in girls. It may become apparent between six mo and six yr of age, although it is more rare after four yr. The bone age of these girls, however, is not advanced, and true puberty happens at a normal age. Premature thelarche may be caused by increased tissue sensitivity to prepubertal estrogen levels or it may be on one side of a spectrum that also includes true precocious puberty. Premature pubarche is the appearance of pubic hair in children usually after 4 yr, possibly owing to early adrenarche or to increased sensitivity of the hair follicles to the low normal level of prepubertal androgens. Again, the bone age of these children is normal, and true puberty happens at a normal age. It is more common in dark-skinned races or obese children. A more uncommon pattern is isolated prepubertal menses. It occurs in girls between one to nine yr of age without breast development and is possibly caused by transient functional ovarian cysts.

SELECTED READINGS

Clayton PE, Gill MS, Hall CM, Tillmann V, Whatmore AJ, Price DA. Serum leptin through childhood and adolescence. Clin Endocrinol 1997;46:727.

Dissen GA, Dees WL, Ojeda SR. Neural and neurotrophic control

of ovarian development. In: Adashi EY, Leung PCK, eds. The Ovary. New York: Raven, 1993:1.

Faiman C, Winter JSD. Gonadotropins and sex hormone patterns in puberty: clinical data. In: Grumbach MM, Grave GD, Mayer FE, eds. Control of the Onset of Puberty. New York: Wiley, 1974:32.

Greulich WS, Pyle SI. Radiographic Atlas of Skeletal Development of the Hand and Wrist. Stanford, CA: Stanford University Press, 1959.

Grumbach MM, Styne DM. Puberty: ontogeny, neuroendocrinology, physiology, and disorders. In: Williams RH, Foster DW, Kroenenberg H, Larsen PP, Zorab R, eds. Williams Textbook of Endocrinology, 9th ed. Philadelphia, PA: WB Saunders, 1998:1509.

Herman-Giddens ME, Slora EJ, Wasserman RC, Boudony CJ, Bhapkar MV, Koch GG, Hasemeier CM. Secondary sexual characteristics and menses in young girls seen in office practice: a study from the Pediatric Research in Office Settings network. Pediatrics 1997;99:505.

Marshall WA, Tanner JM. Variations in patterns of pubertal changes in girls. Arch Dis Child 1969; 44:291.

Marshall WA, Tanner JM. Variations in patterns of pubertal changes in boys. Arch Dis Child 1970; 45:13.

Ojeda SR. The neurobiology of mammalian puberty: Has the contribution of glial cells been underestimated? J NIH Res 1994 6:51.

Ojeda SR, Urbanski HF. Puberty in the rat. In: Knobil E, Neill J, eds. The Physiology of Reproduction, 2nd ed., vol 2. New York: Raven, 1994:363.

Plant TM. Puberty in primates. In: Knobil E, Neill J, eds. The Physiology of Reproduction, 2nd ed., vol 2. New York: Raven, 1994:453.

Terasawa E. Mechanisms controlling the onset of puberty in primates: the role of GABAergic neurons. In: Plant TM, Lee PA, eds. The Neurobiology of Puberty. Bristol: Journal of Endocrinology, Ltd., 1995:139.

Winter JSD, Faiman C. Comparative aspects of animal and human puberty. In: Grumbach MM, Sizonenko PC, Aubert ML, eds. Control of the Onset of Puberty. Baltimore, MD: Williams & Wilkins, 1990:211.

13 Neuroendocrine Regulation of Thyroid Function

William J. DeVito, PHD

CONTENTS

1. INTRODUCTION AND HISTORICAL CONSIDERATIONS

The hypophysis, or pituitary gland, is a small structure that secretes a number of hormones. Despite its size, it is clear that the pituitary gland influences virtually every known biological function in higher animals. Hormones released from the pituitary play key roles in the regulation of growth, maturation, reproduction, lactation, metabolism, and behavior. The physiologic concept of the pituitary gland has a long and interesting history and can be traced back to the early works of Aristotle around 384 to 322 BC. Aristotle considered the brain as a cooling mechanisms that delivered sera and phlegm (or pituita) to the base of the brain where they were excreted through the nose. The function of the pituitary remained unknown, however, for centuries. Galen (130–200 AD) also proposed that the pituitary gland functioned as a "phlegmatic glandute" that removed waste products from the base of the brain. This concept was put to rest by Conrad Schrieber and Richard Lower in the 1600s. Lower suggested that substances contained in the blood passed from the brain to the pituitary where they "distilled" back into the circulation. It would not be until 1963, however, when scientists demonstrated that neurons secreted chemical messengers into the blood, that the concept of neurosecretion was accepted. Lowers' description led to the identification of the pituitary-portal circulation by Joseph Lieutaud and the demonstration that the anterior pituitary was separate from the posterior lobe by Giovanni Domenico Santorini in the early 1700s. The function of the pituitary, however, was still unknown. In 1886, Sir Victor Horsely removed the pituitary from dogs and reported that the dogs survived reasonably well for 6 mo, thus perpetuating the concept that the pituitary served no important functions. Shortly thereafter, the French neurologist Pierre Marie published his papers on "Marie's" acromegaly showing a relationship between the pituitary and acromegaly, and Frohlich described a pituitary tumor with obesity and sexual dysfunction. In 1921, Bailey and Bremer were able to show that lesions made at the base of the brain

From: *Neuroendocrinology in Physiology and Medicine,* (P. M. Conn and M. E. Freeman, eds.),

resulted in a similar syndrome in dogs. The technique of hypophysectomy described by the physiologist Nicholas C.Paulesco in 1907 and later by Harvey Cushing and Bernard Aschner set the stage for studies that would elucidate the function of the pituitary.

In 1921, Evans and Long demonstrated that aqueous extracts prepared from the pituitary restored growth in hypophysectomized animals. By the middle of the 1930s, eight hormones were extracted from the pituitary gland. The mechanisms that regulated the release of pituitary hormones, however, were unknown. Experiments using electrical stimulation of the hypothalamus demonstrated the release of pituitary hormones, thus supporting the concept that the hypothalamus played a major role in the regulation of pituitary hormone secretion. Direct neural innervation of the gland, however, was not apparent. Popa and Fielding in the 1930s described the hypophysial portal vessels and suggested that these vessels function to transport blood from the pituitary to the hypothalamus. Wislocki and King, however, injected Indian ink into monkeys and demonstrated that the direction of blood flow was down, not up, the pituitary stalk. This observation by Wislocki and King, led to the pioneering experiments of Harris and his coworkers, which established that the pituitary gland was controlled by hormones secreted by the hypothalamus and transported to the pituitary by portal vessels. Thus beginning the race for their isolation, which would take 30 yr. In 1969, independent studies by Schally and Guillemin and coworkers identified and characterized the first releasing hormone—thyrotropin-releasing hormone (TRH)—for which they received the Nobel Prize for medicine in 1977.

It is now clear that a number of proteins, peptides, cytokines, and growth factors can modulate thyroid growth and differentiate functions. Normal thyroid function, however, is primarily regulated by thyroid-stimulating hormone (TSH), a glycoprotein hormone synthesized and secreted by thyrotrophs (basophilic cells) in the anterior pituitary gland. In the adult, TSH directly controls thyroid growth and the syntheses of thyroid hormones, in particular T_3 and T_4. TSH synthesis and release is regulated by two opposing mechanisms. TRH, a small tripeptide, synthesized in the hypothalamus, transported by neurons to the median eminence where it is secreted into the hypothalamic-portal-system for transport to the anterior pituitary stimulates TSH synthesis and release. The other major regulatory component is the inhibitory effects of thyroid hormones on TSH release and TSH gene expression in the pituitary and TRH gene expression in the hypothalamus.

The mechanisms involved in neuroregulation of thyroid hormone economy are the subject of thousands of papers, and the topic of numerous reviews. In this chapter, we will examine the current views concerning the neuroendocrine mechanisms involved in maintaining thyroid hormone homeostasis.

2. ENDOCRINE HYPOTHALAMUS

The hypothalamus is a phylogenetically ancient region of the brain located at the base of the diencephalon. The hypothalamus is included among endocrine organs as it produces several hormones, which are secreted into the hypothalamo-hypophyseal blood vessels through which they regulate anterior pituitary functions (*see* Fig. 1). Other hormones are also synthesized by hypothalamic cells and transported down long hypothalamo-hypophysial nerve tracts for storage in the posterior pituitary for subsequent release into the systemic circulation. Because of its location and anatomical connections, the hypothalamus is the major link between the endocrine system and receptors, which test both the internal and external environments. This neuroendocrine system provides the versatility of responses needed to adapt rapidly to changes in our environment. Thus, providing a system through which stimuli from diverse sources can influence a single endocrine gland. The hypothalamus is a bilaterally systemic structure that forms the walls and floor of the lower part of the third ventricle (*see* Fig. 2). It is flanked medially by the third ventricle and laterally by the subthalamus. Rostrally, it is bordered by the lamina terminalis, dorsally by the anterior commissure and the hypothalamic sulcus. Its ventral aspect consists of the preoptic region, optic chiasm, median eminence hypophysis, tuber cinereum, and mammillary bodies. Anatomically, the hypothalamus can be divided into four areas:

1. The preoptic area (telencephalic area), which includes the periventricular, medial preoptic, and lateral preoptic zones.
2. The supraoptic area dorsal to the optic chiasma, which includes paraventricular nucleus, nuclei in the medial hypothalamus, supraoptic nucleus, and nuclei of the lateral zone.
3. The tuberal area dorsal to the tuber cinereum, which includes dorsal medial and ventral medial nuclei.

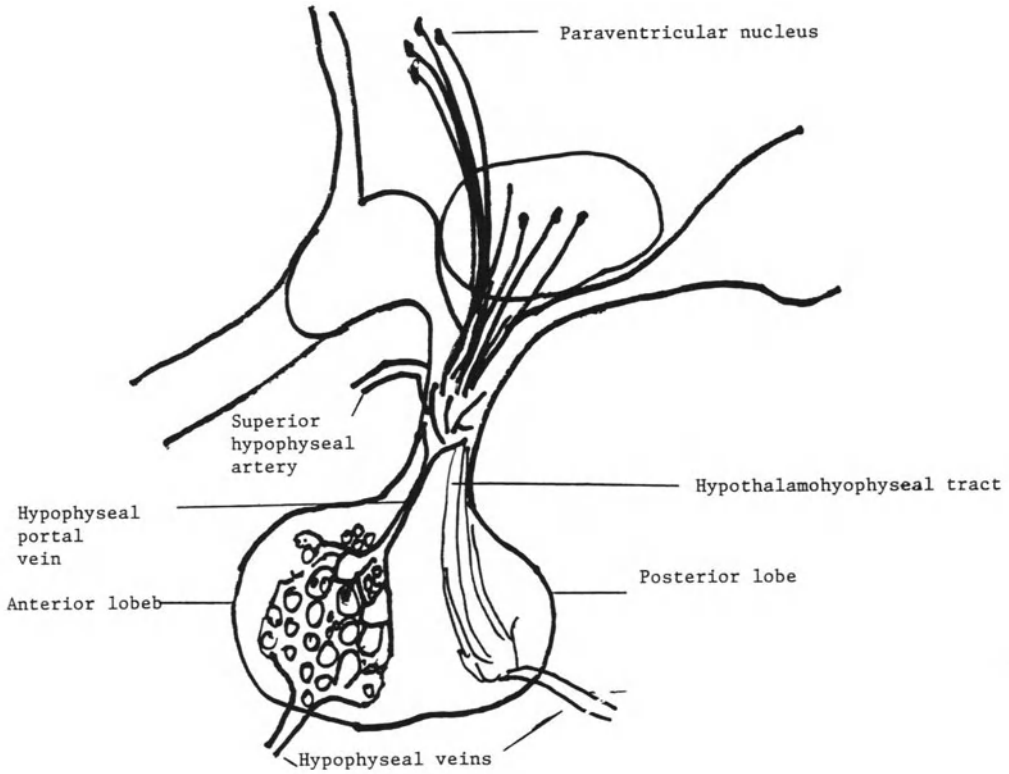

Fig. 1. The hypothalamohypophyseal tract and the hypophyseal portal vein.

4. The mamillary area, which is dorsal to, and includes the mamillary bodies.

The hypothalamic-pituitary-thyroid-axis consists of three main components:

1. The hypothalamus in which regulatory peptides are synthesized.
2. The median eminence in which regulatory peptides are released.
3. The portal system through which the peptides are transported to the anterior pituitary which stimulates the secretion of TSH, which, in turn, regulate thyroid function.

In the following we will examine the morphology of the median eminence and the development of the hypothalamus, TRH expression, and the portal system.

2.1. Median Eminence

The median eminence belongs to the circumventricular organs, which are characterized by a porous blood-brain barrier as it contains fenestrated capillaries, and is considered the "final common pathway" between the nervous and endocrine systems. The median eminence is the neural tissue located beneath the ventral floor of the third ventricle and is continuous with the hypophyseal or pituitary stalk. There are four types of axonal terminations in the median eminence:

1. Axo-axonal terminals in which one axon synaptically influences another axon.
2. Neurosecretory terminals that release neurohormones into the primary portal plexus.
3. Axon terminals in contact with tanycyte ependymal cells, nonneuronal cells that send processes from the ventricular wall and make contacts with blood vessels or neurons.
4. Axons with projections that terminate in the lumen of the third ventricle.

Based on morphological and functional classifications the median eminence has been divided into three layers; an inner ependymal zone, the inner palisade zone, and the outer external zone. The inner ependymal zone, or inner layer, lines the floor of the third ventricle. It consists primarily of cell bodies of tanycyte ependymal cells, which line the ventral part of the third ventricle, and a capillary plexus. Tight junctions, which connect the tanycytes, form a barrier between the ventricular cerebral spinal fluid (CSF) and the extracellular space of the median eminence. In this region, tanycyte ependymal cells send long basal pro-

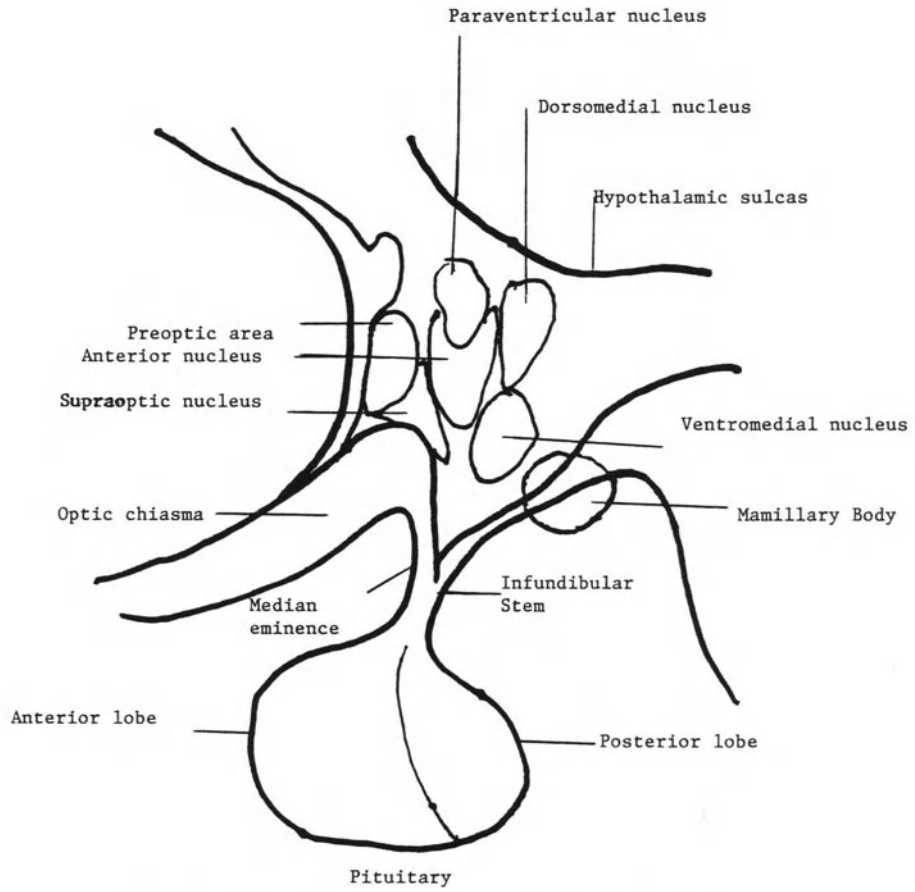

Fig. 2. Orientation of the hypothalamus and of some hypothalamic nuclei.

cesses that pass through the palisade and external zones and connect to capillary walls. In the median eminence, tanycytes also send processes that terminate on primary portal capillaries and neuronal processes. Whereas the functions of axo-axonal and neurosecretory terminal are clearly understood, the functions of tanycytes and interventricular projections remain controversial. Based on the tanycytes morphology, it was originally speculated that tanycytes functioned as transporters of regulatory peptides to the primary portal plexus, thereby regulating pituitary secretions. Further, based on their interventricular projects it was suggested that tanycytes may play a role in the transport of hormones between the CSF and the median eminence. However, there is a lack of strong supporting evidence to support these hypotheses.

The inner palisade zone consists of axons from hypothalamic magnocellular neurons passing through the inner zone and project to the pars nervosa and contain large diameter, electron dense, secretory vesicles. Catecholaminergic- and peptidergic-containing terminals are also found in the middle layer of the median eminence.

The external zone (also refereed to as the outer palisade layer) contains nerve terminals from tuberoinfundibular neurons, glial cells, and processes of tanycytes. Fibers of the tuberoinfundibular tract terminate in this region where they release secretory products, releasing factors, which diffuse from the extracellular space into the portal blood. The external zone also contains catecholaminergic fibers of the tuberoinfundibular system and is rich in dopamine and noradrenaline.

2.2. Hypothalamic-Portal System

The connection between the hypothalamus and the anterior pituitary is the hypothalamic-pituitary-portal system. It is clear that the secretion of TSH, and other anterior pituitary hormones, are regulated by neurohormones, which are transported from the hypothalamus to the pituitary through this portal system.

The system of portal vessels supplying the pituitary is the most highly developed in mammals. Studies have provided descriptions of species variations, in which diverse terms are used for analogous structures. In mammals, the internal carotid artery gives rise to two branches, the superior and inferior hypophyseal arteries. In humans, additional blood is supplied to the pituitary gland from the basilar arteries formed by the vertebral artery branches of the subclavian arteries. The basilar arteries divide into right and left posterior cerebral arteries, which are joined by communicating arteries to the anterior cerebral arteries from the internal carotid, which forms the circle of Willis. The lower portion of the infundibular stem receives blood from the loral, or trabecular, artery, which is a small branch of the superior hypophysial artery. The superior or anterior hypophysial arteries supply blood to the median eminence and infundibular stem. Blood from the upper regions of the median eminence drains into long portal vessels, which descend to the upper and anterior portions of the anterior pituitary. Blood from the lower portion of the stalk drains into short portal vessels, which descend to the lower, more posterior part of the pituitary. In the pituitary, the portal vessels form a second set of capillaries which have a discontinuous, fenestrated epithelium, a continuous basement membrane, and a perivascular tissue space.

2.3. Development of the Hypothalamic-Pituitary Axis

2.3.1. Formation of the Hypothalamus and Median Eminence

The control of normal thyroid function is primarily through the regulation of TSH secretion from the anterior pituitary gland. CNS control of TSH secretion, in turn, is regulated by the release of TRH and other peptides and amines from the hypothalamus that can stimulate or inhibit TSH secretion. Thus, in addition to the normal morphological and biochemical differentiation of the thyroid, regulation of thyroid function requires normal development and differentiation of TSH secreting cells in the pituitary (thyrotrophs), TRH secreting cells in the hypothalamus and the hypothalamic portal system.

The differentiation of the pituitary, hypothalamus, and the hypothalamic portal system occurs independently over a long time period, which extends into the neonatal period. In humans, the development of the neural plate, which leads to the establishment of the central and peripheral nervous system, begins around embryonic day 17 as the thickening of the midline ectoderm. Over the next 10 d, the ectoderm thickens to form the neural tube. Cells from the dorsal region of the neural tube form numerous structures in the peripheral nervous systems and the calcitonin producing parafollicular cells of the thyroid. The hypothalamus originates from the most rostral region of the neural tube, which develops into the ventral caudal prosencephalon around six wk of gestation. The infundibulum, an evagination from the ventral hypothalamus, givies rise to the median eminence and the pituitary stalk. In humans the hypothalamic regions responsible for TRH synthesis, and other TSH regulatory peptides, appear early in gestation. That is, TRH can be detected in the brain around day 30 of gestation. Detection of nuclear groups which synthesize TRH in the hypothalamus appear around nine wk.

Despite the early detection of TRH in the hypothalamus and TSH in the anterior pituitary at 13 wk of gestation there is little support that fetal pituitary TSH content or secretion are regulated by hypothalamic TRH. Further, although relatively high amounts of TRH is synthesized in other tissues, such as pancreatic islet cells as early as six wk, rapid degradation of TRH in the serum precludes any effect of maternal or peripheral TRH on fetal pituitary TSH synthesis or secretion. The lack of TRH-induced regulation of fetal TSH secretion early in gestation is most likely explained by the delayed maturation of the hypophyseal portal system.

2.3.2. Development of Hypophyseal Portal Circulation

The hypophyseal portal system is the conduit through which peptides released by the median eminence are transported to the pituitary. The hypophyseal portal system consists of two capillary plexuses, a primary plexus in the median eminence and a secondary plexus in the pituitary. The precise timing of the development of the portal system has not been established, however, its development lags behind the morphological and biochemical maturation of the peptidergic neurons which it will serve. Early morphological studies suggested that the secondary plexus in the pituitary was the first to develop and was present by the fourth month with the primary plexus in the median eminence of the hypothalamus developing around the sixth mo. Studies using perfused siliconized rubber, however, have shown that in humans a rudimentary portal system is established earlier around 11.5 to 16 wk of gestation. Whether this

rudimentary portal system is sufficient to transport peptides released from TRH fibers in the median eminence to the pituitary is not clear.

3. TSH AND TRH

3.1. TSH

3.1.1. Structure and Synthesis

TSH, like other anterior pituitary hormones (luteinizing hormone, follicle-stimulating hormone, and chorionic gonadotropin) is a glycosylated heterodimer. TSH has an apparent molecular weight of approximately 28 kDa and is secreted by thyrotrophs from the anterior pituitary. TSH is composed of two peptide chains (or subunits), TSH-α and TSH-β, which are linked by noncovalent bonds. As individual proteins, the α-subunit and the TSH-β-subunit display little, if any, biological activity. As with other glycoprotein hormones, TSHα is a common subunit with the β-subunit conferring biological specificity. The α-subunit and the TSH-β-subunit are encoded by seperate genes located on different chromosomes. In humans, the α-subunit is located on chromosome 6q21-23 and the TSHβ-subunit is located on chromosome 1p22. In the human, the TSHα gene is 9.4 kilobases (kb) in length and contains four exons and three introns (*see* Fig. 3). Exon 1 contains one transcriptional start site. Analysis of the 5′-untranslated region shows a TATA box -26 base pair from the start site and a palindromic sequence (TGACGTCA), which confers cAMP responsiveness. The TSHβ-subunit is 4.9 kb in length and contains three exons (37, 163, and 326 bp in length) and two introns (3.9 and 4.5 kb in length). Exon 1 of the human TSHβ gene contains one transcriptional start site. Transcription of TSHβ and α genes are increased by TRH and inhibited by T_3 and dopamine.

Thyroid hormones, predominantly T_3, are the primary negative regulators of TSH production and secretion. Thyroid hormones act by suppressing the transcription of TSHα- and β-subunits. The magnitude and rapidity of suppression of the TSHβ-subunit is more pronounced than the inhibition of the common α subunit. Inhibition of TSH expression by thyroid hormones occurs at the level of transcription. T_3 directly inhibits TSH production by binding to thyroid hormone receptors, which, in turn, bind to DNA sequences known as thyroid hormone response elements (TREs; which bind thyroid hormone receptor proteins) located in the 5′ flanking region of the TSH α- and β-subunit genes. Based on its lower affinity for thyroid hormone receptors and its action in a number of animal models, T_4 was usually considered a prohormone exerting its effects after conversion to T_3 by peripheral monodeiodination. A number of recent studies, however, support a direct effect of T_4, and the concept that T_4 is an active hormone and may directly affect gene transcription.

The major stimulator of TSH production, gene expression, and secretion is TRH, which has direct effects on TSH transcription. Two discreet TRH-responsive regions are present in the TSH gene at −128 to −92 and −28 to +8. The upstream site contains a DNA sequence that shares a high degree of homology to the DNA-binding site for a pituitary-specific transcription factor (Pit-1). The down-stream cis sequence overlaps with the inhibitory THRE, thus suggesting that the regulation of TSH gene expression may involve interactions between the TRH stimulatory- and thyroid hormone inhibitory-elements.

During translation of TSHα- and β-subunits, glycosylation of the individual subunits takes place and is important for the maintenance of biological activity of the TSH heterodimer. Posttranslational modifications of the subunits results in the addition of high-mannose oligosaccharides containing 3 glucose, 9 mannose, and 2 N-acetylglucosamine residues. High-mannose oligosaccharide groups are added in the rough endoplasmic reticulum. It is thought that the function of these high-mannose groups is to facilitate the formation of disulfide bonds and to aid in the structural organization of the protein. Further post-translational processing occurs in the golgi where the carbohydrate groups are modified to form more complex oligosaccharide modifications, which are linked to asparagine resulting in the formation of the intact TSH heterodimer. The proposed functions of these complex oligosaccharides are twofold. Deglycosylation of bovine TSH with endoglycosidase F results in a marked decrease in bioactivity, suggesting that the biological potency of the TSH molecule is in part determined by the degree of glycosylation. Further, deglycosylation of TSH results in a marked increase in the clearance of TSH, indicating the biological half-life of the TSH heterodimer is affected by the degree of its glycosylation.

3.1.2. TSH Receptor

In humans, the TSH receptor gene is more than 60 kb in length and is located on chromosome 14q31. The human gene contains 10 exons and 9 introns,

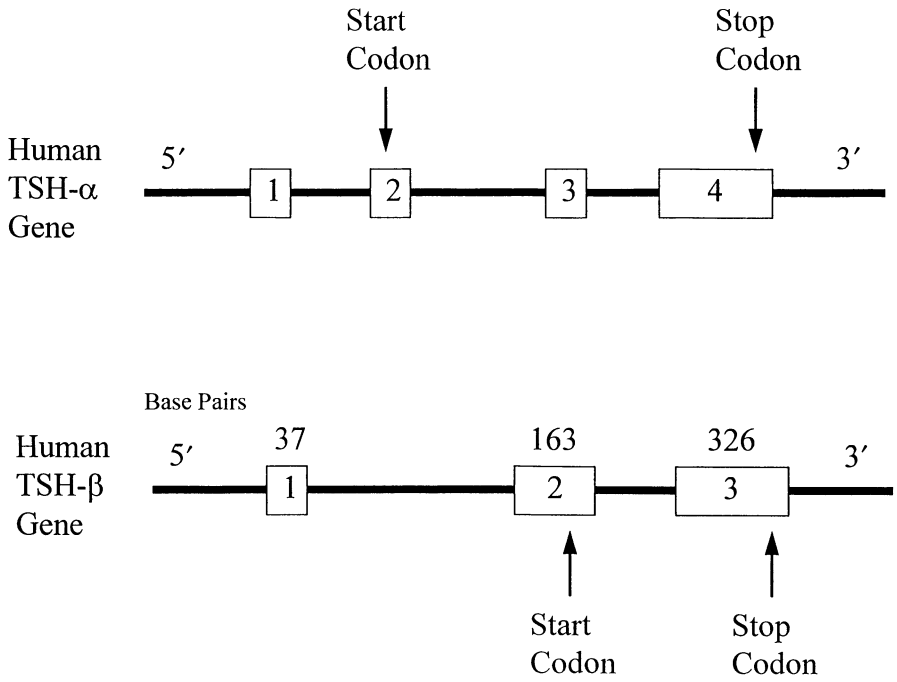

Fig. 3. Organization of the human TSH subunit genes. Exons are represented by numbered boxes. Introns are represented by dark lines. Translational start codon (AUG) and stop codon (TAA) are shown in their relative positions.

which encodes a protein of 764 amino acids in length, including a signal peptide (*see* Fig. 4). After cleavage of the signal peptide, the mature TSH receptor contains a heavily glycosylated 397 amino-terminal extracellular domain and a 344 amino acid carboxyl-terminal. The TSH receptor is a member of a family of receptors that signal to G-proteins, characterized by the common feature of 7 transmembrane domains. The TSH receptor displays an original feature, however, in that the extracellular domain is encoded by exons 1-9 and the first part of exon 10, whereas the total transmembrane and intracellular domains are encoded by a long single domain, exon ten. Analysis of the 5′-untranslated region revealed no sequences corresponding to a consensus CAAT or TATA boxes. The promoter region of the TSH receptor gene is GC-rich and contains multiple binding sites for transcription factors. The human TSH-receptor promoter contains consensus sequences, which could mediate gene transcription by cAMP and phorbol esters, thus raising the possibility that the TSH receptor gene is differentially regulated.

3.1.3. Mechanisms of TSH Action

In the thyroid, the majority of the effects of TSH are mediated by binding to its receptor and the activation of adenylate cyclase, an increase in cellular cAMP levels, and the subsequent serine/threonine phosphorylation of protein kinase A. Newly synthesized cAMP molecules bind to a regulatory subunit

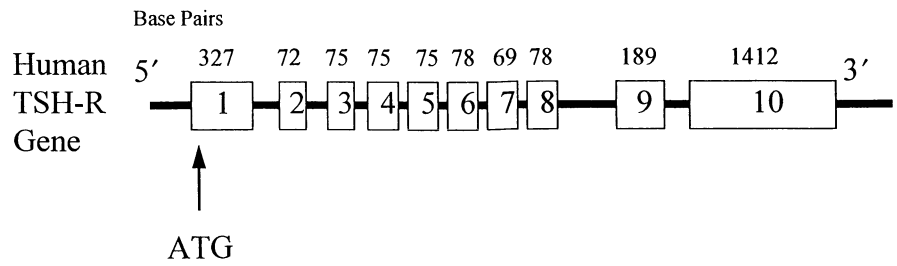

Fig. 4. Organization of the human TSH receptor gene. Exons are represented by numbered boxes. Introns are represented by dark lines. The position of the translational codon (ATG) is indicated in its relative position.

in protein kinase A. There are two different protein kinase A molecules (type I and II), which differ only in the type of cAMP-binding site on the regulatory subunit. The binding of cAMP to two sites on each of the regulatory subunits results in the activation of protein kinase A holoenzymes (which are more resistant to proteolysis) resulting in the release of the catalytic subunit and the subsequent phosphorylation of substrate proteins in the cytoplasm and nucleus. The major nuclear target for free protein kinase A catalytic subunits are CREB (CRE-binding proteins), a family of leucine zipper DNA binding proteins, which bind to cAMP response elements (CREs) located in the promoter region of most, if not all, TSH responsive genes.

Several lines of evidence, suggest that other intracellular signal transduction pathways, including an increase in intracellular calcium, activation of protein kinase c (PKC) and protein tyrosine phosphorylation, are involved in the regulation of thyroid cell proliferation and differentiated functions. TSH stimulates autophosphorylation and kinase activity of insulin and insulin-like growth factor I (IGF-1) receptors and potentiates the IGF-I dependent tyrosine phosphorylation of a 175 kDa cytoskeleton-associated protein in rat thyroid cells (FRTL5 cells). Studies in porcine thyroid cells suggest that tyrosine phosphorylation is involved in the regulation of TSH-induced cell spreading and focal adhesion formation. Further, a possible role of tyrosine phosphorylation in the regulation of thyroid cell proliferation is suggested by studies showing that inhibitors of tyrosine phosphorylation blocks TSH-induced DNA synthesis and TSH-induced thyroid specific gene expression in rat thyroid cells.

3.2. TRH

3.2.1. Characterization and Gene Expression

Reports describing the presence of a thyrotropin-releasing factor, now known as TRH, in the CNS were obtained in the early 1950s. It was not until 1970, however, that the chemical characterization of ovine TRH and bovine TRH were described. TRH is a basic tripeptide pyro-His-Glu-Proamine, which like other hypothalamic factors, lacks phylogenetic specialization (*see* Fig. 5). Recent studies using molecular cloning technology have revealed that TRH is not synthesized enzymatically, as originally thought, but is derived by posttranslational processing of a large TRH precursor (proTRH) and by enzymatic modifications. In the rat, proTRH has an apparent molecular weight of 26 kDa and contains five potential TRH sequences, each of which are processed in the CNS to produce five copies of the mature prohormone. In humans, the preproTRH gene has been cloned and is a single-copy gene located on chromosome 3. The human TRH gene shows a high degree of sequence homology to the rat gene, contains six TRH coding sequences. The human prepoTRH transcriptional unit is 3.3 kb in length and contains three exons interrupted by 2 introns (Fig. 5). Exon 1 encodes the 5′ untranslated region of the mRNA. Exon 2 encodes a signal peptide and the initial portion of the first TRH coding sequence, whereas exon 3 encodes the remainder of the translated sequences including all of proTRH.

In the CNS, TRH gene expression is limited to specific regions, including the hypothalamus and olfactory bulb. In the paraventricular nucleus of the hypothalamus, it is clear that the synthesis and expression of the preproTRH is negatively regulated by thyroid hormones. The precise mechanisms involved in the regulation of TRH gene expression, however, are poorly understood. Recent molecular analyses of the promoter regions of human and rat preproTRH have shown that the TRH gene is a member of a family of T_3-responsive genes, including the α and β-subunits of the TSH gene whose expression is negatively regulated by thyroid hormones (T_3) at the transcriptional level. The thyroid hormone receptors function as ligand-dependent transcription factors with can activate or inhibit gene expression by binding to cis-acting DNA sequences, known as thyroid regulatory elements in the 5′-flanking regions of thyroid hormone responsive genes. In the unliganded state, thyroid hormone receptors can suppress the activity of promoters that contain positively regulated hormone response elements (in the absence of thyroid hormone gene expression is suppressed). Addition of thyroid hormone reverses this suppression stimulating transcription. In contrast, negatively regulated genes are activated by unliganded receptors and transcription is repressed after thyroid hormone binding to the receptor. Analysis of the 5′ region of the TSH gene has identified a number of regulatory sequences, such as TATA and GC box sequences found in the promoter region of other genes, and consensus sequences for cAMP response elements, which could be involved in the regulation of TRH gene expression. Consistent with the inhibitory effect of T_3 on TRH gene expression, the 5′-flanking region of the human preproTRH gene contains two negative TRE ele-

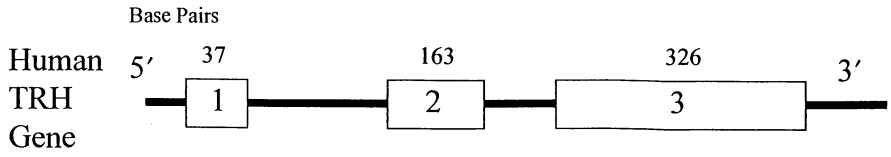

Fig. 5. Organization of the human TRH gene. Exons are represented by numbered boxes. Introns are represented by dark lines.

ments, which are homologous to thyroid regulatory elements sequences found in rat and human TSH-β genes. More recently, studies in transgenic mice indicate that sequences located between +6 and +84 in the first exon of the rat gene are crucial elements involved in the regulation of TRH gene expression. Accordingly, it now appears that the regulation of the TRH synthesis involves an interaction between upstream and downstream elements in the TRH gene.

3.2.2. TRH Metabolism

The plasma half-life of TRH is short and maybe dependent on thyroid status. In tissues and serum, TRH is rapidly degraded to a stable free-acid cyclized metabolite histidyl-proline-diketopiperazine (cyclo [His-Pro]). The metabolism of TRH occurs through the hydrolysis of the pyroGlu-His bond by the enzyme pyroglutamyl aminopeptidase type 1 present in the membranes, synaptosomes and pituitary, and pyroglutamyl aminopeptidase type 2 in the plasma. The cyclized metabolite of TRH, cyclo(His-Pro), has a number of pharmacological actions. It has been suggested that TRH may function as a prohormone for cyclo(His-Pro), however, recent studies indicate that biologically relevant amounts of this molecule can be obtained from absorption from some foods and from its independent production in other tissues.

3.2.3. TRH Receptor

The TRH receptor is a member of a family of G-protein-coupled receptors containing seven transmembrane domains. The human TRH receptor gene is located on chromosome 8. In the human, the TRH receptor gene is more that 30 kb in length, and contains 3 exons and two introns (Fig. 6). Exon 1 encodes the 5′-untranslated region. Exon 2 is more than 25 kb in length and begins encoding 88 base pairs upstream of the translational initiation site and encodes up to the beginning of the sixth transmembrane domain. Exon 3 encodes the remainder of the coding sequence and the entire 3′-untranslated region. Recent studies in human pituitary have revealed a novel transcript of the TRH receptor gene. Cloning and sequence analyses revealed a TRH receptor messenger RNA with a deleted sequence corresponding to the 5′-sequence of exon 3, indicating alternative splicing resulting in a frame shift, yielding a C-terminal truncated protein. Functional analysis of the truncated protein, however, revealed no significant TRH binding or cellular responses to TRH. Whereas this truncated receptor does not appear to be responsive to TRH, it may be a useful tool to evaluate pituitary tumors. In the rat, two forms of the TRH receptor cDNA encoding a long and a short form of the TRH receptor have been identified. Although the human gene has a high degree of homology with those of the mouse and rat, the regions corresponding to the carboxyl terminus differ completely between these species. Further genetic analyses have revealed that the human TRH gene and the mouse gene differ in their organization. That is, although the mouse TRH receptor gene possesses an intron at the same location in the 5′-untranslated region as the human gene, it does not have an intron in the transmembrane region, but possesses an intron close to the carboxyl terminus and one in the 3′-untranslated region.

Sequence analysis of the promoter region of the human TRH receptor gene revealed that, similar to other G-protein-coupled receptors (β-adrenergic, α_{1b}-adrenergic, TSH, luteinizing hormone, and gonadotropin-releasing hormone) there is no typical consensus TATA and CAAT boxes or GC-rich sequences in close proximity to the transcriptional start site. Several cis-acting regulatory elements were identified including two binding sites for Pit-1 (an anterior pituitary-specific transcription factor involved in the regulation of several anterior pituitary hormones, including TSH), two TREs and a palindromic site of the glucocorticoid response element. Thus, suggesting that the regulation of TRH receptor gene expression may share some common regulatory mechanisms with other pituitary genes.

3.2.4. Mechanisms of Action

As described above, the TRH receptor is a member of a family of G-coupled protein receptors containing a seven membrane spanning domain. In the pituitary,

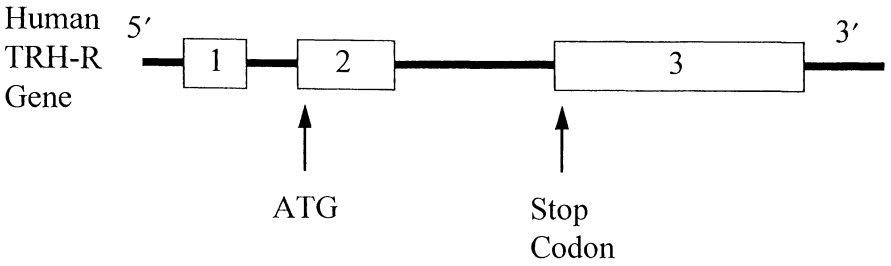

Fig. 6. Organization of the human TRH receptor gene. Exons are represented by numbered boxes. Introns are represented by dark lines. The position of the translational codon (ATG) is indicated in its relative position.

TSH binds to high-affinity binding sites on the receptor. Little is known about the cellular mechanisms activated by TRH in human thyrotrophs. In vivo and in vitro animal studies have helped elucidate the signal transduction pathways activated by TRH. Incubation of rat or mouse pituitary cells with TRH stimulates the activity of phospholipase C. Activation of phospholipase C, in turn, results in the rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-bisphosphate and 1,2-diacylglycerol, which can activate another signal transduction pathway through protein kinase C. It has been proposed, based on pharmacological studies using PKC inhibitors, that the activation of PKC plays a role in the desensitization of hormonal responses that occur because of chronic TRH exposure. Further, studies suggest that PKC may play a role in the TRH-induced decrease in TRH receptor mRNA levels found in cultured rodent pituitary cells.

In response to TRH there is a biphasic increase in intracellular free calcium. Following TRH stimulation there is an immediate, but transient, increase in intracellular free calcium. This is followed by a prolonged increase in intracellular free-calcium concentrations. In cultured rodent pituitary cells this biphasic increase in intracellular free calcium concentrations is directly related to increased secretion and electrical activity.

3.2.5. Hypothalamic Distribution of TRH

Early studies using TRH specific radioimmunoassays and microdissection of specific hypothalamic nuclei revealed that TRH content was the greatest in specific hypothalamic regions localized to the paraventricular nucleus. These early finding were confirmed, and greatly extended, by immunocytochemical analyses and by the cloning of the TRH gene. Consistent with its role as a hypophysiotropic hormone, TRH is found in great abundance in axon terminals in the median eminence and in the tuberoinfundibular system. Immunohistochemical studies have revealed that TRH positive axon terminals are primarily present in the midregion of the external zone of the median eminence. These terminals extend throughout the rostral-caudal extent of the median eminence and are juxtaposed to portal capillaries. In addition, some axons extend to the posterior pituitary where TRH can be transported to the anterior pituitary through short portal vessels.

Neurons of the parventricular neurosecretory system, which synthesize polypeptide neurohormones are localized in the medial basal hypothalamus and comprise the infundibular nucleus (the arcuate nucleus in nonprimates), periventricular zone, and parts of the ventromedial hypothalamus. There is a distinct localization for each of the hypothalamic hormones that regulate the secretion of anterior pituitary hormones. The hormones are carried by axoplasmic transport to the median eminence where they are discharged into the perivascular spaces surrounding the portal capillaries.

The neuronal cell bodies (perikarya), which give rise to TRH terminals in the median eminence, originate from the hypothalamic paraventricular nucleus. The hypothalamic paraventricular nucleus is divided into two major areas, the magnocellular and parvocellular regions. TRH producing cells are localized in small- to medium-size cells in the parvocellular region, primarily in the anterior, medial, and periventricular areas. The highest density of TRH containing cells is in the medial and periventricular areas. Many neurons in the paraventricular nucleus contain more then one peptide. TRH producing neurons, however, do not appear to contain other known peptides. The reports in the literature of coexistence of TRH with other neurohormones appears to be caused by the presence of nonspecific cross-reactive material, which interfere with immunohistochemical analysis.

Axons from TRH producing perikarya originating

in the paraventricular nucleus descend to the median eminence first by projecting laterally dorsal to the fornix and proceed through the lateral retrochiasmatic area. In addition, a small population of TRH containing neurons have been identified descending along the wall of the third ventricle to the median eminence.

4. REGULATION OF THYROTROPIN SECRETION

4.1. Neuroendocrine Regulation

The normal function of the thyroid is closely regulated in order to maintain a constant metabolic milieu. Unlike most of the regulatory systems described in this text, however, the regulation of thyroid homeostasis is more complex. Like other anterior pituitary hormones, TSH release is spontaneously episodic. Furthermore, there is a circadian rhythm with plasma levels increasing at night. The regulation of TSH secretion, and the maintenance of a euthyroid state involves an integrated response of thyrocytes to multiple signals. The current schema of the regulatory systems involved in maintaining constant concentrations of T_4 and T_3 are illustrated (Fig. 7). The primary positive regulator of TSH biosynthesis and secretion is through the release of TRH from the hypothalamus. In addition, there is an inhibitory effect on TSH release exerted by somatostatin (see below). In animal studies, administration of TRH induces a marked increase in TSH release, whereas immunoneutralization of endogenous blood-borne TRH decreases serum TSH concentrations. In contrast, administration of somatostatin decreases basal- and TRH-stimulated TSH release, whereas immunoneutralization of somatostatin increases basal- and TRH-stimulated TSH release. As with other endocrine glands, such as the adrenal cortex and the gonads, the thyroid participates in the regulation of TSH secretion. Thyroid hormones secreted in response to TSH, exert direct-negative feedback effect at the level of the pituitary and indirect effects at the hypothalamus. Estrogens also have a positive effect on serum TSH levels through an increase in TRH receptors on thyrotrophs resulting in an increase in TSH release in response to TRH. Further, several CNS structures outside of the hypothalamus have been implicated in the regulation of TSH secretion. These include limbic system structures such as the epithalamus, which may play an inhibitory role on TSH release.

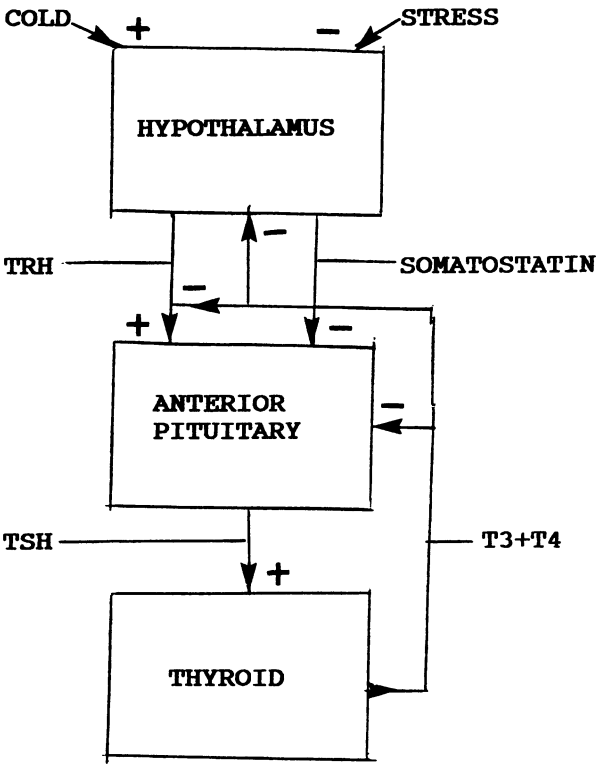

Fig. 7. This diagram illustrates functional interaction among TRH, somatostatin, and thyroid hormones (T_3 and T_4) that are involved in the neuroendocrine regulation of thyroid function.

4.2. Negative Feedback by Thyroid Hormones

There is abundant evidence for the negative-feedback effect of thyroid hormones at the pituitary and hypothalamic levels. The inhibitory effect of thyroid hormones on TSH secretion is exerted at several levels. Acute administration of thyroid hormones results in a rapid decrease in plasma TSH concentration. Chronic treatment with thyroid hormones further decreases plasma TSH concentrations and pituitary TSH content. Thus, indicating that the effect of thyroid hormones on serum TSH concentrations involves an initial inhibition of TSH secretion followed by inhibition of TSH synthesis. In the pituitary, the inhibitory effects of T_3 are directly associated with an increase in nuclear T_3 content. Administration of T_4 also decreases serum TSH concentration, which involves the intrapituitary conversion of T_4 to T_3. Quantitative analyses indicate that in the euthyroid state, about half of the nuclear T_3 concentrations are derived from the intracellular 5′-monodeiodination of

T_4 to T_3. Recent studies, however, support the concept that T_4 may have direct effects on gene transcription.

4.3. Role of Somatostatin

Hypothalamic regulation of TSH release is controlled primarily by two opposing factors—stimulation by TRH and inhibition by somatostatin. Somatostatin was original identified as an inhibitor of growth-hormone secretion. It is clear, however, that in mammals somatostatin is involved in the regulation of a number of organ systems, including the regulation of TSH secretion. In the CNS, somatostatin is secreted in two forms—a 14 amino-acid peptide (somatostatin-14) and somatostatin-28, which is a 14 amino-acid extension of somatostatin-14. Still larger forms of somatostatin-like proteins have been identified ranging in molecular weight from 11.5 to 15.7 kDa. Somatostatin is produced by posttranslational processing of a 116 amino-acid precursor, preprosomatostatin. Studies in rodents have shown that both forms of somatostatin are released into the portal blood in biologically relevant concentrations. In the hypothalamus, somatostatin containing neurons that project to the median eminence arise primarily from the preoptic region, and, to a lesser extent, from neurons in the suprachiasmatic and retrochiasmatic regions. In rodents, immunocytochemical studies have also revealed somatostatin positive cells in the ventromedial nucleus, arcuate nucleus, and lateral hypothalamus. It is unclear, however, if somatostatin in these areas plays a role in the regulation of TSH secretion.

Studies using animal models clearly show that incubation of cultured pituitary cells with somatostatin inhibit basal and TRH-induced TSH release. Further, the inhibitory effect of somatostatin is enhanced in the presence of low levels of thyroid hormones. Similarly in humans, administration of somatostatin inhibits TSH release induced by TRH or dopamine antagonist drugs, and in patients with primary hypothyroidism. Direct physiological effects have also been demonstrated in rodent models. Administration of antisomatostatin increases basal TSH concentrations and enhances TSH release in response to TRH or cold stress.

5. OTHER FACTORS REGULATING TSH SECRETION

5.1. Catecholamines

The catecholamine family is composed of dopamine, norepinephrine and epinephrine. They are formed from the dietary amino acid, tyrosine, by the following sequence of reactions:

L-tyrosine-- L-dihydroxyphenylalanine (DOPA)-- dopamine -- L-norepinephrine-- L-epinephrine

Historically, epinephrine was the first "hormone" to be isolated in crystalline form. Several lines of evidence indicate a role of catecholamines in the regulation of TSH release at the hypothalamic level, through interactions with TRH containing neurons, and at the level of the pituitary. There is clear evidence from studies in rodents indicating synaptic association of catecholamine containing neurons with TRH neurons in the periventricular nucleus. In rodents, immunohistochemical studies, using antiserum to phenylethanolamine-N-methyltransfere (PNMT), a marker specific for epinephrine, show dense innervation of PNMT positive axon terminal in the medial and parvocellular subdivisions. Similarly, immunohistochemical studies using a marker for dopamine reveal strong positive staining in the same regions. These catecholaminergic projections appear to originate from cell bodies in the medullary region. Consistent with this finding, discrete stimulation of noradrenergic cell groups in this region results in activation of tuberoinfundibular neurones in the periventricular nucleus.

Pharmacological studies using catecholamine agonists and antagonists are consistent with a role of catecholamines in the regulation of the hypothalamic-pituitary-thyroid axis. There are, however, multiple and differential effects of catecholamines on the hypothalamic-pituitary-thyroid axis. Systemic administration of adrenergic agonists or direct administration into the third ventricle, potentiate cold-induced TSH release, whereas adrenergic antagonists, or depletion of catecholamines inhibits TSH release.

In hypothalamic organ cultures, norepinephrine stimulates TRH release, thus suggesting a direct stimulatory role of norepinephrine on TRH release. In contrast, in rodent models, systemic administration of dopamine inhibits TSH release, an effect mediated through the DA_2 receptor. In cultured pituitary cells, dopamine inhibits, whereas adrenergic activation stimulates TSH secretion. Further, incubation of cultured pituitary cells with dopamine decreases TSHα- and TSHβ-subunit gene expression, thus suggesting a direct transcriptional effect of dopamine on TSH production. In humans, it is clear that dopamine inhibits TSH secretion. Whereas the site of action is unknown, studies using dopamine receptor antagonists, which have limited assess to the CNS, indicate that dopamine has a direct effect at the level of the

pituitary or the median eminence, both of which are outside of the blood-brain barrier.

In addition to effects of catecholamines at the pituitary and hypothalamic levels, studies indicate that norepinephrine, or other catecholamines can have a direct effect on thyroid function. In rodent models, in which there are no detectable levels of serum TSH, electrical or pharmacological stimulation of norepinephrine release results in histological changes in the thyroid consistent with an increase in thyroid-hormone secretion. Unilateral sympathetic nerve stimulation results in histological changes in thyroid-hormone secretion, which are restricted to the site of innervation. The effect of sympathetic-adrenergic activation most likely is a direct effect on follicular cells resulting from the release of norepinephrine in the gland. Whereas the physiological importance of sympathetic-adrenergic regulation of thyroid function is unclear, such a system would provide a means for rapid adaptation of thyroid hormone secretion to stimuli.

5.2. Serotonin

The influence of serotonin on the regulation of the hypothalamic-pituitary axis is unclear. In rodent models, serotonin fails to induce changes in basal TSH secretion. In contrast, cold-induced TSH release can be blocked by serotonin, an effect that may involve the activation of both the type 1 and type 2 serotonin receptors. In humans, there is no clear indication that serotonin agonists or receptor antagonists can affect TSH secretion. There are reports that suggest that serotonin may play a role in TRH-induced TSH release, as well as the increase in TSH secretion in primary hypothyroidism. Further, indirect evidence suggests that serotonin may be involved in the regulation of nyctohemeral TSH periodicity, however, these studies need further clarification.

5.3. Cytokines

Nonthyroidal illnesses ("Euthyroid Sick Syndrome") are associated with changes in serum thyroid hormone concentrations, which include decreased serum T_3 and increased serum reverse T_3. In its most severe form, nonthyroidal illness is associated with decreased serum T_4 concentrations. The pathogenesis of nonthyroidal illness is not well understood, however, several lines of evidence suggest that cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin, may play a role in the suppression of thyroid functions. There is clear evidence that cytokines play a role in the regulation of thyroid-hormone economy at the level of the thyroid. TNF-α is a polypeptide produced by activated macrophages and monocytes in response to various stimuli. In humans, administration of TNF-α results in decreases in serum T_3 and TSH concentrations. Administration of TNF-α to mice or rats decreases serum T_3, T_4, and TSH concentrations, iodide uptake, and TSH-induced T_3 and T_4 release. Whereas administration of TNF-α results in decreased serum T_3 concentrations, similar to those seen in patients with sick euthyroid syndrome, its mechanism(s) of action is not clearly understood. In cultured human and rat thyroid cells, TNF-α has a number of effects on thyroid function. In human thyroid cells, TNF-α decreases TSH-induced ^{125}I-incorporation and release of ^{125}I-T_3 and ^{125}I-T_4 into the medium.

Interferon-gamma also blunts the TSH-induced increases in TSH receptor mRNA, receptor number, cell growth, and the content and mRNA levels of thyroid peroxidase and thyroglobulin. In FRTL-5 rat thyroid cells, TNF-α decreases TSH-induced iodide uptake, inhibits cell growth, and enhances TSH-induced cAMP accumulation. These, in vivo and in vitro studies suggest that TNF-α and interferon-gamma have direct effects on thyroid hormone synthesis and metabolism.

It is clear that TNFα has an impact on the thyroid, however, there are no clear effects at the pituitary or hypothalamic level. In cultured rodent pituitary cells, TNFα has been reported to increase basal and TRH-induced TSH release. Other reports, however, found that TNFα inhibited, or had no effect on, TSH secretion in cultured pituitary cells. The reasons for the conflicting reports are unclear. Similarly, in vitro studies using cultured rat hypothalamic slices failed to find an effect of TNFα on basal TRH release or stimulated TRH release. Thus, current studies suggest that the impact of TNFα on thyroid-hormone economy is primarily the result of a direct effects on thyroid functions.

Interleukins (IL), together with TNF, play a major role in the inflammatory cascade. IL-1 receptors and IL-1 receptor messenger RNA are found in anterior pituitary cells of mice and in a mouse anterior pituitary tumor cell line, thus, suggesting a possible regulatory role of ILs on pituitary function. Several interleukins, such as IL-6, are putative mediators of the effects of the immune system on the endocrine axis. Several clinical trials have reported thyroid dysfunction in patients treated with IL-2. Clinical trials also found that IL-2 therapy, in combination with interferon-α, induces thyroid dysfunction, which may be associated

with TSH suppression. In cultured pituitary cells, IL-1 decrease TRH-induced TSH release. In vivo rodent models show that systemic administration of IL-1 decreases plasma TSH and T_4 concentrations. In addition, administration of IL-1 into the central nervous system decreases pro-TRH gene expression in periventricular neurons. In the rat, acute and chronic IL-1 treatment decreases TSH secretion, pro-TRH, and TSHβ messenger RNA levels. Interestingly, the acute decrease in serum TSH concentrations following administration of IL-1 occurred before decreases in pro-TRH or TSHβ messenger mRNA were observed. This suggests that the decrease in serum TSH concentration is not caused by decreases in hypothalamic TRH or pituitary TSH gene expression. Further, in hypothyroid rats, IL-1 decreased plasma TSH levels, thus indicating that the effect of IL-1 on TSH secretion is not the result of increased T_3 uptake or increased intrapituitary deiodination of T_4 to T_3. Together, current studies support a role of ILs in the regulation of thyroid function and suggest that ILs may play a regulatory role in nonthyroidal illnesses. The cellular mechanisms involved, however, remain to be established.

5.4. Neuropeptide Y

Neuropeptide Y is a member of the pancreatic polypeptide family, which also contains a specific molecule known as pancreatic polypeptide. The difference between pancreatic polypeptide and neuropeptide Y is that the pancreatic peptide contains a tyrosine residue at the C-terminal, whereas neuropeptide Y contains tyrosine residues at the C- and N-terminal ends. Neuropeptide Y can function as both neuronal and hormonal messengers. In the brain, neuropeptide Y is concentrated in limbic structures, including the hypothalamus and in many cells is colocalized with catecholamines. In the hypothalamus, neuropeptide Y nerve terminals innervate TRH-containing neurons in the periventricular nucleus where they are in contact with dendrites and perikarya. Electron microscopy has shown that neuropeptide Y terminals form synapses in the periventricular nucleus. The neuropeptide Y projections which terminate in the periventricular nucleus arise from cell bodies in the medulla oblongata and arcuate nucleus. Interestingly, neuropeptide Y terminals originating from the medulla coexist with catecholamines, but not terminals from the arcuate nucleus. The association of neuropeptide Y with TRH containing neurons thus suggests a possible role of neuropeptide Y on the regulation of TRH synthesis and release. Consistent with this hypothesis central administration of neuropeptide Y inhibits TSH secretion.

6. EFFECTS OF TEMPERATURE AND FASTING ON THE HYPOTHALAMIC-PITUITARY-THYROID AXIS

6.1. Temperature

In rodent models, cold exposure results in rapid increases in serum TSH concentration, TRH gene expression in the periventricular nucleus, and TRH release. In human neonates, similar responses to cold exposure are observed, however, in the adult a similar response is limited to extreme exposure to cold. The release of TSH in response to cold exposure in rodents is the result of increased TRH release, which is mediated by adrenergic mechanisms. Hypothalamic regulation of cold-induced TSH release is supported by studies showing that antiserum directed against TRH or α-adrenergic blockade inhibits cold-induced TSH release.

6.2. Fasting

Studies in rodents show that prolonged fasting has profound effects on the hypothalamic-pituitary thyroid axis. During fasting, serum T_4, T_3, and estimated free T_4 and T_3 concentrations are reduced, thus reducing thermogenesis and preserving nitrogen stores. Despite the changes in serum thyroid-hormone levels, serum TSH concentrations are unchanged or reduced and TRH-induced TSH secretion is enhanced during fasting. The lack of a compensatory rise in basal plasma TSH concentrations in the face of decreased plasma thyroid-hormone concentrations, together with an increased TSH response to TRH indicate that in rodents, food deprivation results in tertiary hypothyroidism. This could be a result of a decrease in the stimulatory input by TRH, or some other agent, and not by inhibition of TSH release at the pituitary. If inhibition was at the level of the pituitary, for example increased sensitivity to thyroid-hormone feedback or somatostatin, then the response to exogenous TRH would be inhibited. Consistent with fasting-induced tertiary hypothyroidism, in the rodent, fasting results in decreased proTRH messenger RNA levels in hypothalamic paraventricular neurons and decreased portal blood TRH concentration. Recent studies suggest a mechanism for the induction of tertiary hypothyroidism in fasting animals. Leptin is a protein hormone of the ob/ob gene and is synthesized and secreted by adipocytes in proportion to

adiposity in rats and humans. Leptin receptor messenger RNA is present in the CNS and administration of leptin increases cfos levels in many hypothalamic neurons, including the paraventricular nucleus. Interestingly, whole cell recording techniques show that leptin depolarizes neurons in the paraventricular nucleus. Further, aministration of leptin blunts fasting-induced decreases in thyroid hormones, increases proTRH messenger RNA in hypothalamic paraventricular neurons, and inhibits hypothalamic somatostatin secretion and somatostatin mRNA levels. Whereas these studies suggest a direct effect of leptin at the hypothalamic level, it remains to be determined if leptin acts directly at hypothalamic neurons to decrease proTRH gene expression, or indirectly through the activation of another hypothalamic neurohormone.

In humans, the fasting-induced changes in the hypothalamic-thyroid axis are different then those described in rodents. As a result of fasting in humans, serum T_3 decreases, serum rT_3 increases, and T_4 remains unchanged or decreases only slightly. Conflicting reports on the effects of fasting on basal and TRH-induced TSH release have been presented, however, with the majority of studies indicate that basal and TRH-induced TSH secretion are decreased during fasting. Thus, in contrast to studies in rodents, fasting-induced inhibition in TSH secretion in humans appears to occur at the level of the pituitary. The reasons for this difference are unclear, but may be the result of the depletion of adipose tissue store which is more severe in the rodent. This is consistent with recent studies showing that leptin is involved in the regulation of fasting-induced changes in thyroid function.

SELECTED READINGS

Aristotle. On the parts of animals. Book II, Chap. 7. Berlin Nos. 652a, 24; 652b, 25-35, 653a, 1–5.

Bailey P, Bremer F. Experimental diabetes insipidus, Arch Internal Med 1921; 28:773–781.

Balkan W, Tavianinni MA, Gkonos PJ, Roos BA. Expression of rat thyrotropin-releasing hormone (TRH) gene in TRH-producing tissues of transgenic mice requires seequences located in exon 1. Endocrinology 1998; 139:252–259.

Bogazzi F, Bartalena L, Brogioni S, Burelli A, Grasso L, Dell'Unto E, Manetti L, Martino E. Mol Cell Endocrinol 1997; 134:23–31.

Burgus R, Dunn TF, DeSiderio D, Ward DW, Vale W, Guillemin. Characterization of ovine hypothalamic hypophysiotropic TSH-releasing factor. Nature (London) 1970; 226, 312.

Frohlich A. Ein Fall von Tumor der Hyophysis Cerebri ohne Acromegalie. Wien, Klin, Ruundsch., 47–48, 1901.

Galen, De Usu Partium. Book 9, Chap. 4. De Usu Pulsum. Chap. 2.

Gross B, Misrahi M, Sar S, Milgrom E. Composite structure of the human thyrotropin receptor gene. Biochem Biophy Res Comm 1991; 177:679–687.

Iwasaki T, Yamada M, Satoh T, Konaka S, Ren Y, Hashimoto K, Kohga H, Kata Y, Mori M. Genomic organization and promoter function of the human thyrotropin-releasing hormone receptor gene. J Biol Chem 1996; 271:22,183–22,188.

Legradi G, Emerson CH, Ahima RS, Flier JS, Lechan RM. Leptin prevents fasting-induced suppression of prothyrotropin-releasing hormone messenger ribonucleic acid in neurons of the hypothalamic paraventricular nucleus. Endocrinology 1997; 138:2569–2576.

Lower R. Dissertatio de origine catarrhi in qua ostenditur illum non provenire a cerebro. In: Tractatus de corde. Londini, typ. J. Redmayne, 1670.

Marie P. Sur deux cas d'acromegale. Hepertrophie singuliere nooncongenitale des extremite superieures inferieures et cephaliques. Rev Med 1886; 6:297–333.

Nair RMG, Barrett JF, Bowers CY, Schally AV. Structure of porcine thyrotropin releasing hormone. Biochemistry 1970; 9:1103.

Quintela M, Senaris R, Heiman ML, Casanueva FF, Dieguez C. Endocrinology 1997; 138:5641–5644.

Scharrer E, Scharrer B. Neuroendocrinology, New York: Columbia University Press, 1963.

Schreiber CV. Dissertatio de osse cribriforme, et sensu ac organo odoratus. Wittebergae, Mevii, 1655.

Wondisford FE, Randovick S, Moates MM, Usala S, Weintraub BD. Isolation and characterization of human thyrotropin β-subunit gene. J Biol Chem 1988; 263:12,538–12,542.

Yamada M, Radovick S, Wondisford FE, Nakayama Y, Weintraub BD, Wilber JF. Cloning and structure of human geneomic DNA and hypothalamic cDNA encoding human prepro thyrotropin-releasing hormone. Mol Endocrinology 1990; 4:551–556.

14 The Neuroendocrinology of Fluid Balance

The Kidney

Leonard Share, PhD and Edward G. Schneider, PhD

CONTENTS

1. INTRODUCTION

As was first pointed out by Claude Bernard over 120 years ago, the volume and composition of the body fluids (the "milieu intérieur") is maintained remarkably constant. This homeostasis of the body fluids is achieved through control of both intake and renal excretion of salt and water. Yet, even though intake may vary considerably because of availability and social factors, the volume and sodium concentration of the extracellular fluid are still maintained relatively constant because of the multiple factors that control the renal handling of water and sodium.

In this chapter, we will focus on those factors that govern the renal handling of water and sodium that are under neuroendocrine control. Primary attention will be given to the control and renal actions of vasopressin (ADH, AVP, the antidiuretic hormone), which largely determines the ability of the kidney to conserve water, and the renin-angiotensin-aldosterone system, a major element in determining the renal excretion of sodium. Although the atrial natriuretic peptide also plays an important role in the renal handling of sodium, it does not appear to be under neural control and will not be considered here.

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2. VASOPRESSIN

2.1. Overview

Vasopressin is a nonapeptide with a molecular weight of 1084. It is synthesized in the cell bodies of magnocellular neurons in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus, where it is packaged along with neurophysin into neurosecretory vesicles. These vesicles are transported along the axons of these neurons to axon endings in the posterior lobe of the pituitary where they are stored. Stimuli for vasopressin release, acting via neural inputs to the PVN and SON, result in depolarization of these neurons and the release of vasopressin and its accompanying neurophysin into the circulation via the process of exocytosis. A physiological role for circulating neurophysin has not been demonstrated. The primary physiological stimuli for the release of vasopressin are an increase in the osmotic pressure of the plasma and reductions in blood pressure and volume. Other stimuli, such as nausea and "stress," may also affect vasopressin release. Vasopressin is also synthesized in extrahypothalamic sites in the brain, apparently for action as a neurotransmitter or neuromodulator. The role of extrahypothalamic vasopressin will not be considered in this chapter.

Vasopressin can increase reabsorption of water by

the kidney by activating V_2 receptors on the distal nephron. Vasopressin is also a potent vasoconstrictor as a result of activation of V_{1a} receptors on vascular smooth muscle. These receptors have been cloned, and much is known about their intracellular signaling pathways.

2.2. What Are the Elements That Participate in the Control of Vasopressin Release?

2.2.1. Vasopressin Release is Extremely Sensitive to Changes in Plasma Osmolality

The ability of the kidney to conserve water, i.e., to excrete a urine that has a greater solute concentration than the plasma, is due primarily to vasopressin. When the ability to synthesize vasopressin is impaired, usually because of disease or trauma to the head, the kidney excretes a copious dilute urine and water turnover is greatly increased. This condition is called diabetes insipidus. It can be corrected by treatment with an analog of vasopressin that has antidiuretic activity but little or no cardiovascular activity, e.g., desmopressin (dDAVP).

The release of vasopressin from the posterior pituitary is directly related to the osmolality of the plasma. The basic elements in our current concepts of the osmotic control of vasopressin release were largely provided by the early work of E. B. Verney and his colleagues, culminating in a report by Verney in 1947. He demonstrated that increases in the osmotic pressure of the plasma of as little as 1–2% stimulated the release of vasopressin from the posterior pituitary sufficiently to cause an antidiuresis and that the receptors that sense these changes in plasma osmolality, the osmoreceptors, reside in the head. Research in more recent years has focused on identifying the specific location of the osmoreceptors, quantifying the relationship between plasma osmolality and the rate of vasopressin release, and examining factors that can modulate this relationship.

2.2.1.1. Central Osmoreceptors Play an Essential Role in the Control of Vasopressin Release. Although the magnocellular neurons of the PVN and SON are osmosensitive, studies of the effectiveness of different types of solutes in plasma in stimulating vasopressin release have demonstrated that the primary osmoreceptors in the brain lie outside the blood-brain barrier. Thus, increases in plasma concentrations of impermeant solutes such as sodium ions and nonmetabolizable sugars stimulate vasopressin release, whereas an increase in the plasma concentration of urea, which readily penetrates cell membranes but not the blood-brain barrier, is ineffective. Physiologically, it is primarily the plasma sodium chloride concentration that determines the "effective" plasma osmolality and the osmotic stimulus for vasopressin release.

The demonstration that lesions of the anterior wall of the third ventricle (the AV3V region) disrupt the osmotic control of vasopressin release indicates that the osmoreceptors reside in that region. Subsequent studies, involving discrete electrolytic lesions of the organum vasculosum of the lamina terminalis (OVLT) and the subfornical organ (SFO), circumventricular organs that lack a blood-brain barrier, suggest that the osmoreceptors are in these structures. Because a reduction in plasma osmolality from basal levels results in a decreased release of vasopressin and a reduction in urine osmolality, basal plasma osmolality is above the threshold for activation of the osmoreceptors and stimulation of vasopressin release.

There is now considerable evidence that the median preoptic nucleus, a midline structure dorsal to the AV3V region, may serve as a way station and integrating center for the transmission of information from osmoreceptors in the AV3V region to the vasopressinergic magnocellular neurons in the SON and PVN. Lesions of the median preoptic nucleus impair osmotic stimulation of vasopressin release. Electrophysiological and neuroanatomical studies have demonstrated direct projections from the OVLT and SFO to the median preoptic nucleus and from this nucleus to the SON and PVN.

2.2.1.2. Do Peripheral Osmoreceptors Participate in the Control of Vasopressin Release? For some years there has been interest in the possibility that there are osmoreceptors in the portal or splanchnic vasculature that can sense the reabsorption of water and solute from the gastrointestinal tract and affect vasopressin release. Early studies were controversial and, in some cases, were hampered by inadequate methods for the measurement of the plasma vasopressin concentration. More recently, Baertschi et al. have presented more convincing evidence that these receptors exist and may participate in the control of vasopressin release. Thus, in conscious, chronically instrumented rats under circumstances in which systemic

plasma osmolality was unchanged, the infusion of hypertonic saline solutions into the stomach increased the plasma vasopressin concentration, whereas the intragastric infusion of hypotonic salt solutions decreased vasopressin release. The splanchnic osmoreceptors that sense increased osmolality are innervated by the right and left major splanchnic nerves. Central pathways involve the area postrema, the nucleus tractus solitarius (NTS), the ventral noradrenergic bundle, and the median preoptic nucleus. Splanchnic receptors that sense decreased osmolality resulting from absorption of water from the gut are innervated by the vagus. The splanchnic osmoreceptors apparently signal the release of vasopressin in response to the tonicity of fluids absorbed from the gut.

When dehydrated subjects are allowed to drink, there is an initial, very rapid fall in the plasma vasopressin concentration that occurs before there is any change in systemic plasma osmolality. Experiments in animals with a gastric fistula indicate that this initial vasopressin response to drinking is because of activation of oropharyngeal receptors, rather than to receptors in the stomach. This response is independent of the taste, osmolality, or temperature of the solutions ingested and is so rapid as to suggest complete inhibition of vasopressin release. More specific characterization of these receptors remains to be accomplished.

In dehydrated humans, there was an initial rapid fall in plasma vasopressin levels after both ad libitum drinking and ad libitum drinking and simultaneous removal of the ingested water from the stomach by a nasogastric tube. The latter effect was smaller than the former, suggesting the involvement of factors in addition to oropharyngeal receptors in the initial inhibition of vasopressin release. The nature of these "other factors" is uncertain because the installation of water directly into the stomach was without effect.

The splanchnic and oropharyngeal osmoreceptors, along with the central osmoreceptors, provide redundancy in the osmotic control of vasopressin release. However, because it is only the central osmoreceptors that sense the osmolality of the extracellular fluid, it is these receptors that bear the primary burden for the osmotic control of vasopressin release and the maintenance of the constancy of the osmotic pressure of the body fluids.

2.2.1.3. What Are the Quantitative Relationships Between Plasma Osmolality and the Plasma Vasopressin Concentration? There have been numerous studies in humans and experimental animals of the relationship between the plasma vasopressin concentration and plasma osmolality, when the latter is varied by the IV or intraperitoneal administration of hypertonic sodium chloride solutions, water deprivation, or the administration of a water load. Although it is questionable as to whether this relationship is rectilinear, particularly at the extremes, linear regression analysis of the data has proven to be practical and useful. When this is done, the intercept on the plasma osmolality axis is taken to be the threshold for the osmotic stimulation of vasopressin release, and the slope of the relationship is considered to be the sensitivity of osmotic control of vasopressin release. The osmotic threshold in humans is between 280 and 285 mOsm/kg H_2O, but estimates of osmotic sensitivity are quite variable. The measured osmotic threshold and sensitivity are influenced by many factors, e.g., species, gender, phase of the estrous or menstrual cycle, hydrational status of the subject, age, the method used to change plasma osmolality, and the rate of change of plasma osmolality. Regardless of the exact quantitative relationships, relatively small changes in plasma osmolality result in changes in the plasma vasopressin concentration that are significant in determining water reabsorption by the kidney. The methods that are currently available for the measurement of the plasma vasopressin concentration are not sufficiently sensitive to determine whether vasopressin release from the pituitary is completely turned off below the osmotic threshold. Although the methods available for the determination of osmotic threshold and sensitivity have limitations, these measurements are useful in the study of physiological and pathophysiological factors that affect the osmotic control of vasopressin release.

The relationship between the plasma vasopressin concentration and urine osmolality is extremely steep. In humans, maximum urine osmolality is achieved at a plasma vasopressin concentration of approximately 4 pg/mL. One pg is approximately equal to one fM and 0.4 μU.

2.2.2. Changes in Blood Pressure and Blood Volume Contribute to the Control of Vasopressin Release

The regulation of extracellular volume and composition and the control of the cardiovascular system are intimately interconnected. One common element in these regulatory systems is vasopressin, which both

promotes water reabsorption by the kidney and is a potent vasoconstrictor. In the latter context, reductions in arterial blood pressure and blood volume are potent stimuli for the release of vasopressin. A reduction in blood volume of at least 10–20% is necessary to increase vasopressin release. Although an increase in plasma osmolality of only 1–2% is an effective stimulus, the increase in plasma levels of vasopressin after hemorrhage can be many times that after even very large increases in plasma osmolality.

The effects of changes in blood pressure and blood volume on vasopressin release have been studied repeatedly over many years in many species and in a variety of experimental situations. The issues of interest are determination of whether changes in blood volume independent of changes in blood pressure can effect changes in vasopressin release, identification of the receptors that are involved in the blood pressure–blood volume control of vasopressin release, and determination of the physiological role of the vasoconstrictor action of vasopressin release. The last of these issues is beyond the purview of this chapter.

2.2.2.1. What Are the Receptors That Are Involved in the Blood Pressure-Blood Volume Control of Vasopressin Release? There are two groups of receptors in the cardiovascular system that can affect vasopressin release, the arterial baroreceptors and the cardiac or cardiopulmonary receptors, sometimes referred to as the high- and low-pressure receptors. The arterial baroreceptors are divided into two main groups, receptors in the carotid sinuses, innervated by the glossopharyngeal nerve, and in the arch of the aorta, innervated by the vagus. Unloading the carotid sinus baroreceptors in experimental animals, e.g., by occluding both common carotid arteries results in an increased release of vasopressin, but only when the vagi are sectioned. It is likely that when the vagi are intact, the increase in arterial blood pressure that results from carotid occlusion activates the aortic arch baroreceptors, which counteract the effects on vasopressin release of decreased activity of the carotid sinus baroreceptors. Conversely, activating the carotid sinus baroreceptors by an increase in arterial pressure inhibits vasopressin release. Because of their location, it is difficult to study the aortic arch baroreceptors, but it is likely that they function in a manner similar to carotid sinus baroreceptors in affecting vasopressin release. The arterial baroreceptors are tonically active in their inhibition of vasopressin release, so that a reduction in arterial blood pressure from basal levels will result in an increased secretion of vasopressin.

The question of the role of the cardiac receptors has generated considerable controversy. The evidence is convincing that activation of stretch receptors in the left atrium of the dog inhibits vasopressin release. It is uncertain, however, whether these receptors are tonically active, so that decreased left atrial volume, a consequence of a reduced blood volume, will result in decreased inhibition of vasopressin release and an increase in the plasma vasopressin concentration. The observation that there is an increased release of vasopressin when the cardiac nerves are blocked and the resulting increase in arterial blood pressure is prevented suggests that the cardiac receptors are tonically active. Attempts to unload selectively the cardiac receptors by maneuvers such as constriction of the thoracic inferior vena cava in dogs and lower body negative pressure and head-up tilt in humans have produced conflicting results. In some studies, selective unloading of the cardiac receptors resulted in increased plasma vasopressin levels without a concomitant fall in arterial blood pressure. In other reports, plasma vasopressin increased only when there was a reduction in mean arterial pressure or arterial pulse pressure, suggesting that the vasopressin response was because of unloading of the arterial baroreceptors rather than the cardiac receptors. Be that as it may, it does seem likely that under certain circumstances, for example, reduced blood volume owing to dehydration, changes in activity of cardiac receptors can contribute to changes in vasopressin release.

Although it is generally considered that the cardiac receptors that are involved in the control of vasopressin release are left atrial stretch receptors, receptors in the left ventricle may also be involved.

2.2.2.2. What Are the Relative Roles of the Arterial and Cardiac Receptors in the Blood Pressure–Blood Volume Control of Vasopressin Release? A question of considerable physiological interest and one that has been debated for many years is which group of receptors is responsible for the increased release of vasopressin that results from a reduction in blood volume. This issue has been studied most extensively in the dog, and the findings are conflicting. In some studies utilizing continuous or stepwise hemorrhage, the plasma vasopressin concentration

increased before arterial blood pressure fell and was accompanied by a reduction in central venous pressure. This suggests that the vasopressin response was triggered by the cardiac receptors, responding to decreased left atrial volume and stretch. In other studies, plasma vasopressin did not begin to rise until arterial pressure fell, suggesting an essential role for the arterial baroreceptors. Findings in studies in which the cardiac or arterial receptors were selectively denervated have also been conflicting. Wang et al. (1983) reported that cardiac denervation almost completely blocked the stimulation of vasopressin release by hemorrhage; but in other studies, acute blockade of the cardiac nerves or chronic surgical denervation of the heart had no effect on the vasopressin response to hemorrhage. Shen et al. (1991) reported that neither denervation of the sinoaortic baroreceptors nor denervation of the heart individually had a significant effect on the stimulation of vasopressin release by hemorrhage. Surprisingly, when these two procedures were combined, the vasopressin response was blunted, but not eliminated. Thus, at least under the conditions of these experiments, the presence of either the arterial baroreceptors or the cardiac receptors was sufficient to maintain the vasopressin response to a reduction in blood volume. It would appear that elements in addition to unloading of the arterial and cardiac receptors may participate in the stimulation of vasopressin release when blood volume is reduced.

Redundancy in the blood pressure–blood volume control of vasopressin release makes it difficult to evaluate the role of the cardiac and arterial receptors in this control. It is likely, however, that both groups of receptors can contribute to this control, but that their relative importance may vary with species, the specifics of experimental design, and the status of the subjects with respect to body fluid volume and osmolality. The possibility that factors in addition to the arterial and cardiac receptors may have a significant role in the blood pressure–blood volume control of vasopressin release has not been adequately studied.

2.2.3. The Renin-Angiotensin System Participates in the Control of Vasopressin Release

The renal actions of vasopressin and the renin-angiotensin-aldosterone system are major elements in the neuroendocrine regulation of water and electrolyte metabolism. Although the question of interactions between these two systems at the level of the kidney has received little attention, they do interact in the control of their release. Thus, circulating vasopressin, at physiological levels, can inhibit the release of renin from the kidney, and the renin-angiotensin system participates in the control of vasopressin release. For the latter, both the brain renin-angiotensin system and circulating angiotensin II (Ang II) need to be considered.

2.2.3.1. What Is the Role of the Brain Renin-Angiotensin System in the Control of Vasopressin Release? Several lines of investigation indicate that Ang II generated within the brain participates in the control of vasopressin release. First, it has been shown repeatedly that the intracerebroventricular administration of Ang II stimulates vasopressin release. Second, all of the components of the renin-angiotensin system have been found in the brain. Third, angiotensinergic pathways have been demonstrated to project from regions in the brain that contain the osmoreceptors to the PVN and SON via the median preoptic nucleus (Tanaka, 1989). Fourth, angiotensin AT_1 receptors have been demonstrated in brain sites that are involved in the control of vasopressin release, e.g., the subfornical organ (SFO), the organum vasculosum of the lamina terminalis (OVLT), the SON, the PVN, the NTS, and the area postrema. Apparently, angiotensin generated in the brain does not entirely act directly on the vasopressinergic magnocellular neurons of the PVN and SON to stimulate vasopressin release, but may involve in part a catecholaminergic link. Thus, the microinjection of an alpha-1 adrenoreceptor antagonist into the PVN markedly reduced the vasopressin response to intracerebroventricular Ang II, and there is no expression of AT_1 receptor mRNA in vasopressinergic neurons of the PVN and SON.

The brain renin-angiotensin system participates in the osmotic control of vasopressin release. Thus, angiotensin receptor antagonists given intracerebroventricularly attenuate the stimulation of vasopressin release by increased plasma osmolality. Both AT_1 and AT_2 receptors appear to be involved in this effect, but how these two receptor types interact is uncertain. On the other hand, the preponderance of evidence indicates that central blockade of components of the renin-angiotensin system is without effect on the stimulation of vasopressin release by a reduction in blood volume or blood pressure.

2.2.3.2. What Is the Role of Circulating Angiotensin II in the Control of Vasopressin Release? Whether circulating Ang II plays a role in the control

of vasopressin release is uncertain. Thus, there is considerable disagreement as to whether the IV administration of Ang II stimulates vasopressin release. Contributing factors to the disagreement are the use of anesthesia, hydrational status, and species. Even in experiments in conscious dogs, in which IV infusion of Ang II did increase plasma levels of vasopressin, large, pathophysiological doses of Ang II were required. The possibility that the pressor response to the angiotensin, by activating baroreceptors, attenuated its stimulatory action on vasopressin release was not supported in experiments in dogs that had been subjected to denervation of the cardiac and arterial baroreceptors.

The stimulation of vasopressin release by hemorrhage is not attenuated by IV converting enzyme blockade. We are not aware of any studies of the effects of blockade of the systemic renin-angiotensin system on the osmotic stimulation of vasopressin release.

Thus, on the one hand, brain angiotensinergic pathways are clearly essential elements in the osmotic control of vasopressin release. On the other hand, there is little evidence to support an important role for circulating angiotensin in vasopressin control, even though the circumventricular organs, which lack a blood-brain barrier, have Ang II receptors and have neuronal connections to the vasopressin neurosecretory cells in the PVN and the SON.

2.2.4. Glucocorticoids Participate in the Control of Vasopressin Release

It is well known that in adrenal insufficiency, there is an impaired ability to excrete a water load. The clinical picture is similar to that of the syndrome of inappropriate secretion of antidiuretic hormone (SIADH). In both experimental animals and humans with untreated adrenal insufficiency, the plasma vasopressin concentration is elevated relative to plasma osmolality. When subjects with untreated adrenal insufficiency are given a water load, the fraction of the load excreted is decreased, and the abilities to reduce the plasma vasopressin concentration and to dilute the urine are impaired. These defects are corrected by treatment with glucocorticoids, suggesting that glucocorticoids can inhibit vasopressin release. Indeed, in animals with normal adrenal function, treatment with glucocorticoids attenuated the stimulation of vasopressin release by increased plasma osmolality and by hypotension. Glucocorticoids can decrease the sensitivity of the osmotic control of vasopressin release and may also increase the osmotic threshold. Thus, the impaired water metabolism in adrenal insufficiency may be largely due to an impaired ability of the osmoreceptors to respond appropriately to a reduced plasma osmolality as a consequence of glucocorticoid deficiency. Other, nonosmotic factors, e.g., decreased blood volume and hypotension, may also contribute to the inappropriately elevated plasma vasopressin concentration.

The mechanisms by which glucocorticoids affect vasopressin release are uncertain. Whereas there are glucocorticoid receptors in the parvocellular vasopressinergic neurons of the PVN, which project to the median eminence and which participate in the control of ACTH secretion, most investigators have failed to demonstrate glucocorticoid receptors in the vasopressinergic magnocellular neurons of the PVN and SON that are responsible for the release of vasopressin into the circulation. On the other hand, Berghorn et al. (1995) found that chronic hyposmolality in the rat induced the expression of glucocorticoid receptors in hypothalamic magnocellular vasopressinergic neurons of the rat. Although glucocorticoids can inhibit vasopressin release from the posterior pituitary, their effect on vasopressin synthesis in magnocellular neurons is uncertain.

Thus, the ability of glucocorticoids to inhibit vasopressin release may not necessarily involve a direct action on vasopressinergic magnocellular neurons of the SON and PVN, but rather actions at other sites in the neuronal circuitry responsible for the control of vasopressin release, perhaps, for example, the osmoreceptors. Furthermore, although the elevated plasma vasopressin concentration in adrenal insufficiency is due in part to a lack of glucocorticoid inhibition of vasopressin release, the physiological role of glucocorticoids in the control of vasopressin release in individuals with normal adrenocortical function is not readily apparent.

2.3. What Are the Renal Actions of Vasopressin?

2.3.1. What Are the Effects of Vasopressin on Urine Output?

The ability of vasopressin to adjust urine flow and osmolality to the water balance requirements of an

individual has allowed us to roam the far corners of the earth and even venture out into space; but we never venture too far from a source of water because the kidney can only conserve water; it cannot generate new water. It is remarkable that one hormone, acting on the water and urea permeability characteristics of the terminal portion of the distal nephron and collecting ducts, is responsible for determining how our kidneys respond to whatever water challenges exist.

In the absence of vasopressin, the kidney will produce a large volume of dilute urine. This occurs whether the low levels of vasopressin are the response to an excess intake of water or some other enjoyable dilute solutions or are the result of some pathophysiological suppression of the ability to secrete vasopressin (central diabetes insipidus). In the former case, the renal response to the low levels of vasopressin quickly helps the individual excrete the excess water and prevents any significant alterations in either total body water or plasma osmolality. In the latter case, the defect causes the loss of a large volume of dilute urine, leading to body fluid volume contraction and an elevation in plasma osmolality. A similar situation can also occur when the kidneys lack the ability to respond to vasopressin (nephrogenic diabetes insipidus). Only by ingesting water will individuals with these defects be able to compensate for the loss of water and maintain total body water and osmolality near normal levels.

In the presence of high levels of vasopressin, the kidney will produce a small volume of concentrated urine. This occurs whether the high levels of vasopressin are the result of a physiological response to water deprivation or of pathophysiological increases in vasopressin because of either severe plasma volume depletion or the inappropriate secretion of vasopressin (SIADH) from some source. In the former case, the renal response to the high levels of vasopressin helps the individual minimize the further loss of water until water ingestion can correct the dehydration. In the latter cases, however, the defect causes the individual to retain ingested water and leads to an increase in total body water and a decrease in plasma osmolality (hyponatremia). Thus, the concept of neuroendocrine regulation of water balance involves an understanding not only of the factors that control vasopressin secretion but also of how vasopressin affects the kidney.

2.3.2. The Countercurrent Mechanism Makes It Possible for the Kidney to Excrete a Dilute or Concentrated Urine

The ability of the kidney to vary urine osmolality from less than 100 mOsm/kg of H_2O at a high rate of urine flow to levels in excess of 1200 mOsm/kg of H_2O at a low rate of urine flow requires a renal countercurrent mechanism and a variable level of water and urea permeability in the late distal nephron and collecting ducts. Figure 1 illustrates the essential features of these renal processes. The left side of the figure shows the effect of a low level of vasopressin on the kidney; the right-hand side presents the effect of a high level of vasopressin. Before continuing, it is first important to review the basic characteristics of each of the major nephron segments and the roles that they have in the formation of both dilute and concentrated urines.

2.3.2.1. What Are the Characteristics of the Individual Nephron Segments That Contribute to the Dilution or Concentration of the Urine?

2.3.2.1.1. The Glomerulus and Proximal Tubule. The glomerulus and the proximal tubule, although essential for normal urine formation, do not contribute uniquely to the formation of either a dilute or a concentrated urine because they maintain the osmolality of the tubular fluid isosmotic to systemic plasma under both circumstances.

2.3.2.1.2. Thin Descending Limb of the Loop of Henle. This segment of the nephron is permeable to water and impermeable to sodium, chloride, and urea. Thus, as the tubular fluid travels down the descending limb through an interstitium that is increasingly hypertonic, water is reabsorbed, causing the sodium and chloride concentrations to increase until the osmolality of the fluid is similar to the osmolality of the adjacent interstitial fluid.

2.3.2.1.3. Ascending Limb of the Loop of Henle. The ascending limb is more complex because it has two parts, a thick and a thin segment. All nephrons have a thick ascending limb of the loop of Henle that begins at the juncture of the inner and outer medulla and ends at the macula densa, which is in the cortex where the distal tubule touches its glomerulus. The thin ascending limb of Henle's loop is found in the juxtamedullary nephrons (approx 20% of the nephrons in the human kidneys); it begins at the bend of

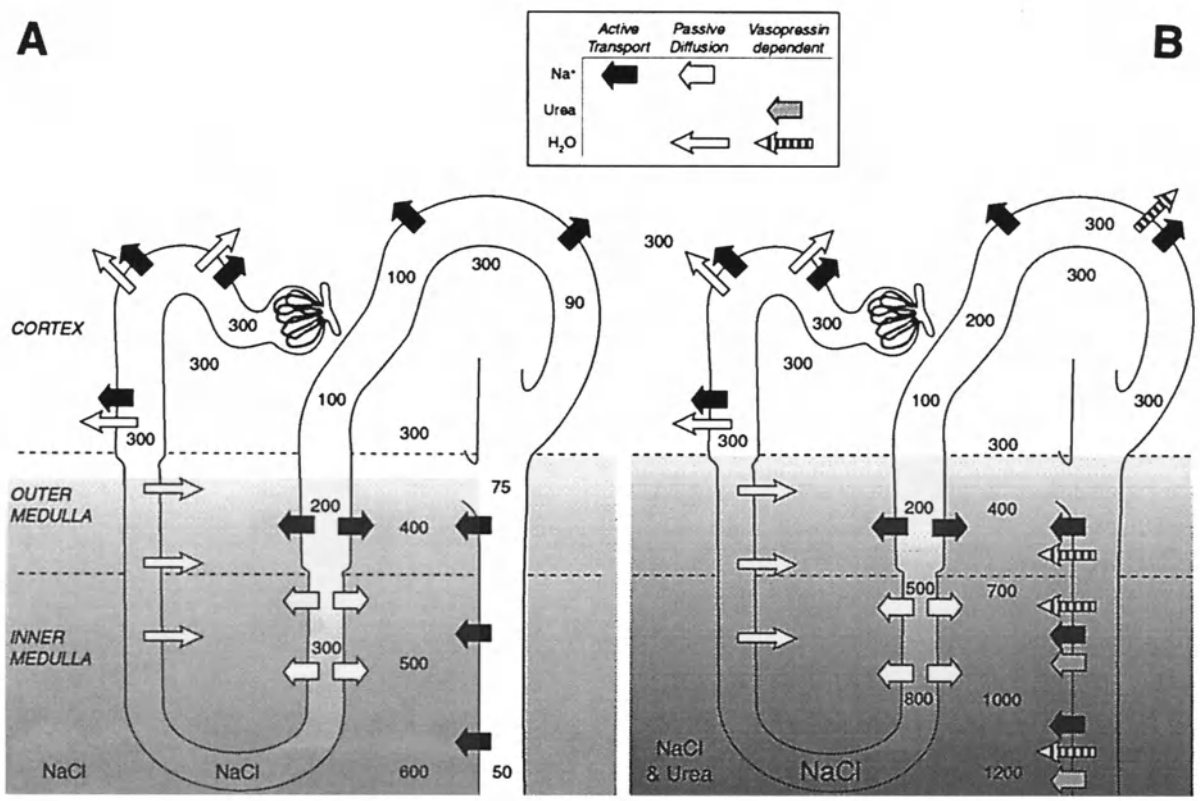

Fig. 1. Panel A shows the osmotic profile of the kidney during the production of a dilute urine. The absence of vasopressin causes the late distal nephron and the entire collecting duct to be impermeable to water and urea. Under this circumstance, the osmotic gradient in the inner medulla is due primarily to NaCl with little contribution made by urea. Panel B shows the osmotic profile of the kidney during the production of a concentrated urine. The presence of vasopressin causes the distal nephron and entire collecting duct system to be permeable to water and the inner medullar collecting duct to be permeable to urea. Under this circumstance, the osmotic gradient in the inner medulla is caused by both NaCl and urea.

the loop of Henle and ends at the juncture of the inner and outer medulla by becoming the thick ascending limb. The precise point at which the bend is located within the inner zone of the medulla varies, with only a few nephrons having their bend at the very tip of the papilla. All ascending limbs have one feature in common: they are impermeable to water even in the presence of vasopressin. This segment also transports sodium and chloride (osmotic particles) from the lumen into the interstitium. This is the driving force for the generation of the medullary osmotic gradient and is referred to as the "single effect."

The mechanism responsible for transporting sodium and chloride from the lumen into the interstitium is different for the thin and thick ascending limbs. No active transport process in the thin limb of Henle's loop has been found for moving sodium and chloride from the lumen into the interstitium. The thin segment, however, is permeable to sodium and chloride, but relatively impermeable to urea and water. Thus, sodium and chloride diffuse passively down the chemical gradient that exists between the lumen and the interstitium, but no significant movement of urea or water occurs. For the thick ascending limb, there is a Na-K-2 Cl cotransporter located on the apical membranes. Via this cotransporter, sodium, potassium, and two chloride ions enter the cells, using the steep electrochemical gradient for sodium as the driving force. The sodium is actively pumped into the interstitium via the basolaterally located Na:K ATPase. The chloride and potassium ions are extruded into the interstitium either via chloride or potassium channels or via K:Cl cotransporters. A more detailed description of the ionic and electrical processes associated with this segment of the nephron is beyond the scope of this chapter.

2.3.2.2. Distal Tubule and Collecting Duct Within the cortex, the distal convoluted tubules join together, forming the cortical collecting ducts. The collecting ducts then course straight down through the medulla (medullary collecting duct) and papilla (papillary or inner medullary collecting duct) and empty into the pelvis. There are two important features of this segment of the nephron: 1) vasopressin changes the late distal tubule and the entire collecting duct system from a water-impermeable to a water-permeable segment and 2) vasopressin changes the inner medullary collecting duct, but not the distal tubules or outer medullary collecting ducts from a urea-impermeable to a urea-permeable segment. In addition, this segment of the nephron also has a variety of sodium, potassium, chloride, hydrogen, and other ion transport processes, and it makes important contributions to regulation of the urinary excretion of all of these ions.

2.3.2.3. What Is the Role of Vasopressin in the Formation of a Dilute Urine (Lack of Vasopressin) or a Concentrated Urine (Presence of Vasopressin)? The two views of the kidney shown in Fig. 1 illustrate how the kidney produces the two extremes—dilute urine and concentrated urine—and present the osmolality of the renal interstitium and the tubular fluid at various sites along the nephron in the presence and absence of vasopressin. Because alterations in neither glomerular filtration rate nor fractional reabsorption of fluid along the proximal tubule are significantly affected by vasopressin's actions, these variables are not markedly different when a large volume of dilute urine (a water diuresis) or a small volume of concentrated urine is produced. In both situations, approximately 70% of the original glomerular filtrate is reabsorbed, and the osmolality of the fluid remains equal to that of the systemic plasma and the original glomerular filtrate. As the remaining 30% of the filtrate enters the descending limb of the loop of Henle, water is reabsorbed because the renal interstitial fluid osmolality increases progressively as it descends into the medulla. Because the tubular fluid becomes isotonic to the medullary interstitium because of water reabsorption and not because of the movement of either NaCl or urea, the fluid at the bend of the loop of Henle is NaCl-rich and has an osmolality identical to that of the adjacent interstitial fluid. It is the countercurrent flow of fluid down the descending limb (countercurrent to the flow direction in the neighboring ascending limb of the loop of Henle) that traps the osmotic particles transported into the interstitium from the ascending limb of the loop of Henle and thus generates the osmotic gradient characteristic of the renal medulla.

The interstitial environment of the renal medulla is different during a water diuresis than during the production of a concentrated urine. During water diuresis, the osmolality of the medullary interstitium is almost entirely because of NaCl. Without vasopressin, urea cannot diffuse into the inner medullary interstitium from the adjacent nephrons; hence, a high-urea concentration in the inner medullary region cannot be maintained because urea is being removed without being replaced. In contrast, in the presence of vasopressin, the inner medullary collecting duct is permeable to urea, and as will be explained, this causes the urea concentration in the inner medullary interstitium to increase significantly (approximately half of the interstitial osmolality near the tip of the papilla is because of urea; the other half is because of NaCl). In summary, fluid leaves the thin descending limb of the loop of Henle hypertonic to systemic plasma because of water reabsorption; thus, the tubular fluid is primarily a NaCl solution.

As the fluid enters the ascending limb of Henle, NaCl from the lumen is transported into the interstitium. This is the nephron segment that is called the "diluting segment" because NaCl is reabsorbed but water cannot follow even in the presence of vasopressin. Whether NaCl is being reabsorbed by passively diffusing down its concentration gradient along the thin ascending limb cells or by being transported actively by the thick ascending limb cells, the osmolality of the fluid in the ascending limb is hypotonic to the adjacent interstitial fluid. In fact, as the fluid leaves the ascending limb and enters the distal nephron within the cortex, the fluid is hypotonic to systemic plasma whether vasopressin is present or not. It is at this point in the process that vasopressin begins to exerts its effects on the water and urea permeability of the nephron, resulting in the formation of either a concentrated or a dilute urine.

The simpler case to understand is that of a water diuresis where the absence of vasopressin makes the entire distal tubule and collecting duct system water-impermeable. Because water is not reabsorbed, the osmolality of the fluid remains below that of plasma and the volume flow remains high (*see* Fig. 1A). In the absence of water reabsorption, the urea concentra-

tion of the tubular fluid remains low. The inability to generate a tubular fluid with a high-urea concentration within the cortical and outer medullary regions prevents the normal delivery of urea to the inner medullary region. Because the removal of urea from this region via the vasa recta continues, a decrease in the urea concentration and content within this region of the renal interstitium occurs (Fig. 1A).

Depending on the factors responsible for reabsorbing sodium, sodium reabsorption along the distal tubule and collecting ducts continues. The final urine, in fact, can have an osmolality approaching 50 mOsm/kg of H_2O, significantly lower than that of the fluid leaving the ascending limb (*see* Fig. 1A). In fact, a decrease in plasma vasopressin levels has little effect on the rate of sodium excretion. Therefore, the rate of sodium excretion is not affected during a water diuresis despite the large increase in urine flow.

The effects of vasopressin on the water and urea permeability of the terminal portion of the nephron and the consequences that this has on the composition of the interstitial environment within the inner medullary region of the kidney are illustrated in Fig. 1B. In the presence of vasopressin, hypotonic fluid leaves the ascending limb; when it reaches the late distal tubule, which is now permeable to water, water is reabsorbed and the tubular fluid becomes isosmotic to plasma. Because this segment is impermeable to urea, the urea concentration of the fluid increases. Because sodium can still be reabsorbed, there is a continued reabsorption of water that elevates the urea concentration even further. In fact, by the time the fluid reaches the inner medullary collecting duct, the urea concentration approaches 500 to 700 mM and the rate of fluid delivery is very slow.

As this fluid with a high-urea content reaches the inner medullary region, the papillary collecting duct (which is water-permeable in the presence of vasopressin) now becomes permeable to urea. Because of the high concentration of urea within the inner medullary collecting duct, urea diffuses into the inner medullary interstitium, raising the osmolality of this region to levels approaching 1200 mOsm/kg of H_2O at the tip of the papilla. Because this terminal segment of the nephron is both water- and urea-permeable, the continued reabsorption of water along this segment of the nephron is driven by the osmotic activity of the electrolytes (~600 mOsm/kg of H_2O) and not by that of urea.

It is now apparent that it is the vasopressin-dependent reabsorption of water without the concomitant reabsorption of urea by the distal tubule and collecting ducts within the cortical and outer medullary regions of the kidney that is ultimately responsible for the generation of the inner medullary urea gradient. This urea gradient serve two important roles: 1) it accounts for a significant amount of water reabsorption along the thin descending limb of the juxtamedullary nephrons as it passes through the inner medullary region and 2) it creates the large NaCl concentration gradient between the interstitium and the fluid within the thin ascending limb that drives the diffusion of osmotic particles (NaCl) into the inner medullary interstitium.

The basic features of this process are reasonably well established and accepted. However, current models of kidney function, which use experimentally determined values for the various parameters (such as water and urea permeability of the nephron segments) do not provide realistic profiles of the interstitial osmotic environment. Until physiologically determined parameter values can be used in system models to generate successful fits, our understanding of this system cannot be considered a *fait accompli.*

2.3.2.4. How Does Vasopressin Alter Water and Urea Permeability? The water channel induced by vasopressin is now known to be aquaporin-2 (Knepper, 1997). The aquaporins are transmembrane proteins approx 300 amino acids long (six membrane-spanning regions) that form channels that are selectively permeable to water (not urea). Aquaporin-1 is located on both apical and basolateral membranes of the proximal tubules and thin descending limbs, whereas aquaporin-3 and 4 are on the basolateral surfaces of principal cells of the collecting duct. Aquaporin-2 is found on the apical surface and in small subapical vesicles of both cortical and medullary collecting duct principal cells. After activation by vasopressin of the V_2 receptors on the basolateral surface of principal cells, the subsequent increase in cAMP (long known as the second messenger for induction of the increase in water permeability) causes aquaporin-2 in the submembrane vesicles to fuse with the apical membrane, thus increasing water permeability. Hence, an increase in the number of water channels on the apical membrane (rather than activation of channels already in the membrane) causes the increased water permeability throughout the collecting duct system, including that within the cortex.

The precise series of events that accounts for the insertion of these water channels is not known. Recently, several intracellular proteins similar to those

thought to be involved in the fusion of synaptic vesicles with neuronal cell membranes have been found within the principal cells. It is possible that a process very similar to fusion of synaptic vesicles is employed to insert aquaporin-2 into the apical membrane of the principal cells. In addition to the acute rapid insertion of aquaporin-2 into the apical membrane after an increase in plasma levels of vasopressin, chronic exposure to high levels of vasopressin (such as what occurs with water deprivation) leads to the up-regulation of aquaporin-2. Thus, chronic dehydration increases the number of aquaporin-2 channels available for insertion along the entire collecting duct system.

These water channels are not permeable to urea; however, vasopressin causes an increase in urea permeability along the late segments of the inner collecting ducts by inducing a different protein, sometimes referred to as the vasopressin-regulated urea transporter (VRUT) or urea transporter-1 (UT-1). This VRUT increases the apical membrane's permeability to urea. The lag time for the activation (or insertion) of both VRUT and aquaporin-2 are identical after exposure to either vasopressin or 8-bromoadenosine 3′5′-cyclic monophosphate, a cell membrane permeable analog of cAMP. Thus, it is possible that the same intracellular process is responsible for inserting both water and urea channels.

2.3.2.5. The Vasa Recta is Important in Maintaining the Medullary Interstitial Environment. The integrity of the concentrating mechanism also depends on the ability of the microcirculation within the medullary region of the kidney (the vasa recta) to supply the metabolic needs of the nephrons and maintain the osmotic gradient. With the anatomical arrangement of the vasa recta as a countercurrent exchanger, the blood flowing down into the hyperosmotic environment of the medullary region rapidly equilibrates with the interstitial environment. As this hyperosmotic blood returns toward the cortex, it again rapidly equilibrates with the now decreasing osmotic gradient. This equilibration involves both the diffusion and filtration of water and osmotic particles. Although the rates of filtration and diffusion across the capillary membranes are rapid, both the volume and osmolality of the blood are increased as it leaves the medullary region and reenters the cortex. This is necessitated by the laws of mass balance. The reabsorption of 10–15 mL/min of fluid within the medullary region, mostly along the thin descending limb of the loop of Henle, but also along the medullary portion of the collecting ducts, causes the flow of blood out of this region to exceed the inflow by 10–15 mL/min. Similarly, the net transport of osmotic particles into the medullary interstitium (primarily by the ascending limb but also by the medullary portion of the collecting ducts when there is not an accompanying proportional amount of water reabsorption) causes the blood leaving this region to have a higher osmolality. When osmotic particles and water from the various tubular segments are added into the interstitium at the same rate at which they are removed by the vasa recta, the system is in a steady state.

Although the vasa recta does not directly establish the medullary osmotic gradient, it is essential for maintaining it. Factors that cause an inappropriate increase in blood flow through the medullary region can "wash out" the osmotic gradient. Hence, when the rate at which osmotic particles and water are removed from the medullary region exceeds the rate at which they are being added, the osmotic gradient can be destroyed. Similarly, if blood flow is inappropriately low, the gradient will be destroyed, partly because of an inadequate supply of nutrients.

2.3.2.6. Multiple Factors Can Affect the Concentrating Mechanism, Producing Symptoms Indicative of Neuroendocrine Disorders of Vasopressin Function. The concentrating mechanism of the kidney is dependent on multiple factors besides vasopressin and its ability to affect water and urea permeability along the collecting ducts. The rate of medullary blood flow, availability of urea, and rate of fluid delivery to each segment of the nephron can all affect the concentrating and diluting ability of the kidney. Hence, when patients with suspected neuroendocrine disorders involving hyper- or hyposecretion of vasopressin are evaluated, the urinary output data must be considered in relation to the overall status of the urinary concentrating mechanism before it can be concluded that the problem is caused by a defect in the secretion of vasopressin. For example, a variety of factors besides a lack of vasopressin can produce symptoms similar to those of diabetes insipidus (polyuria, polydypsia, and a hyperosmotic plasma). These include starvation (a lack of urea), inflammation within the medullary region (washout of gradient), inability of the collecting duct cells to respond to vasopressin (excess medullary prostaglandin production or genetic defects in the vasopressin receptor—nephrogenic diabetes insipidus), diuretic therapy causing blockade of

sodium transport by the ascending limb, osmotic diuresis caused by the inability of the proximal tubule to reabsorb glucose (diabetes mellitus), and electrolyte abnormalities (hypokalemia and hypercalcemia, both acting at multiple sites) are all known to cause polyuria, polydypsia, and a hyperosmotic plasma. On the other hand, the inappropriate secretion of vasopressin, whether of central or ectopic origin or caused by intense stimulation of the volume-sensing mechanisms (*see* Section 2.2.2.) secondary to severe fluid volume depletion or cardiovascular diseases, can cause the production of a concentrated urine and the inappropriate retention of water, leading to hyponatremia (hyposmolality).

Finally, the ability of vasopressin to alter urine flow and osmolality without affecting the urinary excretion of sodium allows the body to regulate water balance independent of its ability to regulate sodium balance. Thus, alterations in vasopressin secretion affect body water content and plasma osmolality but have little effect on total body sodium. The ability of vasopressin to alter urine output, however, can be modified by a variety of peripheral factors that affect the ability of the kidneys to respond to neuroendocrine-induced changes in vasopressin secretion.

3. THE RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM

Sodium balance, unlike water balance, is not controlled by a classical neuroendocrine system but rather by endocrine factors in the periphery. A variety of endocrine factors such as the renin-angiotensin-aldosterone system, atrial natriuretic peptide (ANP), "natriuretic hormone"/endogenous ouabain, and adrenomedullin are either established as or hypothesized to be involved in regulating sodium balance. The renin-angiotensin-aldosterone system is clearly the primary endocrine regulator of sodium balance. Although interest has historically been focused on the importance of systemic and intrarenal factors as the primary signals affecting the secretion of renin, there is an abundance of evidence that the central nervous system (CNS) via sympathetic innervation of the kidneys contributes importantly to the overall control of the renin-angiotensin-aldosterone system. In this section, a brief review will be given of the nature of the components of the renin-angiotensin-aldosterone system, the factors responsible for the control of this system, the importance of the CNS in regulating this system, and the effects that the components of this system have on the sodium-retaining processes of the kidney (Inagami, 1994).

3.1. What Are the Components of the Renin-Angiotensin-Aldosterone System?

Few endocrine systems involve a more diverse tissue distribution than that associated with the renin-angiotensin-aldosterone system. The juxtaglomerular cells (JG cells) of the kidney, primarily distributed along the afferent arterioles near the glomerulus, are the site for the synthesis, storage, and release of the enzyme renin. Upon release from the JG cells, renin reacts with angiotensinogen or renin substrate, a 56- to 60-kDa protein produced by the liver that has the decapeptide angiotensin-I (Ang-I) as its N-terminus sequence. The circulating level of angiotensinogen is not considered a regulated variable, but a variety of factors is known to stimulate its release by stimulating its synthesis (increasing mRNA expression). These factors include cortisol and, thus, adrenocorticotropic hormone (ACTH), estrogen, thyroid hormone, and Ang-II.

After renin's enzymatic action of splitting a leucyl peptide (leu-val) bound in angiotensinogen, Ang-I is released from angiotensinogen. Ang-I is immediately acted on by angiotensin-converting enzyme (ACE), which clips a his-leu peptide from the C-terminus of Ang-I to form Ang-II, an octapeptide that is the biologically active component of this cascade. ACE is primarily an endothelial cell membrane-bound enzyme that belongs to the family of neutral metalloendopeptidases, hence, it is found throughout the body. In contrast to renin, which is a highly specific enzyme, ACE is not that specific and is known to cleave dipeptides from various substrates (bradykinin, enkephalins, and substance P). Ang-II acts on a variety of end-organs such as vascular smooth muscle, adrenal glomerulosa, and proximal tubule cells to produce vasoconstriction, the release of aldosterone, and the stimulation of proximal tubule sodium transport. Each of these contributes to an integrated homeostatic response that maintains extracellular volume and arterial blood pressure. The rate-limiting step in this cascade of events is, however, the release of renin from the JG cells. Thus, the release of renin regulates the circulating levels of Ang-II and aldosterone, the biologically active end-products of this cascade.

The final component of this system is aldosterone. Aldosterone is a steroid hormone produced by the glomerulosa cells of the adrenal gland. It is controlled by factors that stimulate the conversion of cholesterol to pregnenolone by the P450 side chain cleavage

enzyme. Pregnenolone is rapidly converted to corticosterone through a cascade of reactions catalyzed by a family of P450 enzymes. Corticosterone is then converted to aldosterone by aldosterone synthase, a P450 enzyme that is unique to the glomerulosa cells. Like most steroid hormones, aldosterone is not stored and then released. Rather, stimuli cause the release of aldosterone by activating the synthetic pathway. The enzyme aldosterone synthase can also be regulated, which can then enhance or suppress the rate at which corticosterone is converted to aldosterone.

3.2. What Are the Elements That Participate in the Control of Renin Release?

3.2.1. What Are the Intrarenal Processes That Are Responsible for Renin Release?

The signals responsible for stimulating the release of renin are linked to variables that reflect the blood or extracellular volume. One of these signals is the perfusion pressure to the kidney—in essence, the arterial blood pressure. A reduction in arterial pressure (renal perfusion pressure) evokes the release of renin by several mechanisms. First, the JG cells are sensitive to stretch and can behave as intrarenal baroreceptors. As arterial pressure decreases, the decreased stretch of the JG cells directly causes an increase in renin release. Second, a decrease in arterial pressure may activate prostacyclin formation by endothelial cells. The prostacyclin then activates renin secretion via a cAMP-sensitive pathway within the JG cells. Third, changes in perfusion pressure affect the rate of glomerular filtration. A decrease in glomerular filtration rate results in a decreased delivery of fluid to the macula densa, resulting in increased renin release. The macula densa appears to be particularly sensitive to the Cl^- concentration of the tubular fluid, which affects the activity of the Na^+, K^+, 2 Cl^- cotransporter (furosemide-sensitive transporter), leading, somehow, to the release of renin from the JG cells. The intracellular processes associated with these mechanisms continue to be areas of active investigation, but a detailed discussion of these processes is beyond the scope of this chapter. Finally, Ang-II inhibits renin secretion by a direct action on the JG cells.

3.2.2. The Renal Nerves Stimulate Renin Release

Stimulation of the renal nerves can increase renin release. Anatomical evidence clearly shows that the JG cells are innervated by sympathetic nerves. However, because nerve stimulation also causes decreases in glomerular filtration rate and sodium excretion, there was concern that at least part of the effect of renal nerve stimulation might be because of activation of the macula densa mechanism or some other intrarenal process. Hence, it was difficult to assign the precise contribution that the renal nerves may have in the overall control of renin release.

An important role for the sympathetic nervous system in controlling renin release, however, has now been clearly established. Low-level stimulation of renal sympathetic nerves that causes no change in glomerular filtration rate or sodium excretion causes a significant increase in renin release. This effect is mediated by the activation of beta-2 adrenergic receptors that then causes a cAMP-dependent increase in renin secretion. Pharmacological blockade of these receptors has been shown to block the release of renin after a variety of acute stress paradigms, such as head-up tilt, or a small hemorrhage. Experimental studies using unilateral renal denervation (differential release of renin by the innervated vs the denervated kidney) have shown that renin release after carotid occlusion, unloading of cardiac receptors (low-pressure receptors), or hemorrhage has a major neural component linked to the baroreceptor-mediated reflexes. In a recent review, Thrasher concluded that signals from atrial (cardiac) baroreceptors associated with sensing of alterations in blood volume contributed significantly to renin release via renal sympathetic nerve activity. Renin release is also clearly affected by alteration in arterial baroreceptor activity.

The importance of the renal nerves in the control of renin release has also been summarized by Osborn and Johns (1989) and by Kopp and DiBona (1993). In addition, these authors pointed out that there is considerable evidence that the level of renal nerve activity can influence the release of renin in response to nonneuronal signals. Renal denervation or blockade of beta-adrenergic receptors by propranolol reduced the increase in renin release induced by a decrease in renal perfusion pressure, for example.

More recently, studies evaluating the importance of the autonomic nervous system in the control of renin release have suggested that the renal nerves are a major contributor to the release of renin in man. Acute pharmacological blockade of the efferent limb of the autonomic nervous system in humans blocked a variety of expected reflex responses to head-up tilt. In these individuals, tilt caused an acute decrease in blood pressure that was not associated with an increase in heart rate or in circulating levels of norepinephrine. During the tilt, the decreased blood pressure was associated with a marked increase in plasma

vasopressin levels, but not in plasma renin activity. The lack of an increase in renin was surprising because the decrease in blood pressure would have been expected to increase renin release via direct activation of the various intrarenal mechanisms. These latter results are compatible with data obtained in patients with autonomic failure, who have a depressed renin response when going from the supine to the upright position. Taken together, these studies suggest that the reflex activation of the sympathetic nervous system is a major regulator of renin release. The signals from both atrial (low pressure) and arterial (high pressure) baroreceptors, in response to alterations in blood volume and blood pressure, are integrated within the CNS and result in the activation of the renin-angiotensin-aldosterone system via the sympathetic nervous system. Thus, the CNS plays an important role in regulating the renin-angiotensin-aldosterone system and, hence, in regulating sodium balance.

3.3. What Are the Elements That Participate in the Control of Aldosterone Release?

Aldosterone is considered an intimate part of the renin-angiotensin system because Ang-II is the primary stimulus for the secretion of aldosterone. The primary effects of aldosterone are the stimulation of sodium reabsorption and of potassium secretion by the principal cells of the collecting duct. In addition, the transport of sodium and potassium by many other epithelial cells (colon, sweat glands, and salivary glands) is also responsive to aldosterone. Thus, aldosterone reduces the sodium and increases the potassium content of urine, feces, sweat, and saliva.

The primary control of aldosterone secretion resides with Ang-II via the AT_1 receptors that activate the cascade of events resulting in the synthesis and release of aldosterone. Because renin release is controlled by renal sympathetic nerve activity linked to both atrial and arterial receptors, aldosterone's stimulation by Ang-II must also be considered to be controlled by the same stimuli that affect these receptors. However, the effect on aldosterone is mediated strictly through the renin-angiotensin system and not by direct neural stimulation of the adrenal glomerulosa cells. In addition, aldosterone secretion is sensitive to small changes in plasma potassium concentration and to moderate changes in sodium concentration (an osmolality effect) resulting from direct effects on the glomerulosa cells. Both potassium and sodium ions are thought to affect aldosterone secretion by altering the influx of extracellular Ca^{2+} and inducing changes in the intracellular calcium concentration. Although changes in sodium concentration (osmolality) can also enhance the aldosterone response of the glomerulosa cells to Ang-II and potassium, the fact that the vasopressin/thirst system maintains plasma sodium concentration within a very narrow range minimizes the role of sodium concentration in the physiological regulation of aldosterone secretion under most circumstances.

ACTH, which primarily controls the production of cortisol by the zona fasciculata, also acutely stimulates aldosterone via a cAMP-dependent process. However, chronic administration of ACTH does not produce a sustained increase in aldosterone secretion. ACTH is not considered an important physiological regulator of aldosterone secretion for several reasons. First, the glomerulosa cells are not as sensitive to ACTH as are the fasciculata cells. Second, when ACTH causes a large increase in cortisol secretion, some of the steroid intermediates (e.g., deoxycorticosterone) that have a low level of mineralocorticoid activity can be increased in the plasma. This increase in mineralocorticoid activity results in salt and water retention, increasing the extracellular volume, which causes reflex (feedback) inhibition of the renin-angiotensin-aldosterone system. Third, chronic administration of ACTH is associated with an inhibition of aldosterone synthase activity, which dampens ACTH's ability to cause a sustained increase in aldosterone secretion. Although ACTH is not considered a physiological controller of aldosterone secretion or an important component of the sodium regulatory system, pathophysiological increases in ACTH can induce increased plasma mineralocorticoid (not aldosterone) activity, resulting in symptoms of salt retention, hypokalemia, and hypertension.

3.4. What Are the Biological Actions of Angiotensin II and Aldosterone?

Both Ang-II and aldosterone affect a diverse number of cell types. Ang-II and/or aldosterone are able to adjust the rate of sodium excretion by the kidneys, as well as the sodium and potassium content of the feces, sweat, and saliva, so that the rate of sodium retention is adjusted to meet the needs of the body.

3.4.1. Angiotensin

Angiotensin acts by interacting with a seven-transmembrane domain receptor called the AT_1 receptor. This receptor is known to interact with three G proteins, one that activates phospholipase C (G_q), another that stimulates the influx of Ca^{2+} via Ca^{2+} channels (G_o), and another that inhibits the formation of cAMP (G_i). Although three other isoforms of the Ang-II receptor (AT_2, AT_3, and AT_4) have been identified (pharmacologically and from actual cloning of the receptors), the biological importance of these other three isoforms has not been established. Hence, the classical effects of Ang-II are mediated by the AT_1 receptors. There are multiple ways by which Ang-II acts to help the body regulate sodium balance and arterial blood pressure. Those directly associated with regulating sodium balance are 1) stimulation of aldosterone secretion by the glomerulosa cells, 2) stimulation of the Na:H exchanger along the proximal tubule, which enhances the rate of sodium reabsorption by this segment, and 3) inhibition of renin release from the JG cells, which prevents large increases in plasma renin activity. In addition to exerting these actions, Ang-II stimulates contraction of vascular smooth muscle, producing peripheral vasoconstriction, and stimulates the release of norepinephrine from peripheral nerve endings, epinephrine from the adrenal medulla, and ACTH and vasopressin from the pituitary. These latter processes are important for the maintenance of arterial blood pressure, which in itself is important for the maintenance of sodium balance. Finally, angiotensin has trophic and remodeling effects on the vasculature that may be important for the maintenance of vascular function over long periods of time.

3.4.2. Aldosterone

Aldosterone interacts with cytosolic receptors that induce (transcriptional) production of several proteins referred to as aldosterone-induced proteins. These proteins then direct the cells to produce the effects on sodium and potassium transport. One of the earliest effects (30–60 min) of aldosterone is the insertion of sodium channels (amiloride-sensitive channels) into the apical membrane from a submembrane, much like the insertion of water channels caused by vasopressin. These channels increase the apical membrane's permeability to sodium, allowing more sodium to diffuse into the cell, where it is then pumped (actively) out of the cell by the basolaterally located sodium pump (Na:K ATPase). Aldosterone also stimulates (up-regulates) the synthesis of new sodium channels, but this process requires a longer time period. Besides exerting its effects on sodium permeability, aldosterone also increases the number of sodium pumps, which enhances the transport of sodium into the interstitial compartment (reabsorption of sodium). This process also maintains a low-intracellular sodium concentration; thus, the gradient for the influx of sodium into the cell is maintained. In some species, aldosterone increases the number of potassium channels on the apical membrane. An increase in the number of luminal potassium channels enhances the ability of the cells to secrete potassium. Even without an increase in the number of potassium channels, the enhanced influx of sodium that increases the activity of the sodium pump will increase the influx of potassium, although the increase in sodium permeability slightly depolarizes the apical membrane. Both of these factors increase the electrochemical gradient for potassium, which facilitates its secretion. In summary, aldosterone affects sodium and potassium transport by the principal cells of the collecting duct as well as by most other transporting epithelial cells. Hence, any increase in aldosterone secretion results in a reduction in the sodium content and an increase in the potassium content of urine, feces, saliva, and sweat.

4. SUMMARY OVERVIEW

The maintenance of the volume and composition of the body fluids within narrow limits is essential for the optimal function of the individual organ systems. This constancy of the internal environment is largely dependent upon the endocrine control of the renal excretion of water and sodium. The classic neurohormone vasopressin is the sole hormone that is responsible for matching the renal excretion of water and urine osmolality to the needs of the body. The central osmoreceptors, responsible for the osmotic control of vasopressin release, are extremely sensitive to osmotic stimuli. Relatively large changes in blood volume and blood pressure, acting via cardiovascular receptors, can also affect vasopressin release. A variety of system factors can influence how efficiently vasopressin acts on the kidney to alter urine output.

Sodium balance employs several systemic endocrine factors to adjust sodium excretion to match the needs of the body. Of primary importance here is the renin-angiotensin-aldosterone system. Renin excretion and therefore aldosterone secretion, are extremely sensitive to small changes in blood volume and pressure, acting via atrial and arterial baroreceptor-mediated reflexes. Only large changes in plasma osmolality can affect aldosterone, acting directly on the cells of the zone glomerulosa.

Clearly, integration of the control of the secretion of vasopressin and renin is required to maintain the constancy of the internal environment. This integration occurs to some extent at the level of the peripheral receptor systems, but must be largely dependent upon the CNS.

SELECTED READINGS

Aguilera G, Kiss A, Luo X, Akbasak B-S. The renin angiotensin system and the stress response. Ann NY Acad Sci 1995; 771:173.

Baertschi AJ, Pence RA. Gut-brain signaling of water absorption inhibits vasopressin in rats. Am J Physiol 1995; 268:R-236.

Baylis PH. Osmoregulation and control of vasopressin secretion in healthy humans. Am J Physiol 1987; 253:R671.

Berghorn KA, Knapp LT, Hoffman GE, Sherman TG. Induction of glucocorticoid receptor expression in hypothalamic magnocellular vasopressin neurons during chronic hypoosmolality. Endocrinology 1995; 136:804.

Bichet DG. Vasopressin receptors in health and disease. Kidney Intl 1996; 49:1706.

Brooks VL, Klingbeil CK, Quillen EW, Keil LC, Reid IA. Effect of baroreceptor denervation on vasopressin and cortisol responses to angiotensin II infusion in conscious dogs. Am J Physiol 1989; 257:R1175.

Ceccatelli S, Cintra A, Hökfelt T, Fuxe K, Wikström A-C, Gustafsson JÅ. Coexistence of glucocorticoid receptor-like immunoreactivity with neuropeptides in the hypothalamic paraventricular nucleus. Exp Brain Res 1989; 78:33.

Choi-Kwon S, McCarty R, Baertschi AJ. Splanchnic control of vasopressin secretion in conscious rats. Am J Physiol 1990; 259:E19.

Claybaugh JR, Share L. Vasopressin, renin, and cardiovascular responses to continuous slow hemorrhage. Am J Physiol 1973; 224:519.

Davies R, Slater JDH, Forsling ML, Payne N. The response of arginine vasopressin and plasma renin to postural change in normal man, with observations on syncope. Clin Sci Molec Med 1976; 51:267.

Figaro MK, Mack GW. Regulation of fluid intake in dehydrated humans: role of oropharyngeal stimulation. Am J Physiol 1997; 272:R1740.

Hogarty DC, Tran DN, Phillips MI. Involvement of angiotensin receptor subtypes in osmotically induced release of vasopressin. Brain Res 1994; 637:126.

Inagami T. The renin-angiotensin system. Essays Biochem 1994; 28:147.

Jordan J, Shannon JR, Black BK, et al. N(N)-nicotinic blockade as an acute human model of autonomic failure. Hypertension 1998; 31:1178.

Keil LC, Summy-Long J, Severs WB. Release of vasopressin by angiotensin II. Endocrinology 1975; 96:1063.

Kishore BK, Teris J, Fernandez-Llama P, Knepper MA. Ultramicrodetermination of vasopressin-regulated urea transporter protein in microdissected renal tubules. Am J Physiol 1997; 272:F531.

Knepper MA. Molecular physiology of urinary concentrating mechanism: regulation of aquaporin water channels by vasopressin. Am J Physiol 1997; 272:F3.

Kopp UC, DiBona GF. Neural regulation of renin secretion. Sem Nephrol, 1993; 13:543.

Lenkei Z, Corvol P, Llorens-Cortes C. Comparative expression of vasopressin and angiotensin type-1 receptor mRNA in rat hypothalamic nuclei: a double in situ hybridization study. Molec Brain Res 1995; 34:135.

Matsukawa S, Keil LC, Reid I A. Role of endogenous angiotensin II in the control of vasopressin secretion during hypovolemia and hypotension in conscious rabbits. Endocrinology 1991; 128:204.

McKinley MJ, Congiu M, Denton DA, et al. The anterior wall of the third cerebral ventricle and homeostatic responses to dehydration. J Physiol 1984; 79:421.

Moses AM. Osmotic thresholds for AVP release with the use of plasma and urine AVP and free water clearance. Am J Physiol 1989; 256:R892.

Norsk P, Ellegaard P, Videbæk R, et al. Arterial pulse pressure and vasopressin release in humans during lower body negative pressure. Am J Physiol 1993; 264:R1024.

O'Donnell CP, Keil LC, Thrasher TN. Vasopressin responses to unloading arterial baroreceptors during cardiac nerve blockade in conscious dogs. Am J Physiol 1992; 262:R51.

O'Donnell CP, Keil LC, Thrasher TN. Vasopressin, renin and cortisol responses to hemorrhage during acute blockade of cardiac nerves in conscious dogs. Am J Physiol 1993; 265:R220.

Oldfield BJ, Miselis RR, McKinley MJ. Median preoptic nucleus projections to vasopressin-containing neurones of the supraoptic nucleus in sheep: a light and electron microscopic study. Brain Res 1991; 542:193.

O'Neil RG. Aldosterone regulation of sodium and potassium transport in the cortical collecting duct. Sem Nephrol 1990; 10:365.

Osborn JL, Johns EJ. Renal neurogenic control of renin and prostaglandin release. Miner Electrolyte Metab 1989; 15:51.

Papanek PE, Raff H. Chronic physiological increases in cortisol inhibit the vasopressin response to hypertonicity in conscious dogs. Am J Physiol 1994; 267:R1342.

Phillips MI, Shen L, Richards EM, Raizada MK. Immunohistochemical mapping of angiotensin AT_1 receptors in the brain. Regul Pept 1993; 44:95.

Raff H, Skelton MM, Cowley AW Jr. Cortisol inhibition of vasopressin and ACTH responses to arterial hypotension in conscious dogs. Am J Physiol 1990; 258:R64.

Roy DR, Layton HE, Jamison RL. Countercurrent mechanism and its regulation. In Seldin DW, Giebisch G, eds. The Kidney–Physiology and Pathophysiology, 2nd ed., vol. 2. New York: Raven, 1992:1649.

Sands JM, Kokko JP. Current concepts of the countercurrent multiplication system. Kidney Intl Suppl 1996; 57:S93.

Schneider EG, Radke K, Ulderich D, Taylor R. Effect of osmolality on aldosterone secretion. Endocrinology 1985; 116:1621.

Seif SM, Robinson AG, Zimmerman EA, Wilkins J. Plasma neurophysin and vasopressin in the rat: response to adrenalectomy and steroid replacement. Endocrinology 1978; 103:1009.

Share L. Role of vasopressin in cardiovascular regulation. Physiol Rev 1988; 68:1248.

Share L, Levy MN. Cardiovascular receptors and blood titer of antidiuretic hormone. Am J Physiol 1962; 203:425.

Shen Y-T, Cowley AW Jr, Vatner SF. Relative roles of cardiac and arterial baroreceptors in vasopressin regulation during hemorrhage in conscious dogs. Circulation Res 1991; 68:1422.

Tanaka J. Involvement of the median preoptic nucleus in the regulation of paraventricular vasopressin neurons by the subfornical organ in the rat. Exp Brain Res 1989; 76:47.

Thrasher TN. Baroreceptor regulation of vasopressin and renin secretion: low-pressure versus high-pressure receptors. Frontiers Neuroendocrinol 1994; 15:157.

Thrasher TN, Brown CJ, Keil LC, Ramsay DJ. Thirst and vasopressin release in the dog: an osmoreceptor or sodium receptor mechanism? Am J Physiol 1980; 238:R333.

Thrasher TN, Nistal-Herrera JF, Keil LC, Ramsay DJ. Satiety and inhibition of vasopressin secretion after drinking in dehydrated dogs. Am J Physiol 1981; 240:E394.

Unger T, Badoer E, Ganten D, Lang RE, Rettig R. Brain angiotensin: pathways and pharmacology. Circulation 1988; 77:140.

Veltmar A, Culman J, Qadri F, Rascher W, Unger T. Involvement of adrenergic and angiotensinergic receptors in the paraventricular nucleus in the angiotensin II-induced vasopressin release. J Pharm Exp Ther 1992; 263:1253.

Verney EB. The antidiuretic hormone and the factors which determine its release. Proc R Soc London Biol Sci 1947; 135:25.

Wang BC, Sundet WD, Hakumäki MOK, Goetz KL. Vasopressin and renin responses to hemorrhage in conscious, cardiac-denervated dogs. Am J Physiol 1983; 245:H399.

Zerbe RL, Feurstein G, Kopin IJ. Effect of captopril on cardiovascular, sympathetic and vasopressin responses to hemorrhage. Eur J Pharm 1981; 72:391.

PART III NEUROENDOCRINE CORRELATES OF STRESS, BEHAVIOR, AND BIOLOGICAL PROCESSES

15 Regulation of the Stress Response by Corticotropin-Releasing Factor Receptors

Richard L. Hauger, MD and
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CONTENTS

1. DEVELOPMENT OF THE FIELD OF STRESS RESEARCH

1.1. Early Concepts of the Physiological Origin of Emotion

Late in the 19th century, William James, the American philosopher and psychologist, and the Danish scientist Carl Lang, independently concluded that human emotion is the perception of material changes in bodily states generated by the impact of external stimuli. James believed that one could not think of fear unless ". . . feelings of quickened heart-beats [or] of shallow-breathing, . . . of trembling lips [or] of weakened limbs, . . . of goose-flesh [or] of visceral stirring, were present." This idea that cognition can be influenced by "hot" emotional experiences can be traced from Aristotle to Descartes to James to Walter Cannon. From this general concept, the theory evolved that physiological systems mediate emotion. In a classic paper published in the *American Journal of Insanity* in 1913, Harvey Cushing expanded the concept of the interdependence of bodily and emotional states by suggesting that a "primary derangement of the nervous system" generates "glandular hyperplasias" and, reciprocally, that a "primary secretory derangement" of the pituitary gland causes "psychic disturbances." Cushing's ground-breaking hypotheses constituted an important cornerstone of contemporary psychoneuroendocrine research.

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1.2. Stress Theories of Walter Canon and Hans Selye

Walter Canon, a contemporary of Cushing's, also helped to lay the groundwork for the field of stress research by proposing the concept of "the emergency reaction," a fight-or-flight response provoked by a stressor of sufficient magnitude to swiftly activate the release of epinephrine from the adrenal medulla and norepinephrine from sympathetic nerve endings. In elaborating his "emergency reaction" hypothesis, Canon further posited that mammals would perish if subjected to stress of a magnitude and duration that surpassed the capacity of the sympatho-adrenomedullary axis to respond.

While Canon's work helped to characterize the mechanisms mediating physiological responses to acute stress, it was Hans Selye, in the mid-1900s, who first described the human response to chronic stress in his theory of the "general adaptation syndrome." Selye's "general adaptation syndrome" consists of several, distinct stages. The first stage, an "alarm reaction" resembling Canon's "emergency reaction," begins in the central nervous system (CNS), then spreads to the anterior pituitary where increased adrenocorticotrophic hormone (ACTH) secretion stimulates the adrenal cortex to release glucocorticoid and mineralocorticoid hormones. The "alarm reaction" constitutes an immediate response to an acutely stressful situation. If exposure to a stressor is prolonged, Selye hypothesized, a second "stage of resistance" or adaptation develops during which synthesis and release of glucocorticoids and catecholamines increases, facilitating metabolic, and other physiological processes crucial for survival, whereas inhibiting inessential ones. The third stage posited by Selye is a stage of "exhaustion" during which declining adaptational resistance and increasing vulnerability to stress engenders pathophysiological responses.

1.3. The Contemporary Definition of Stress

Today, a stressor is defined as a physical or psychological stimulus that disrupts an organism's homeostatic equilibrium. Physical stressors pose a material threat to an organism's survival (e.g., hemorrhage, hypoglycemia, high, or low extremes of environmental temperature, physical injury, etc.). Psychological stressors, on the other hand, are stimuli such as fearful thoughts or memories which perturb emotion and cognition, processes regulated by the CNS. Both types of stressor activate neuroendocrine stress responses or "cascades" within the CNS and the periphery that serve to reestablish homeostasis in two stages. The first stage consists of an immediate neuroendocrine response to an acutely stressful stimulus. If repeated exposure to stress occurs, however, one of two outcomes ensues: the organism either adapts to its stressful environment or, if it is unable to adapt, develops pathophysiological responses.

Today, it is widely believed that an organism's ability to adapt to a particular stressor is determined by two factors: (1) the magnitude and duration of the stressor and (2) the sensitivity to stress of the individual organism, a characteristic which is determined by the interplay of genetic diatheses, biological development, and environmental conditions. Understanding of how genetic abnormalities contribute to the molecular pathogenesis of psychiatric diseases is advancing, spurred by information generated by the human genome project. Recent research suggests that repeated exposure to psychosocial stressors such as excessive work demands, the loss of a family member, or the loss of a job, impairs physical health. Further, there is now evidence that exposure to either an acute or chronic stressor may initiate onset of, and relapse into, serious psychiatric disorders such as major depression, bipolar disorder, posttraumatic stress disorder, anxiety disorders, and schizophrenia.

1.4. The Discovery of Corticotropin-Releasing Factor

A critical link in understanding neuroendocrine responses to stress was provided, during the 1940s and 1950s, by Geoffrey Harris who concluded, based on a series of landmark experiments, that stimulation and inhibition of the secretion of anterior pituitary hormones such as ACTH is regulated by humoral factors released from the median eminence of the hypothalamus into the hypophysial-portal circulatory system. In 1955, Roger Guillemin and Andrew Schally confirmed that ACTH secretion from anterior pituitary tissue increases in the presence of hypothalamic tissue fragments or extracts. During the next three decades, Guillemin and Schally—who were awarded the Nobel Prize for their efforts in 1978—attempted to identify the elusive humoral factor responsible for stimulating the release of ACTH. It was not until 1981, however, that Wylie Vale and his colleagues at the Salk Institute forged a major breakthrough in the field of stress research by isolating, sequencing, and characterizing the neuroactive peptide responsible for ACTH release: corticotropin releasing factor (CRF). Following their successful isolation of ovine CRF (oCRF) from sheep hypothalamic tissue, the

Vale group proceeded to sequence and characterize human and rat forms of the CRF peptide (hCRF and rCRF). To date, however, the hypothalamic inhibiting factor envisioned by Harris has not been identified.

The Vale group's discovery of CRF sparked widespread interest in the study of "transmitter neuropeptide" regulation of cerebral responses to stress. Today, a considerable body of evidence suggests that CRF plays an important role in generating neuroendocrine and behavioral responses to stress by acting at two different receptors, CRF receptor type 1 (CRF-R1) and type 2 (CRF-R2), that are differentially expressed on pituitary corticotropes and brain neurons located in neocortical and limbic regions of the brain. Evidence also suggests that the recently discovered CRF binding protein (CRF-BP) may modulate stress responses by limiting CRF receptor activation. In addition to generating neuroendocrine and behavioral responses to stress, CRF mediates immunological and other processes implicated in both the immediate stress reaction and adaptation to stress by binding to various subtypes of the CRF receptor. Further, the most important neuroendocrine hypothesis concerning the pathogenesis of affective illness posits that stress-induced CRF hypersecretion in hypothalamic and limbic neurocircuits generates both HPA axis and CNS dysregulation. Abnormal HPA functioning results in hypercortisolism, decreased ACTH responsiveness to CRF, and dysregulation of the glucocorticoid negative feedback mechanism. Malfunctioning brain CRF systems, on the other hand, play a role in the genesis of depression, anxiety, and associated "neurovegetative" symptoms such as sleep disruption, appetite loss, abnormal psychomotor behavior, loss of energy and libido, and other physiological parameters mediated by mood. The neuroendocrine hypothesis of affective illness is supported by a number of psychoneuroendocrine studies showing that patients suffering from major depression exhibit both HPA dysregulation and abnormally high levels of CRF in cerebrospinal fluid.

2. THE CRF FAMILY OF PEPTIDES

2.1. Introduction

To date, the following species-specific variants of the CRF peptide have been identified: human and rat CRF, which share an identical amino acid sequence; ovine CRF from sheep; human and rat urocortin; and two nonmammalian CRF peptides: urotensin, present in the teleost fish, and sauvagine, identified in two frog species.

2.2. Human, Rat, and Ovine Variants of Corticotropin-Releasing Factor

2.2.1. Mammalian CRF Genes

The two-exon CRF precursor gene is located on chromosome 8 (8q13) of the human genome. The proximal, 330-bp segment of the human CRF gene's 5′-flanking region contains promotor sites controlling gene transcription. Because CRF gene regulation is critical for survival, this region is highly conserved in genes encoding human, rat, and ovine CRF peptides. Consistent with data showing that the protein kinase A (PKA) pathway regulates CRF promoter activity, the 5′-flanking region of the human CRF gene contains a consensus cyclic AMP response element (CRE). Evidence suggests that protein kinase C (PKC) may also regulate CRF gene transcription at eight consensus AP1 binding sites (possibly representing a TRE) located upstream of the CRE in the 5′-flanking region. Finally, a GRE in the 5′-flanking region is the site of negative glucocorticoid regulation of CRF gene transcription.

2.2.2. Biosynthesis and Structure of Human, Rat, and Ovine CRF

Human, rat, and ovine CRF are 41-amino acid, single-chain peptides cleaved at positions 154 and 194 of the C-terminus of pre-pro-CRF, the 196-amino acid CRF precursor polypeptide. Human and rat forms of CRF (h/rCRF) share an identical amino acid sequence. Ovine CRF (oCRF) shares 83% amino acid sequence homology with h/rCRF (*see* Table 1).

2.2.3. Biological Activity of Human, Rat, and Ovine CRF

In CRF peptides, the side chains of amino acids 5–19 in the N-terminus largely determine CRF receptor binding and other biological activity (*see* Table 1). The N-termini of h/rCRF and oCRF peptides closely resemble one another. Fully processed (i.e., mature) and biologically active CRF peptides are amidated at their C-terminus (CRF[1–41]NH_2). The finding that two forms of oCRF possessing altered C-termini (deamidated oCRF with a C-terminal free acid and CRF-(1,3,9)-NH_2) minimally stimulate cyclic AMP accumulation and ACTH release in anterior pituitary cells, suggests that the C-terminal domain also contributes importantly to the intrinsic activity of CRF peptides. Further, CRF peptides containing oxidized methionines possess little biological activity. Although oCRF and h/rCRF are equipotent in their ability to stimulate ACTH secretion, oCRF is significantly

Table 1
Amino acid sequences of the CRF neuropeptide family

CRF Peptides	*Amino Acid Sequences*	*% Homology to h/rCRF*
	N-terminus 1 5 10 15 20 25 30 35 40 41 C-terminus	
Human CRF	H_2N-S-E-E-P-P-I-S-L-D-L-T-F-H-L-L-R-E-V-L-E-M-A-R-A-E-Q-L-A-Q-Q-A-H-S-N-R-K-L-M-E-I-Ile-$CONH_2$	100%
Ovine CRF	H_2N-S-Q-E-P-P-I-S-L-D-L-T-F-H-L-L-R-E-V-L-E-M-T-K-A-D-Q-L-A-Q-Q-A-H-S-N-R-K-L-L-D-I-Ile-$CONH_2$	83%
Human Urocortin	H_2N-D-N-P-S-L-S-I-D-L-T-F-H-L-L-R-T-L-L-E-L-A-R-T-Q-S-Q-R-E-R-A-E-Q-N-R-I-I-F-D-S-Val-$CONH_2$	42%
Rat Urocortin	H_2N-D-D-P-P-L-S-I-D-L-T-F-H-L-L-R-T-L-L-E-L-A-R-T-Q-S-Q-R-E-R-A-E-Q-N-R-I-I-F-D-S-Val-$CONH_2$	45%
Frog Sauvagine	H_2N-E-G-P-P-I-S-I-D-L-S-L-E-L-L-R-K-M-I-E-I-E-K-Q-E-K-E-K-Q-Q-A-A-N-N-R-L-L-L-D-T- Ile-$CONH_2$	48%
Carp Urotensin	H_2N-N-D-D-P-P-I-S-I-D-L-T-F-H-L-L-R-N-M-I-E-M-A-R-N-E-N-Q-R-E-Q-A-G-L-N-R-K-Y-L-D-E-Val-$CONH_2$	54%

The underlined amino acids in human CRF is a sequence that has been determined to be crucial for binding of CRF agonists to the CRF receptor, type 1 (CRF-R1).

longer acting because the oCRF $t_{1/2}$ is ten times longer than the h/rCRF $t_{1/2}$. Since oCRF binds with weak affinity to the CRF binding protein (CRF-BP), a regulator of CRF bioavailability, it is less susceptible to degradation and clearance than hCRF.

2.2.4. Distribution and Functions of Mammalian CRF

In mammals, the major locus of CRF synthesis is a group of hypothalamic neuronal cell bodies, projecting to the median eminence, that are situated within the parvicellular division of the paraventricular nucleus (PVN). Following its release into the hypophyseal portal circulation system, CRF is carried to corticotrope receptor sites in the anterior pituitary. CRF signaling at these receptor sites constitutes the dominant mode of ACTH secretion regulation. To date, pleiotropic actions of CRF have been implicated in neurotransmitter, autocrine, and paracrine processes occurring in both the brain and periphery. In mammals, low levels of CRF expression have been detected in the adrenal medulla, spleen, ovary, the Leydig cells of the testis (where CRF inhibits androgen release), gastrointestinal tract (i.e., stomach, duodenum, pancreas, and enterochromaffin cells of the colon), skin, lung, and liver. During the last 60 days of the third trimester of pregnancy, CRF expression by placental trophoblast cells increases exponentially. Falling CRF-BP levels cause the amount of free CRF in fetal circulation to rise and bind to CRF-R1 receptors on fetal adrenocortical cells. This process influences the timing of parturition by instigating hypersecretion of dehydroepiandrosterone-sulfate which is converted to estrogen in the placenta.

2.3. Urocortin: A Novel Mammalian CRF Receptor Agonist

In 1995, after screening a cDNA library of the rat Edinger-Westphal nucleus, Wylie Vale and colleagues at the Salk Institute isolated a novel, 40-amino acid, urotensin-like CRF peptide which they called urocortin. Amino acid sequences for human and rat forms of urocortin differ by only two amino acids (*see* Table 1). Urocortin shares 45% amino acid sequence homology with h/rCRF. Although urocortin is 63% homologous to nonmammalian urotensin, it shares only 35% sequence homology with sauvagine, the other nonmammalian form of CRF (*see* Table 1). Acting at the CRF receptor type 1 (CRF-R1), urocortin is slightly more potent than h/rCRF in stimulating ACTH release from anterior pituitary corticotropes. In addition, intravenous injection of urocortin produces a prolonged hypotensive response, while central urocortin administration results in a brief period of hypertension. Recent data suggest that urocortin is markedly less potent than other CRF peptides in increasing anxiety-like behavior in rats.

2.4. Nonmammalian Forms of CRF: Sauvagine and Urotensin

To date, two non-mammalian variants of the CRF peptide have been identified: sauvagine (present in two frog species, *Phyllomedusa sauvagei* and *Xenopus*) and urotensin (present in the teleost fish, *Catostomus commersoni).* Both nonmammalian forms of CRF exhibit 50% amino acid sequence homology with h/rCRF (*see* Table 1). Interestingly, the peptide conformations of urotensin and sauvagine resemble the peptide conformation of h/rCRF even more

closely than the amino acid sequences of these mammalian and nonmammalian peptides resemble one another. Similarities in amino acid sequence and conformation between sauvagine and urotensin, on the one hand, and h/rCRF, on the other, are evidence that the CRF peptide has been conserved in species inhabiting distant rungs of the evolutionary ladder. Studies show that type 1 and type 2 CRF receptors (CRF-R1 and CRF-R2) exhibit strong affinities for sauvagine, urotensin, and urocortin. When used as radioligands, oCRF and h/r CRF bind with strong affinities to CRF-R1, but exhibit weak affinities for CRF-R2. Because sauvagine and urocortin bind with low nanomolar K_D values to CRF-R1 and CRF-R2, they are used as radioligands to detect the presence of both CRF receptors. Sauvagine and urotensin are as potent as h/rCRF in their ACTH-releasing and anorexigenic properties. Further, these two nonmammalian peptides induce a greater degree of vasodilation and hypotension than does h/rCRF.

2.5. Synthetic CRF Receptor Antagonists

Because a substantial body of evidence implicates CRF in the pathophysiology of severe anxiety and depression, development of CRF receptor antagonists to treat these disorders is currently underway. The discovery that CRF peptides signal by assuming an α-helical tertiary conformation when binding to CRF receptors enabled investigators to synthesize the first CRF receptor antagonist, α-helical CRF-(9–41), an N-terminally shortened form of h/rCRF. At high concentrations, α-helical CRF-(9–41) potently inhibits the following processes: (1) CRF-stimulated adenylate cyclase activity in cerebral cortical membranes; and (2) the anxiogenic and hypotensive responses produced by central CRF administration. Even at high concentrations, however, α-helical CRF-(9–41) only partially blocks in vivo and in vitro CRF-stimulated ACTH secretion. This discrepancy may be explained by the tenfold stronger affinity displayed by α-helical CRF-(9–41) for the CRF-R2 over the CRF-R1.

At the high concentrations required to produce antagonist potency at brain CRF receptors, α-helical CRF-(9–41) exhibits limited solubility and produces stress-like (i.e., agonist) behavioral effects. Consequently, a new antagonist devoid of agonist properties has been synthesized, [D-Phe^{12},Nle^{21,38}]h/rCRF-(12–41). Because [D-Phe^{12},Nle^{21,38}] h/rCRF-(12–41) possesses a more stable α-helix than α-helical CRF-(9–41), its action is significantly more potent and longer-lasting. Recently, a new CRF receptor antagonist named Astressin, cyclo(30–33)[D-Phe^{12}, Nle^{21,38}] Glu^{30},Lys^{33}]h/rCRF-(12–41), was synthesized by introducing a lactam ring into the α-helix of [D-Phe^{12},Nle^{21,38}]h/rCRF-(12–41). In addition to being 30-fold more potent in inhibiting ACTH secretion, Astressin also exhibits a longer duration of action than [D-Phe^{12},Nle^{21,38}] h/rCRF-(12–41).

A number of pharmaceutical and biotechnology companies including Janssen/Neurocrine, Dupont, Pfizer, and Park Davis have obtained patents on nonpeptidic, high affinity CRF-R1 receptor antagonists that possess a pyrolo[1,5-alpyrimidine structure and very little affinity for CRF-R2. Pyrrolopyrimidine compounds (e.g., Antalarmin and CP154,526) exhibit a strong affinity for the CRF-R1 receptor and inhibit both CRF-stimulated adenylate cyclase activity and ACTH release in anterior pituitary cells. Presently, a selective CRF-R2 antagonist has not been synthesized although Alanex in San Diego has developed a nonpeptidic compound that binds to CRF-R2 receptors. Although the blood-brain barrier restricts the entry of peptide antagonists into the brain, nonpeptide CRF receptor antagonists enter the central nervous system when administered peripherally. If future research confirms the hypothesis that particular effects of CRF are mediated by specific CRF receptor subtypes, nonpeptide antagonists that selectively target brain CRF-R1 and CRF-R2 variants may prove to be useful anxiolytic and antidepressant medications.

3. BRAIN CRF SYSTEMS, THE STRESS RESPONSE, AND ANXIETY

3.1. Brain Afferents Regulating Hypothalamic Paraventricular CRF Neurons

CRF is synthesized in approximately 2000 parvicellular cell bodies located in the paraventricular nucleus (PVN) of the hypothalamus. Neurons residing in the dorsal aspect of the medial parvicellular subdivision of the PVN are especially important in CRF synthesis. CRF is released into the hypophysial-portal circulation from the external layer of the median eminence, the terminal field for dorsomedial PVN CRF neurons. In addition, a very small collection of CRF-immunoreactive cell bodies situated in the magnocellular division of the hypothalamic PVN contains oxytocinergic neurons. Several neural pathways originating in limbic and brainstem regions of the central nervous system innervate PVN CRF neurons (*see* Table 2). Approximately 70% of A_1 cells present in the caudal ventrolateral medulla and 20% of A_2 cells present in the nucleus of the solitary tract project

to PVN CRF neurons. This ascending noradrenergic pathway relays visceral sensory information from medullary baroreceptors involved in blood pressure regulation to the hypothalamic PVN where CRF release increases in response to hypotension. Angiotensinergic neurons projecting from the subfornical organ to PVN CRF neurons constitute another important stimulatory pathway for elicitation of hypothalamic CRF release. In addition, a pathway arising from ventral portions of the bed nucleus of the stria terminalis (BNST) provides a third important afferent input to PVN CRF neurons. Complex information concerning actual or potential stressors is funneled to PVN CRF neurons by the bed nucleus of the stria terminalis which is innervated by the amygdaloid nuclei and the subiculum and CA1 regions of the hippocampus. The latter three brain regions receive converging projections from the neocortex and limbic system that regulate cognition and emotion, respectively. Because these neural pathways are implicated in perceptual, emotional and cognitive processing of stress-inducing stimuli, their dysregulation may contribute to mood and anxiety disorders. Glucocorticoid, neurotransmitter, and neuropeptide mechanisms modulating PVN CRF neurons are summarized in Table 2.

3.2. General Aspects of Brain CRF Systems

The theory that a broad network of CRF neurons globally regulates neurotransmitter, behavioral, and autonomic responses to stress is supported by the presence of CRF perikarya and CRF terminals (located in the vicinity of CRF receptors) in neocortical and limbic brain regions. Evidence suggests that a continuous series of CRF neuronal cell bodies and terminal fields, constituting both ascending and descending fiber pathways, connects the following brain regions: the central nucleus of the amygdala, the bed nucleus of the stria terminalis, the central gray matter, the parabrachial nucleus, the locus coeruleus, and the nucleus of the solitary tract. In the neocortex, immunostaining reveals the presence of CRF neurons (presumably interneurons) in bipolar cells located mainly in cortical layers II and III. In addition, CRF neurons are especially abundant in prefrontal, cingulate, and insular regions of the cerebral cortex, brain regions which, neuroimaging studies reveal, are abnormal in depressed patients.

3.3. Role of CRF Receptors in Anxiety and Stress Behavior

Studies show that central administration of CRF produces marked, dose-dependent, long-lasting behavioral and autonomic effects ranging from a low dose-induced increase in arousal to high dose-generated anxiety-like behaviors. Central administration of CRF elicits many, if not most, of the endocrine, autonomic and behavioral responses symptomatic of stress. Some of the important behavioral effects elicited in laboratory animals receiving intracerebroventricular (ICV) or intra-limbic injections of CRF are the following:

1. Low dose-induced increases in locomotor activity in a familiar environment.
2. High-dose-induced increases in freezing behavior.
3. Increased grooming, a behavior associated with anxiety.
4. Decreased responding in a shock-motivated operant task.
5. Enhancement of acoustic startle response amplitude.

Central CRF receptor antagonist administration or peripheral benzodiazepine injection block anxiety-like behavior elicited by ICV injection of CRF. Recent studies show that a CRF-R1 knockout mouse exhibits deficient HPA stress responses and is markedly less anxious in plus maze and open field tests than normal, wild-type controls. Further, antisense-induced knockdown of brain CRF-R1 receptors produces a significant anxiolytic-like effect in animals subjected to the defensive withdrawal test. Central administration of CRF-R2 antisense, on the other hand, fails to reduce the anxiety-like responses of animals subjected to the same test. These studies suggest that CRF-R1 plays a more prominent role than CRF-R2 in mediating anxiety-like behavior produced by CRF or stress. Central CRF administration, CRF microdialysis, CRF receptor antisense experiments, and CRF transgenic mouse studies all show that secretion of brain CRF is both necessary and sufficient to to define stress.

4. THE FAMILY OF CRF RECEPTORS

4.1. Characteristics and Distribution of CRF Receptors

In 1983, Greti Aguilera et al. at the National Institutes of Health demonstrated that the CRF radioligand ^{125}I-Tyr-ovine CRF binds to rat anterior pituitary membranes with a strong affinity (K_D = 1 nM) which corresponds to the nanomolar range characteristic of the concentration of CRF present in portal circulation. Subsequent research has shown that CRF receptors are expressed in human, primate and other mammalian POMC-secreting corticotrope cells. Additionally,

Table 2
Regulation of Parvicellular CRF Neurons in Hypothalamic Paraventricular Nucleus

Regulatory Factor	*Hypothalamic Receptor*	*Mechanisms of Action*	*Effect on Hypothalamic CRF Release*
(1) Glucocorticoids (GC)	Type II-glucocortocoid receptor (GR) and Type I-mineralocorticoid receptor (MR)	Variation in circulating GC levels during stress determines the level of activation of low-affinity GRs localized to PVN CRF neurons which, in turn, exert negative-feedback inhibition on the HPA axis. GRs in the hippocampus (possibly CA3 pyramidal cells) and the brain stem also contribute to the regulation of dynamic HPA responses, whereas high-affinity MRs (in the hippocampus or septum) play an important role in the diurnal inhibition of PVN CRF neurosecretory activity via negative-glucocorticoid feedback. Recent evidence suggests that inhibition of stress-induced increases in CRF gene transcription and ACTH secretion by glucocorticoids may not operate or may be overridden until the midpoint of the delayed time domain of GC negative feedback is reached.	↓
(2) Classical Neurotransmitters:			
Catecholamines (NE, E)	α_1-, α_2-, β_1-, and β_2-Adrenergic receptors	Ascending catecholaminergic neurons from the nucleus of the solitary tract and other brain stem nuclei (A_2, C1-C3) project directly to PVN CRF neurons. NE and E released from these presynaptic afferents then act at αAR and βAR expressed on PVN CRF neurons to produce extitatory effects resulting in the release of CRF.	↑
γ-Amino-butyric acid (GABA)	$GABA_A$ receptor	GABAergic projections, possibly originating in the amygdala, bed nucleus of the stria terminalis (BNST) and hypothalamic nuclei (arcuate, medial preoptic, ventromedial, and suprachiasmatic) release GABA from terminals on parvocellular PVN neurons.	↓
Glutamate (Glu)	NMDA, Kainate and AMPA receptors	Glutaminergic afferents from undefined brain regions activate parvicellular PVN neurons expressing Glu receptors.	↑
Acetyl Choline (ACh)	Muscarinic cholinergic receptors (possibly M_4)	ACh increases CRF mRNA expression and CRF release in the hypothalamus, but the cholinergic innervation of PVN CRF neurons is minimal suggesting an indirect mechanism.	↑
Serotonin (5-HT)	$5\text{-}HT_{2A}$, $5\text{-}HT_3$ and, possibly, $5\text{-}HT_7$ receptors in the hypothalamus and $5\text{-}HT_{1A}$ receptors in the hippocampus	Ascending serotonergic afferents from the dorsal and medial raphe nuclei and B9 neurons project to the hypothalamus, but do not appear to directly innervate PVN CRF neurons. As neuronal serotonin release increases during stress, hypothalamic $5\text{-}HT_{2A}$ and $5\text{-}HT_3$ receptors are activated, which helps to promote the secretion of CRF, ACTH, and glucocorticoids. Tonic inhibition of hypothalamic CRF release by glucocorticoids may be mediated, in part, by hippocampal neuron hyperpolarization. Hippocampal neurons can be hyperpolarized by $5\text{-}HT_{1A}$ receptor activation which produces an increase in the conductance of inward rectifying potassium channels coupled to pertussis-sensitive G proteins. Because prolonged release of corticosteroids during chronic stress reduces transcription of the $5\text{-}HT_{1A}$ receptor gene in hippocampal CA3 neurons and, possibly, in dorsal raphe neurons, it may diminish the degree of GC negative feedback. Consequently, brain serotonergic neurotransmission during stress may play a role in both facilitating and terminating HPA responses to stress.	↑ or ↓

(*continued*)

Table 2 (Continued)

Regulatory Factor	*Hypothalamic Receptor*	*Mechanisms of Action*	*Effect on Hypothalamic CRF Release*
(3) Neuropeptides:			
Corticotropin-releasing factor (CRF)	CRF-R1 receptor	Evidence suggests that excitatory limbic inputs from the amygdala (central, medial, and cortical nuclei) and the BNST may express and activate PVN CRF-R1 receptors. Furthermore, stress may increase intrahypothalamic CRF concentrations, thereby increasing CRF-R1 expression in the PVN. CRF-R2α receptor are expressed on PVN neurons, but their function is not known.	↑
Angiotensin II (AII)	AT, angiotensin II receptor	A dense angiotensinergic innervation of PVN CRF neurons may play an important role in the genesis of hypovolemic or inflammatory stress states. Angiotensin II can increase CRF-R1 expression and CRF release, possibly by directly activating AT_1 receptors on parvicellular neurons expressing CRF and CRF + VP. An indirect mechanism whereby increasing circulating levels of angiotensin II activate AT_1 receptors expressed in angiotensinergic terminals of the subfornical organ that projects to PVN CRF neurons may also contribute to this process.	↑
Opioid peptides	Opioid receptors (μ_1 and κ_1)	Opioid afferents to the hypothalamus exert direct and indirect inhibitory actions on PVN CRF neurons. Because naloxone can stimulate hypothalamic CRF release, opioid peptides appear to act at μ_1 receptors when regulating PVN CRF neurons.	↓
Thyrotropin releasing hormone (TRH)	TRH receptors	TRH afferents may directly or indirectly stimulate parvicellular CRF neurons. Circulating levels of thyroid hormone (TH) may act at THα1 and THβ1 receptors to further regulate the effect of TRH on PVN CRF neurons.	↑
Neuropeptide Y (NPY)	NPY Y_4 and NPY Y_5 receptors	NPY afferents from the arcuate nucleus directly synapse on PVN CRF neurons. NPY increases hypothalamic CRF release and HPA secretion. Arcuate NPY expression increases during stressful states associated with high metabolic demand (e.g., food deprivation). Further, activation of central NPY neurons during stress may override glucocorticoid negative-feedback control of the HPA axis at the hippocampus and PVN. Because CRF inhibits feeding behavior and NPY is a potent promotor of orexigenic actions (e.g., increased food consumption), the interplay between these two hypothalamic neuropeptides plays an important role in appetite regulation.	↑
Leptin	Leptin (OB-R1) receptor	Leptin may participate in the regulation of hypothalamic CRF neurons during stress by acting at OB-R1 receptors to signal via the JAK/STAT pathway. In the hypothalamic circuit that inhibits appetite, leptin may modulate CRF and NPY neurons in the PVN. Leptin also increases expression and, possibly, release of POMC peptides in the arcuate nucleus which, in turn, augments the activation of MC4-R receptors that inhibit feeding.	↓
Somatostatin (SST)	SST receptor	Although SST_1 somatostatin autoreceptors appear to inhibit the release of somatostatin from periventricular nerve terminals in the median eminence, the mechanism by which SST inhibits parvicellular CRF neurons has not been defined.	↓
Substance P	Substance P receptor	Substance P acts at its PVN receptors to inhibit hypothalamic CRF expression during the stress states associated with pain, chronic inflammation, or chronic infection.	↓

(*continued*)

Table 2 (Continued)

Regulatory Factor	*Hypothalamic Receptor*	*Mechanisms of Action*	*Effect on Hypothalamic CRF Release*
(4) Cytokines			
Interleukin-1β (IL-1)	IL-1 receptor	Interleukin released from hypothalamic neurons (and/or peripheral interleukin acting at circumventricular receptor sites) stimulates CRF gene expression and CRF release from parvicellular neurons, thereby activating the HPA axis. Interleukin can decrease CRF-R1 receptor expression in anterior pituitary corticotropes, however, independent of changes in CRF and vasopressin levels circulating in hypothysial-portal blood.	↑
Tumor necrosis factor (TNFβ)	?	Unknown mechanism.	↑

CRF receptor binding has been detected in the following mammalian tissues: In the CNS, CRF binding occurs in neocortical and limbic brain regions; in the periphery, CRF binding has been detected in the sympathetic ganglia, adrenal medulla, the renal glomeruli, aortic endothelium, Leydig cells of the testis, ovary, spleen, fetal adrenal cortex, and other peripheral tissues. The following cell lines also exhibit CRF binding: mouse AtT-20 pituitary tumor cells; neuronal CATH cells derived from a transgenic mouse brainstem tumor; and many human tumor cell lines (i.e., retinoblastoma Y-79, neuroblastoma IMR-3, and small cell lung cancer NCI-H82).

4.2. Molecular Size of CRF Receptors

Studies using protein chemical methods such as disuccinimidyl suberate (DSS) cross-linking and SDS polyacrylamide gel electrophoresis, show that the molecular size of the CRF receptor type 1 (CRF-R1) in the mammalian pituitary, spleen, and placenta is 70 to 75 kDa. The molecular size of the CRF-R1 in mouse AtT-20 pituitary cells (66 kDa) and mammalian brain tissues (58 kDa) is slightly smaller. Because CRF-R1 is a glycoprotein, differences in molecular size result from variations in the degree of posttranslational glycosylation of the CRF receptor in a given tissue. When the CRF-R1 is fully deglycosylated, its molecular size shrinks from 40 to 45 kDa, the size predicted by CRF-R1 cDNA cloning (*see* Fig. 1). Use of the Western immunoblot method to analyze CRF receptor structure has been only partially successful because of the difficulty in obtaining a CRF-R1 antibody that exhibits degrees of affinity and specificity sufficient to detect native CRF receptor type 1 expression in cultured corticotropes, brain-derived cell lines, and anterior pituitary and brain tissues.

4.3. Signal Transduction at CRF Receptors

The binding of CRF agonists to CRF receptors in anterior pituitary corticotropes and brain neurons transforms the membrane conformation of the CRF receptor protein, increasing its affinity for the stimulatory heterotrimeric GTP binding protein (G_S protein). As the G_S protein couples to the third intracellular loop of the activated CRF receptor, the α subunit of the G_S protein heterotrimer binds GTP instead of GDP. GTP binding results in dissociation of the α subunit and βγ dimer of the heterotrimeric G_S protein (*see* Figs. 2 and 3). The activated, GTP-bound α subunit moves laterally in the membrane and then binds to the membrane effector enzyme, adenylate cyclase, which, in turn, catalyzes synthesis of the second messenger molecule, cyclic AMP, from cytosolic ATP. The time domain of CRF receptor signaling is short because homologous desensitization shuts off cyclic AMP accumulation within 15–30 min of receptor activation.

In cultured anterior pituitary cells, half-maximum effective concentrations (ED_{50}) of 1 to 3 nM of CRF are sufficient to stimulate intracellular cyclic AMP accumulation and ACTH release. Because maximal ACTH release occurs when CRF occupies approximately 50% of its corticotrope receptor sites, a significant number of "spare" receptors are present on corticotropes. CRF-stimulated cyclic AMP accumulation in the anterior pituitary activates the following cellular processes:

1. Synthesis of postreceptor tertiary messengers (such as protein kinase A) which phosphorylate target cellular proteins and the transcription factor CREB.
2. Target gene transcription, and, possibly.
3. The opening of voltage-sensitive L- and N-type calcium channels (*see* Fig. 2).

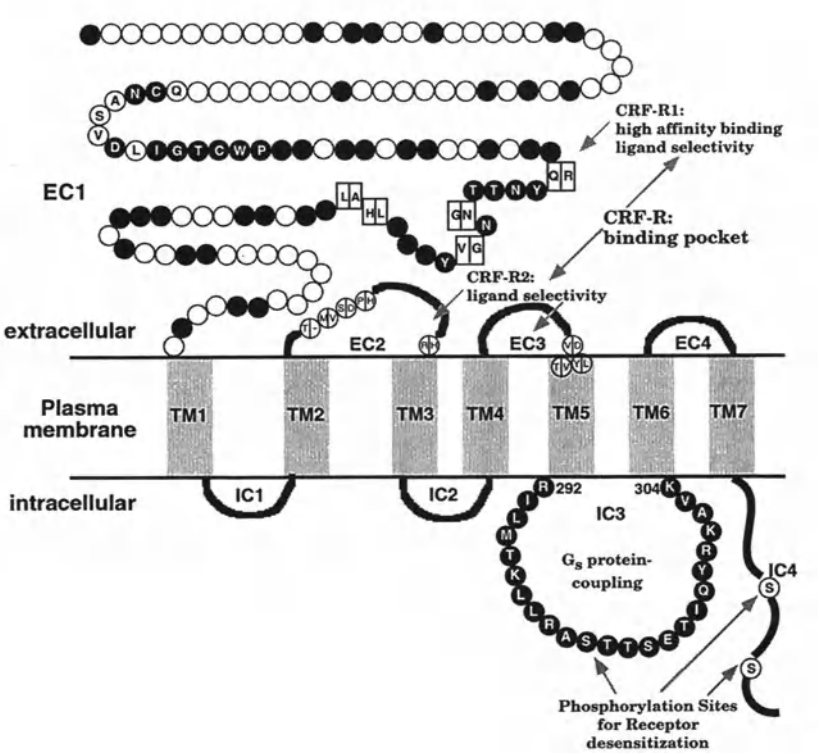


Fig. 1. Schematic representation and comparison of human CRF-R1 and CRF-R2. The conserved amino acids are presented in black circles. Divergent residues are presented in open circles. The amino acids in EC1 that are involved in the ligand specificity of xenopus CRF-R1 are highlighted with the residues of Xenopus CRF-R1 on the left and the residues of human CRF-R1 on the right. The residues in EC2 and EC3 that are important for the ligand selectivity of CRF-R2 are also highlighted with the amino acids of human CRF-R1 on the left and the amino acids of human CRF-R2 on the right. Finally, the IC3 domain, which is believed to be important for G protein-coupling is highlighted. Abbreviations: EC, extracellular domain; TM, transmembrane domain; IC, intracellular domain.

These CRF-induced events culminate in the rapid release of ACTH and β-endorphin from cellular stores, and, after a delay, stimulation of proopiomelanocortin (POMC) gene transcription, a process which serves to replenish stores of ACTH and β-endorphin.

In the CNS, modulation of neuronal ion channels appears to mediate CRF receptor signaling. Brain CRF receptor activation may directly or indirectly (i.e., via G_S proteins) modulate calcium-dependent potassium channels. In vitro experiments suggest that CRF reduces outward potassium current following firing bursts in hippocampal cells, thereby shortening the duration of after-hyperpolarization (AHP) (*see* Fig. 3). In vivo, this process may induce anxiety or stress-related behavior by exciting limbic neurons. Alternatively, a cyclic AMP-dependent mechanism resulting from CRF receptor signaling may modulate calcium-dependent potassium conductance. Finally, in vitro experiments show that CRF stimulates the opening of calcium channels. CRF-stimulated calcium influx into astrocytes is inhibited by the CRF receptor antagonist α-helical CRF-(9–41).

4.4. The Molecular Biology of the CRF Receptor Type 1

In 1993, the Vale lab, using the technique of expression cloning, isolated and sequenced human CRF receptor type 1 protein from a human pituitary corticotropic adenoma cDNA library. Human CRF-R1 cDNA encodes a 415-amino acid protein with the following characteristics:

1. Seven transmembrane spanning domains (TMs).
2. A signal peptide and five potential N-linked glycosylation sites located in the extracellular N-terminus.
3. Three extracellular loops.
4. Three intracellular regions of which the third intracellular loop provides potential sites for G_S coupling and G-protein receptor kinase-induced phosphorylation.
5. An intracellular C-terminal domain containing two serines which may function as consensus sites for protein kinase C phosphorylation (*see* Fig. 1).

Subsequently, cDNAs encoding the CRF-R1 were isolated from brain and pituitary gland tissues of tree shrews *(Tupaia belangeri,* a mammalian species that bears phyllogenetic resemblances to primates), rats, mice, chickens, and the South African amphibian *Xenopus laevis*. The following evidence suggests that CRF-R1 variants share a high degree of primary sequence conservation: (1) The overall amino acid sequence homology among mammalian variants of the CRF-R1 protein is ~97% and (2) the *Xenopus* CRF-R1 is ~80% homologous with mammalian forms of the CRF-R1 (*see* Table 3).

Although the murine CRF-R1 gene has been localized to chromosome 11 of the mouse genome, the location of the human CRF-R1 gene (which contains at

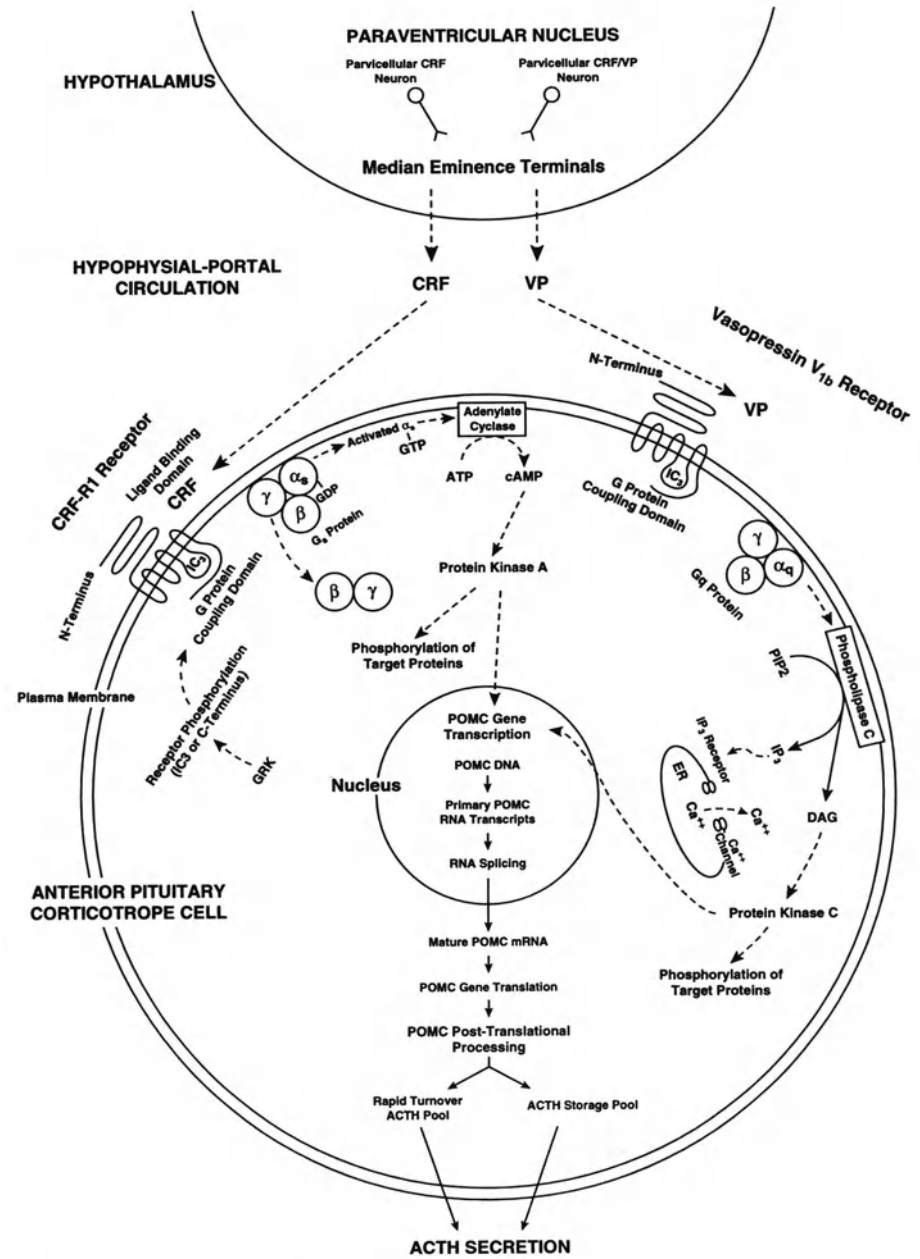


Fig. 2. CRF receptor signaling in anterior pituitary corticotrope cells.

least 14 exons and spans over 20 kilo bases) location in the human genome has not yet been determined. The CRF-R1 is classified as a Class II G protein-coupled receptor (GPCR) because it shares a high degree of sequence homology with receptors for growth hormone releasing factor (GHRF), vasoactive intestinal peptide, pituitary adenylate cyclase activating peptide (PACAP), parathyroid, calcitonin, and glucagon. Amino acid sequences constituting extracellular domains (ECs) and transmembrane spanning domains (TMs) are highly conserved among all Class II GPCRs.

A significant degree of heterogeneity is present among species-specific splice variants of the CRF-R1 (*see* Table 3). In rats, for example, a frame shift mutation responsible for deleting the entire third TM and parts of the fourth TM of the CRF-R1, has produced a 224-amino acid CRF-R1 protein variant that may be dysfunctional, although it is expressed in many brain regions. Because the first intracellular loop of the CRF-R1β contains an additional 29-amino acid sequence, this 444-amino acid receptor is longer than other CRF-R1 variants. Compared to other CRF

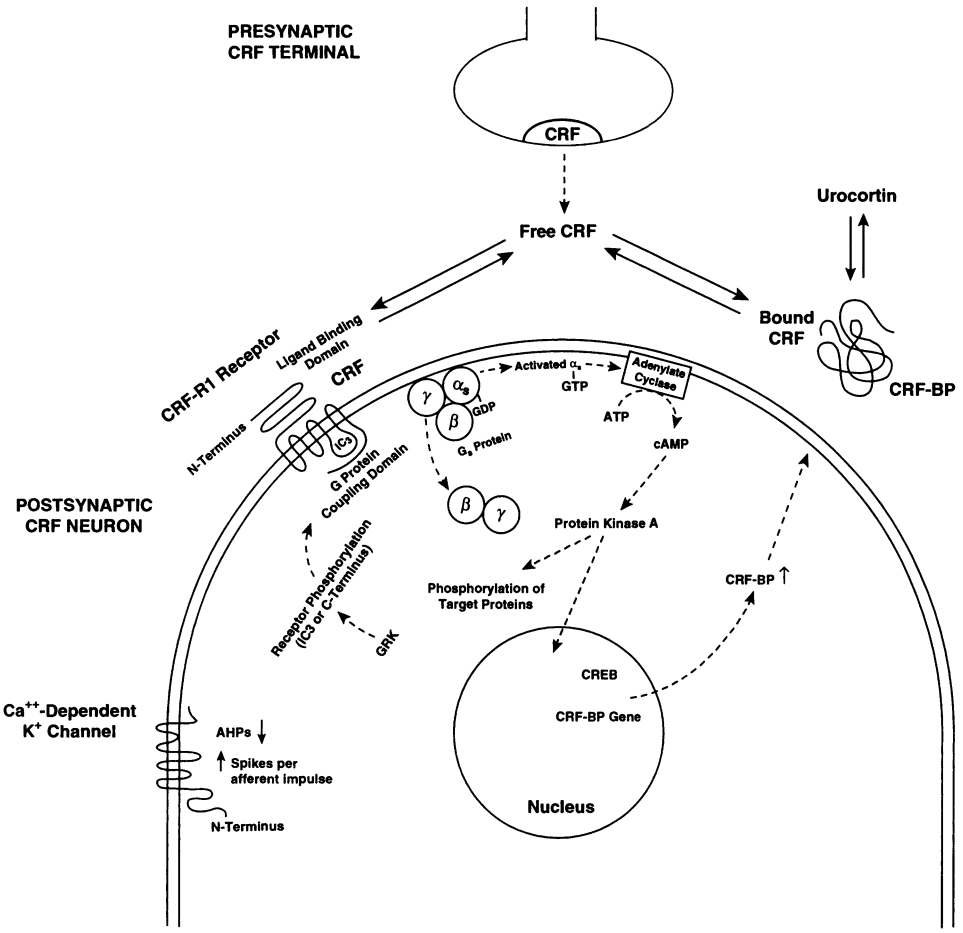


Fig. 3. CRF receptor signaling in brain neurons.

receptors, the CRF-R1β exhibits a significantly weaker affinity for h/rCRF and limited G_S coupling (*see* Table 4). CRF-R1γ is only 375 amino acids in length because it lacks amino acids 41–80 in the N-terminal domain of the CRF-R1. Similarly, CRF-R1δ, 386 amino acids in length, is a short splice variant which lacks N-terminal amino acids 81 to 99 of the CRF-R1. In cells transfected with CRF-R1γ or CRF-R1δ cDNA, cyclic AMP accumulation can be stimulated only by introducing CRF concentrations in the μM range. The CRF-R1γ and the CRF-R1δ possess μM affinities for h/rCRF (*see* Table 4).

4.5. The Remarkable Ligand Selectivity of Xenopus CRF-R1: Mapping the Ligand Binding Domain

Although mammalian CRF and sauvagine both stimulate the secretion of ACTH in human and rat pituitary cells, sauvagine fails to stimulate amphibian ACTH release. Consequently, it has been hypothesized that the pharmacological properties of amphibian and mammalian CRF receptors differ. The cloning and sequencing of CRF receptors expressed in the brain and heart of the *Xenopus laevis* revealed the following important data: (1) The amphibian CRF-R1 is ~80% homologous to the mammalian CRF-R1 and (2) the amphibian CRF-R2 is ~81% homologous to the alpha form of mammalian CRF-R2 (*see* Table 3). Subsequent studies have shown that human/rat CRF, *Xenopus* CRF, urotensin I and urocortin bind to the amphibian CRF-R1 receptor with 10- to 22-fold stronger affinities than do ovine CRF (K_D ~30 nM) and sauvagine (K_D ~50 nM). In contrast, the human CRF-R1 binds human/rat CRF, ovine CRF, sauvagine, urotensin I, and urocortin with very similar degrees of affinity. Remarkably, in HEK293 cells transfected with the amphibian CRF-R1, the EC_{50} for the stimulation of cyclic AMP accumulation by h/rCRF (~1 nM) differs substantially from the EC_{50} for sauvagine (~40 nM). Although the amphibian CRF-R1 is highly selective in binding to CRF ligands, the human CRF-R1 is relatively nonselective. Because the *Xenopus* CRF-R1 exhibits a much stronger affinity for h/rCRF than for sauvagine or oCRF, site-directed

Table 3
Comparison of the protein sequences of vertebrate CRF-R1 and CRF-R2

	human CRF-R1	*human CRF-R2α*	*human CRF-R2β*	*rat CRF-R1*	*rat CRF-R2α*	*rat CRF-R2β*	*xenopus CRF-R1*	*xenopus CRF-R2α*
human CRF-R1	**100**							
human CRF-R2α	**69**	**100**						
human CRF-R2β	**69**	**93**	**100**					
rat CRF-R1	**97**	**69**	**69**	**100**				
rat CRF-R2α	**70**	**94**	**87**	**70**	**100**			
rat CRF-R2β	**69**	**88**	**89**	**70**	**93**	**100**		
xenopus CRF-R1	**80**	**70**	**70**	**81**	**70**	**70**	**100**	
xenopus CRF-R2α	**70**	**81**	**78**	**70**	**81**	**77**	**71**	**100**

The percent amino acid identity is shown.

mutagenesis has been used to synthesize chimeric forms of human and *Xenopus* CRF-R1 receptors or mutant receptors characterized by double or multiple mutations in defined regions of the N-terminus. The substitution of the *Xenopus* CRF-R1 N-terminal sequence Gln76, Gly81, and Val83 for the corresponding human CRF-R1 N-terminal sequence Arg76, Asn81, and Gly83, produced a chimeric receptor with a K_D for h/rCRF that is ~11-fold greater than its affinity for oCRF and sauvagine. Amino acids 68–92 in the N-terminus of *Xenopus* CRF-R1 is the most highly conserved sequence in the EC1 domains of all CRF receptors sequenced to date. It appears that the N-terminal EC1 segment of the CRF-R1 from amino acids 70–89 contains an important ligand binding site (*see* Fig. 1). The EC1 region's importance as a site of CRF-R1 ligand binding has been further corroborated by construction of a second chimeric receptor that substitutes the EC1 sequence of the rat CRF-R1 for the EC1 sequence of the rat growth factor-releasing receptor. The resulting chimeric growth factor-releasing receptor binds astressin and rat urocortin with affinities that are almost identical to the rat CRF-R1's affinity for these two ligands. In addition, evidence suggests that a second region within the EC1 segment of the human CRF-R1 consisting of amino acids 43–55 contributes importantly to CRF binding. A chimeric receptor containing the substitution of human vasoactive intestinal peptide (VIP) receptor type 2 amino acids for amino acids 43–55 of the EC1 region of the human CRF-R1 fails to exhibit a strong affinity for CRF.

4.6. The Molecular Biology of the CRF Receptor Type 2

A second CRF receptor, the CRF receptor type 2 (CRF-R2), has been cloned from brain, heart, lung, and skeletal muscle cDNA libraries for the following species: humans, tree shrews, rats, mice, and frogs. The human CRF-R2 gene is located on chromosome 7 of the human genome, whereas the rat CRF-R2 gene, containing 13 exons and 12 introns, is located on chromosome 6 of the rodent genome. The degree of amino acid sequence conservation among mammalian variants of the CRF-R2 receptor protein is somewhat lower (87–94%) than the extent of sequence conservation among mammalian variants of the CRF-R1 protein (*see* Table 3). A comparison of the primary amino acid sequences of CRF-R1 and CRF-R2 reveals an overall identity of structure of ~70%. Further, important structural elements of the CRF-R1 and the CRF-R2 exhibit the following degrees of homology:

1. Intracellular loops 84%.
2. Transmembrane spanning regions 79%.
3. Extracellular loops 60%. Interestingly, the N-terminal regions of the CRF-R1 and the CRF-R2 share only 40% amino acid sequence homology.

Table 4
Characteristics of CRF Receptor Type 1 Subtypes

CRF Receptor Subtype or Splice Variant	*Receptor Length*	*K_D for h/rCRF*	*Selectivity Profile for CRF Ligands*	*Important Characteristics*
CRF-R1α	415 aa	1-2 nM	h/rCRF=oCRF= sauvagine=urotensin= urocortin	The CRF-R1α variant is the "classic" anterior pituitary CRF receptor. Anterior pituitary corticotropes express variants of the CRF receptor, type 1, but not of the CRF receptor, type 2. In the central nervous system, CRF-R1α is widely expressed in neocortical and limbic regions, brain centers which regulate emotional behavior and the stress response. CRF-R1α is not selective for any of the five CRF ligands.
CRF-R1β	444 aa	20 nM	---	29 additional amino acids are inserted into the first intracellular loop of the CRF-R1β splice variant, resulting in a ~10-fold reduction in this variant's affinity for h/rCRF. The lower K_D of CRF-R1β may be explained by deficient Gs coupling.
CRF-R1γ	375 aa	~1 μM	---	CRF binding cannot be detected at CRF-R1γ, which lacks amino acids 41-80 in the N-terminus of CRF-R1α. Consistent with the very low affinity (K_D) of this CRF-R1 variant, high CRF concentrations (1 μM) are required to stimulate cAMP accumulation in cells transfected with CRF-R1γ cDNA.
CRF-R1δ	386 aa	≥100 nM	---	Although amino acids 81-99 of CRF-R1 have been deleted from the N-terminal domain of CRFR1δ, this variant retains some functional CRF-R1 activity, exhibiting a low affinity for several CRF ligands (K_D values in the range of 80–300 nM). In transfected cells, cAMP accumulation can be stimulated by higher CRF concentrations (~100–200 nM).
Xenopus CRF-R1	415 aa	3 nM	h/rCRF=urotensin= urocortin≥xCRF> oCRF>sauvagine	An unusual CRF-R1 variant possessing a remarkably high degree of selectivity for h/r CRF, urotensin I, and urocortin.The K_D values for the latter three peptides are ~2.5-fold higher than the Kd value for xenopus CRF and ~10- and ~20-fold higher than the K_D values for oCRF and sauvagine, respectively.

This significant divergence in N-terminal structure accounts for differences in ligand selectivity for h/rCRF and oCRF exhibited by the CRF-R1 and the CRF-R2 (*see* Tables 4–6). Importantly, the third intracellular loops of all CRF receptor variants are highly conserved, presumably because this region of the receptor functions as a binding site for the heterotrimeric G_S protein. The ligand selectivity domains of the human CRF-R2 reside within the receptor's second and third extracellular domains (EC2 and EC3).

Three CRF-R2 splice variants have been identified (*see* Table 5). Only the N-terminal domains of the CRF-R2α, a 410 to 413 amino-acid protein, and the CRF-R2β, a 430 to 438 amino-acid protein, differ. The N-terminal differences between these two splice variants may explain differences in their binding affinities for CRF ligands. The N-terminal domain of the rat CRF-R2β splice variant lacks the first 34 amino acids of the N-terminus of the rat CRF-R2α, containing instead a unique 54-amino acid sequence. The human CRF-R2β splice variant, a 438-amino acid protein, possesses a unique 61-amino acid sequence in its N-

Table 5
Characteristics of CRF Receptor Type 2 Subtypes

CRF Receptor Subtype or Splice Variant	*Receptor Length*	*K_D for h/rCRF*	*Selectivity Profile for CRF Ligands*	*Important Characteristics*
CRF-R2α	411–413 aa	13 nM	sauvagine=urotensin= urocortin>xCRF> h/rCRF>oCRF	Unlike CRF-R1α, which is widely distributed in the central nervous system, CRF-R2α is discretely expressed in specific neuronal populations in the amygdala, hippocampus, lateral septum, and hypothalamus, but is not present in the anterior pituitary or periphery. This CRF-R2 subtype binds sauvagine, urotensin I, human urocortin, and rat urocortin with a substantially higher affinity than oCRF and h/rCRF. In contrast, K_D values for the binding of these four CRF peptides to CRF-R1α are similar (~1–2 nM).
CRF-R2β	430–438 aa	17 nM	sauvagine=urotensin= urocortin>h/rCRF> oCRF	Because CRF-R2β is exclusively expressed in heart, lung, intestine, and skeletal muscle in mammals, this variant has been characterized as the "peripheral" CRF receptor.
CRF-R2γ	397 aa	60 pM and 5 nM	urocortin≥sauvagine> urotensin>h/rCRF	Although CRF-R2γ is ›15 amino acids shorter than CRF-R2α, the pharmacological profiles of these two CRF-R2 splice variants are similar. There is little homology between the CRF-R2γ N-terminus and the N-terminal domains of CRF-R2α and CRF-R2β. In human brain,CRF-R2γ is primarily expressed in the septum and hippocampus. No homologs of this variant have been found in lower mammals.

terminus. Although a high degree of sequence conservation is shared by amphibian, human, Tupaia (96%), and rat forms of the CRF-R2α (Table 3), there is no evidence that the CRF-R2β splice variant exists in species other than mammals. The absence of the CRF-R2β in amphibians suggests that this splice variant developed at a later stage of evolution than the CRF-R2α. This hypothesis is further supported by the lower degree of sequence conservation among mammalian CRF-R2β variants, especially with respect to the variable length of the receptor's β-specific domain. The N-terminus of CRF-R2γ, a splice variant that has only been detected in humans to date, lacks any significant degree of homology with the N-termini of the CRF-R2α and the CRF-R2β (*see* Table 5).

4.7. The Role of the CRF Binding Protein in CRF Receptor Regulation

Cloning studies using mammalian brain and liver cDNA libraries show that the CRF binding protein (CRF-BP) is a 322-amino acid protein (~37 kDa) that contains one putative N-glycosylation site and eleven conserved cysteine residues. The 18 kb human CRF-BP gene, located on chromosome 5 (5q), consists of seven exons and six introns. The following are important characteristics of the CRF-BP: (1) Loci for CRF-BP binding activity are amino acids 4 to 28 and the amidated C-terminus, (2) CRF-BP's binding affinity (K_D) for ovine CRF is 300-fold lower than its K_D for h/rCRF, (3) CRF-BP possesses a low K_D for D-Phe12-41-hCRF, but (4) a strong binding affinity for urocortin (*see* Table 6). Studies suggest that by increasing CRF clearance during the third trimester of pregnancy, CRF-BP prevents placental CRF hypersecretion from producing Cushing's syndrome in the mother and fetus. Further, in neocortical and limbic regions of the central nervous system, the CRF-BP may regulate the action of CRF in brain synapses by limiting its release, transport, and bioavailability to CRF receptors. Studies performed in transfected COS cells suggest that a novel molecular mechanism regulates CRF action in the brain: a CRF- and forskolin-induced increase in intracellular cyclic AMP accumulation which binds to a 127-bp cyclic AMP response element, increasing CRF-BP promotor activity (*see* Fig. 3). Consequently, homologous desensitization of CRF receptors by exposure to a CRF agonist may stimulate upregulation of CRF-BP transcription. The

Table 6
Characteristics of CRF-R1α and CRF-R2β Receptors and CRF Binding Protein

	CRF-R1α	*CRF-R2β*	*CRF Binding Protein*
1) Molecular Weight	~70 kDa	?	~37 kDa
2) Binding Affinities for CRF ligands:			
h/rCRF	1-2 nM	~20 nM	0.1-0.2 nM
oCRF	1-2 nM	~30 nM	~450 nM
D-Phe$_{12-41}$-hCRF	~30 nM	~25 nM	~300 nM
3) Brain Expression			
Cerebral Cortex	++++	–	++++
Lateral Septum	+	++++	+
Bed Nucleus of the Stria Terminalis	+++	+++	+++
Amygdaloid Nuclei			
Basolateral	++++	+	++++
Medial	++++	++	+
Cortical	+	++++	+++
4) Anterior Pituitary Expression	++++	–	+++
5) Regulation			
CRF	CRF-R1 desensitization and downregulation CRF-R1 mRNA ↓ (AP)	?	CRF-BP transcription ↑
Vasopressin	CRF-R1 downregulation ↑ CRF-R1 mRNA ↓ (AP)	?	?
Forskolin	CRF-R1 mRNA ↓ (AP)	?	CRF-BP transcription ↑
Glucocorticoids	CRF-R1 downregulation (AP) CRF-R1 mRNA ↓ (AP)	?	CRF-BP mRNA ↓ (AP)

rate of CRF binding to CRF-BP is slower than the rate of CRF binding to CRF receptors. Because CRF receptor activation induces CRF-BP expression, the faster rate of CRF binding to CRF receptors initially favors the process of CRF receptor desensitization. Subsequently, free synaptic CRF binding to CRF-BP limits any further desensitization of brain CRF receptors. Reciprocity between CRF receptor desensitization and CRF-BP upregulation during CRF exposure may play an important role in the regulation of CRF action in brain neurons.

5. CELLULAR AND MOLECULAR MECHANISMS REGULATING CRF RECEPTOR DESENSITIZATION AND DOWNREGULATION

5.1. General Aspects of Homologous Desensitization, Resensitization, and Downregulation of G Protein-Coupled Receptors

5.1.1. Introduction

Homologous desensitization and resensitization are important processes regulating the cellular and neuronal signaling of G protein-coupled receptors. Mechanisms mediating homologous receptor desensitization and resensitization operate at the following cellular levels:

1. Transcription of DNA to RNA.
2. Protein synthesis and degradation.
3. Protein-protein interactions, which pair activated receptors with other signaling molecules and relocate receptors within a cell.

The third-level mechanisms described below constitute the most important and intricate aspects of the homologous desensitization process.

5.1.2. GRK-Induced Phosphorylation and Homologous Receptor Desensitization

GRK-induced phosphorylation of GPCRs in their active, agonist-bound conformation uncouples G protein-coupled receptors from their G proteins. To date, six GRKs have been cloned and sequenced: GRK-1 (rhodopsin kinase), GRK-2 (β-adrenergic receptor kinase or βARK-1), GRK-3 (βARK-2), GRK-4, GRK-5, and GRK-6. Each GRK protein is divided into the following domains: (1) an N-terminus (~185 amino acids) which binds to the GPCR, (2) a centrally-localized domain (~270 amino acids) which catalyzes

receptor phosphorylation, and (3) a C-terminus which undergoes post-translational modification. Protein-protein interactions between the pleckstrin homology domains of the GRK-2 and GRK-3 C-termini, on the one hand, and free βγ dimers, membrane phosphatidylinositol bisphosphate (PIP_2), and activated GPCRs, on the other, anchor GRKs to the cell membrane, creating a GRK scaffold to which the GTPase activating protein, RGS12, is then docked. Importantly, GRK-2, GRK-3, β-arrestin, and RGS proteins are highly localized in postsynaptic neurons in the central nervous system where, evidence suggests, they modulate brain neurotransmission. GRK-, β-arrestin-, and RGS-expressing neurons are distributed in brain monoaminergic pathways and in CRF receptor-expressing limbic brain regions, sites mediating neuronal responses to stress.

Following completion of cyclic AMP accumulation, homologous desensitization of G_S protein-coupled receptors begins with phosphorylation of the agonist-occupied GPCR by a cytosolic GRK, which migrates to the cell membrane following receptor activation (*see* Fig. 4). Phosphorylation alters conformation of the GPCR, uncoupling it from its G protein. Recent studies show that GRK overexpression engenders excessive GPCR phosphorylation and homologous desensitization, a pathogenic process which plays an important role in congestive heart failure and hypertension. Conversely, GRK underexpression may initiate a pathophysiological degree of cellular signaling by limiting phosphorylation and desensitization of GPCRs during prolonged agonist activation. Recent data confirm that GPCRs in cells transfected with a phosphorylation-blocking GRK negative dominant mutant or a GRK antisense construct fail to undergo homologous desensitization.

5.1.3. The Role of β-Arrestin in Receptor Sequestration, Internalization, and Resensitization

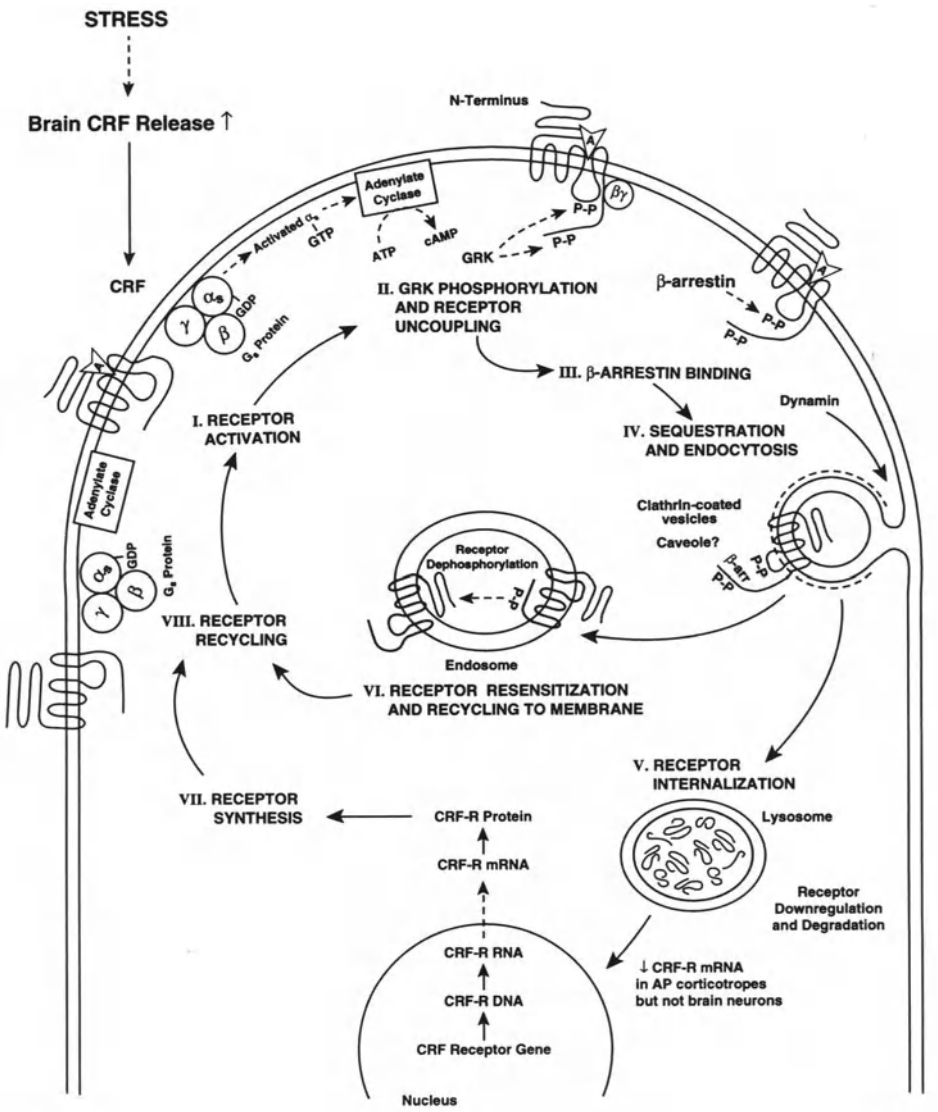
During the second stage of homologous receptor desensitization, constitutively phosphorylated arrestin proteins undergo dephosphorylation, then further supress cellular signaling by binding rapidly and with high affinity (K_D of 1 to 2 nM) to GRK-phosphorylated, agonist-occupied GPCRs (*see* Fig. 4). Importantly, the affinity with which β-arrestin binds to the GPCR increases 10- to 30-fold in response to GRK phosphorylation of the receptor protein. The stoichiometry for GRK-mediated phosphorylation is 2 moles of phosphate per mole of receptor, and each receptor phosphorylated by GRK binds 1 to 2 β-arrestin molecules. Studies show that GPCR sequestration and internalization are substantially reduced in cells transfected with a β-arrestin negative dominant mutant or β-arrestin antisense. β-Arrestin overexpression augments GPCR phosphorylation and desensitization. Both GRK-induced phosphorylation and β-arrestin-mediated cytoplasmic sequestration contribute to the completion of homologous receptor desensitization process.

β-Arrestin also participates in GPCR resensitization. The process of receptor resensitization begins with the binding of β-arrestin to clathrin, a process which translocates the GPCR-GRK-β-arrestin complex into clathrin-coated vesicles. During resensitization, GRK-phosphorylated GPCRs are dephosphorylated by GPCR phosphatase. Interestingly, GPCR phosphatase-induced dephosphorylation does not occur in GPCRs that have been phosphorylated by protein kinase A during heterologous receptor desensitization. Receptor resensitization culminates in the recycling of the dephosphorylated GPCR to the cell membrane (*see* Fig. 4). If cells are subjected to prolonged exposure to high levels of agonist, however, internalized receptors undergo degradation in lysosomes, completing the process of downregulation. Following downregulation, new GPCRs must be synthesized and translocated to the cell membrane to restore normal receptor signaling (*see* Fig. 4).

5.2. Desensitization and Downregulation of Corticotrope CRF Receptors

5.2.1. Acute Homologous Desensitization of CRF Receptors in the Anterior Pituitary

A considerable amount of information exists regarding acute homologous regulation of CRF-R1 receptor sites on pituitary corticotropes during CRF exposure. Studies show that CRF-stimulated cyclic AMP accumulation in mouse AtT-20 pituitary tumor cells and primary cultures of corticotrope cells begins to decrease following 30 min of exposure to low nanomolar levels of CRF, reaching maximal desensitization after three to four hours of CRF exposure. In anterior pituitary cells, homologous desensitization is accompanied by a large reduction in steady-state levels of CRF-R1 mRNA during 1–3 h of exposure to 0.1 to 10 nM h/rCRF. Because desensitization does not reduce POMC mRNA levels, lessen CRF-stimulated calcium uptake, or affect the capacity of heterologous stimulators (i.e., forskolin and β-adrenergic receptor agonists) to increase cyclic AMP accumulation and ACTH release during the period of acute CRF exposure, these corticotrope processes do not play a role in rapid homologous CRF receptor desensi-



tization. CRF receptor function is restored in desensitized corticotropes within 4 h of agonist removal.

5.2.2. Long-Term Downregulation of Anterior Pituitary CRF Receptors by Glucocorticoid Negative Feedback and Adrenalectomy

Glucocorticoid feedback processes are primarily mediated by Type I (mineralocorticoid) receptors on brain neurons within the septal and hippocampal regions of the brain, and by Type II (glucocorticoid) receptors on brain neurons in hippocampus and hypothalamic PVN, and on anterior pituitary corticotropes. Glucocorticoids bind to cytosolic receptors that normally form a complex with heat shock proteins (HSPs), thereby dissociating the activated receptor from HSPs. Next, glucocorticoid-bound GR and MR translocate to the nucleus where they interact with transactivating factors on DNA response elements (GREs), inhibiting transcription of CRF and POMC genes. The degree of glucocorticoid negative feedback is determined by the size of the mRNA pool. Because the POMC mRNA pool is large, the maximal inhibitory effect exerted by glucocorticoids on POMC transcription does not develop for hours to days (i.e., delayed GC negative feedback). The capacity of all levels of the HPA axis, from limbic CRF circuits to anterior pituitary corticotropes are regulated by these genomic effects. At a earlier temporal domain, negative feedback effects of glucocorticoids on the HPA axis are mediated by a rate-sensitive (i.e., dependent on the rate of increase in circulating GC levels) mech-

anism, a process which is important for rapid inhibition of stress- and CRF-stimulated ACTH secretion occurring within minutes of stressor onset. During rapid GC negative feedback, glucocorticoids action occurs at neuron and corticotrope membranes, without producing changes in protein synthesis. Finally, an intermediate GC negative feedback process, emerging from 2 to 12 h following stressor onset, becomes operational when the magnitude of stress is sufficient to decrease ACTH release by depleting corticotrope pools of ACTH. This process does not, however, impede ACTH synthesis.

During delayed glucocorticoid negative feedback, the following glucocorticoid-induced processes occur:

1. Inhibition of POMC gene transcription in the corticotrope cell nucleus.
2. Inhibition of POMC peptide biosynthesis and secretion.
3. Reduction in CRF receptor mRNA expression in anterior pituitary corticotropes.
4. Downregulation of corticotrope CRF receptors.

Administration of corticosterone or dexamethasone in vivo produces a dose-dependent decrease in the number of anterior pituitary CRF receptors which is accompanied by a parallel reduction in ACTH secretion. Because this mode of CRF receptor downregulation requires at least 24 h of exposure to high levels of corticosteroid, its physiological onset coincides with the time domain for delayed glucocorticoid feedback. Further, in vitro experiments show that corticosteroids downregulate CRF receptors on cultured pituitary cells. In summary, a substantial body of evidence suggests that glucocorticoids exert a direct regulatory effect on anterior pituitary CRF receptors. Vasopressin influences HPA regulation by overriding delayed glucocorticoid feedback of ACTH secretion. Because chronic stress increases parvicellular VP expression in the hypothalamus, vasopressin modulates the regulatory effects of glucocorticoids during stress.

Adrenalectomy produces the following physiological changes:

1. An increase in the expression and release of CRF in hypothalamic parvicellular neurons.
2. An increase in PVN CRF-R1 mRNA expression.
3. A 10-fold increase in the expression of POMC mRNA in the anterior pituitary.

In vitro studies show that anterior pituitary cells collected from adrenalectomized rats two days following adrenalectomy exhibit decreases in the sensitivity (EC_{50}) and maximum responsiveness of CRF-stimulated cyclic AMP accumulation and ACTH release. Because administration of a dose of dexamethasone sufficient to suppress hypothalamic CRF hypersecretion reverses HPA changes, it appears that homologous desensitization and downregulation of corticotrope CRF receptors results from exposure to high levels of CRF. The process of homologous desensitization of CRF receptors observed in vitro in anterior pituitary cells prepared from adrenalectomized rat tissue does not occur in vivo. Although CRF-stimulated ACTH release in corticotrope cells collected from adrenalectomized rats decreases in vitro, in vivo ACTH secretion in adrenalecotomized rats injected intravenously with CRF increases threefold in comparison to nonadrenalectomized controls. Evidence suggests that the mechanism responsible for producing this paradox is in vivo potentiation of CRF-stimulated ACTH secretion by vasopressin acting at corticotrope V1b receptors. Because vasopressin expression markedly increases in parvicellular PVN CRF neurons and is hypersecreted into portal circulation in response to adrenalectomy, vasopressinergic potentiation of CRF-stimulated ACTH secretion occurs only in vivo. Increases caused by adrenalectomy in PVN CRF-R1 mRNA expression can be reversed by glucocorticoid replacement.

5.2.3. Long-Term Downregulation of Anterior Pituitary CRF Receptors by Chronic Stress

Studies show that expression of CRF mRNA in the hypothalamic PVN and expression of POMC mRNA in the anterior pituitary increase in response to prolonged stressor exposure (*see* Fig. 5). Consequently, it is widely believed that CRF is hypersecreted by the hypothalamus during chronic stress. Adaptation to chronic stress consists of two processes: (1) desensitization of corticotrope ACTH responses to the primary stressor and (2) maintenance of ACTH responsivity to a novel stressor superimposed on the primary stressor. During the first process of stress adaptation, sustained exposure of the anterior pituitary to high levels of CRF produces desensitization of CRF-stimulated cyclic AMP accumulation and decreased ACTH release in corticotrope cells. Furthermore, downregulation or loss of anterior pituitary CRF receptors promotes adaptational lowering of the in vivo ACTH response to the primary stressor. Studies demonstrate that CRF receptor downregulation develops in the anterior pituitary in vivo when peripheral levels of CRF are increased for several days by CRF infusion. Similarly, CRF receptor loss and increased POMC transcription occur in vitro when AtT-20 cells are incubated with CRF for prolonged time periods. These findings support the hypothesis

that CRF receptor downregulation constitutes a long-term regulatory response to hypothalamic CRF hypersecretion.

5.2.4. The Role of Vasopressin in the Downregulation of Anterior Pituitary CRF Receptors During Adaptation to Chronic Stress

Although basal vasopressin expression is low in the parvocellular PVN, it increases substantially in response to physiologically demanding states such as chronic stress. Alone, vasopressin (VP) is only a weak ACTH secretagogue. In synergy with CRF, however, VP potentiates ACTH release by activating corticotrope vasopressin V1b (V3) receptors that signal via the phospholipase C (PLC) pathway by coupling to Gq proteins (*see* Fig. 2). PLC signaling results from hydrolysis of PIP2 by two second messenger proteins: (1) inositol 1,4,5-triphosphate (IP_3), which stimulates intracellular Ca^{++} release and (2) diacylglycerol (DAG), which, with IP_3, activates and translocates protein kinase C (*see* Fig. 2). Vasopressin-induced potentiation of CRF-stimulated ACTH release may be dependent upon protein kinase C phosphorylation. In addition, evidence suggests that protein kinase C phosphorylates consensus sites in the CRF-R1 C-terminus during CRF receptor desensitization. In contrast to CRF, however, vasopressin does not modulate basal POMC expression in the anterior pituitary or increase POMC transcription. Furthermore, CRF and VP appear to mobilize different pools of ACTH in the anterior pituitary. One important function of vasopressin is maintainence of corticotrope responsiveness to novel stressors following repeated activation of the HPA axis. Vasopressin also influences HPA regulation during stress by overriding glucocorticoid feedback inhibition of ACTH release.

The second adaptational response to stress is maintainence of HPA responsiveness to a novel stressor superimposed on a primary stressor. In vitro experiments show that concomitant incubation of anterior pituitary corticotropes collected from chronically stressed animals with CRF plus vasopressin restores cyclic AMP accumulation and ACTH release to levels observed in corticotrope cells harvested from non-stressed controls. This finding suggests that simultaneous release of CRF and vasopressin from the hypothalamus determines ACTH responsiveness to a novel, second stressor. This hypothesis is supported by the observation that vasopressin expression in hypothalamic PVN parvicellular CRF neurons increases during chronic stress. As the ratio of vasopressin to CRF in hypothysial-portal circulation increases during stress, synergism of action between vasopressin and CRF allows the corticotrope to initiate a strong ACTH response in the presence of a novel stressor, despite concomitant CRF receptor downregulation.

Although vasopressin (100 nM for 3 h) decreases CRF-R1 mRNA levels in anterior pituitary cells in vitro, acting alone it cannot induce desensitization of CRF receptors. Vasopressin can, however, augment in vitro CRF-induced desensitization of corticotrope CRF receptors. In vivo experiments show that a combined peripheral infusion of vasopressin and CRF causes a degree of downregulation of anterior pituitary CRF receptors that is similar in magnitude to adrenalectomy-induced CRF receptor downregulation, and considerably greater than the degree of downregulation produced by infusion of CRF alone. Vasopressin increases CRF-induced downregulation of corticotrope CRF receptors, however, only if the circulating level of infused VP is equivalent to the physiological level of VP present in hypophysial-portal circulation. Studies show that infusion of both a vasopressin V1b receptor antagonist and CRF blocks the augmentation of CRF-induced downregulation of anterior pituitary CRF receptors produced by chronic stress. In concert with hypothalamic CRF hypersecretion, the parvicellular vasopressinergic system plays an important role in regulating CRF receptors during chronic stress.

5.3. Human Retinoblastoma Y-79 Cells: A Model System for Studying Brain CRF Receptor Desensitization

Because the molecular mechanisms regulating brain CRF receptors are difficult to investigate in vivo, recent studies of homologous CRF receptor desensitization have been performed utilizing human Y-79 retinoblastoma cells. Retinoblastoma cells constitute a well-controlled model of CRF receptor regulation in the human brain because they express three important cellular proteins:

1. High-affinity CRF receptors coupled via G_S to adenylate cyclase.
2. Both isoforms of βARK (GRK-2 and GRK-3).
3. β-arrestin.

When retinoblastoma cells are exposed to physiological concentrations of CRF for a relatively brief preincubation period, a time-dependent reduction in CRF-stimulated cAMP accumulation begins ten minutes

following exposure, becoming maximal at 4 h. Although the time course of CRF-R1 receptor desensitization is similar in retinoblastoma cells and anterior pituitary corticotropes, desensitized retinoblastoma CRF receptors undergo a prolonged period of recovery ($t_{1/2}$ of 13 h) that differs significantly from the more rapid course of corticotrope CRF receptor resensitization. Differences in the process of β-arrestin-mediated sequestration in retinoblastoma and corticotrope cells may account for this phenomenon. Although steady-state levels of CRF-R1 mRNA decline rapidly in corticotrope cells during several hours of exposure to CRF, CRF-R1 mRNA levels do not decrease in Y-79 cells desensitized with CRF for up to 24 h, despite a 70% decrease in the number of CRF receptors present on the retinobastoma cell surface. Recent experiments show that inhibition of GRK-3 activity causes CRF receptor desensitization to decrease substantially (60–70%) in retinoblastoma cells. These findings are consonant with the observation that the CRF-R1 protein possesses consensus sites for GRK phosphorylation (*see* Fig. 1).

5.4. CRF Receptor Downregulation in the Central Nervous System

In vivo, CRF receptor downregulation occurs in the central nervous system when intracerebral CRF levels are increased by repeated, central administration of CRF for several days. Similarly, in vitro experiments show that CRF receptors are downregulated in primary cultures of fetal rat extrahypothalamic cells incubated with CRF for 1 to 3 days. In murine brainstem tumor CATH.a cells, CRF-R1 mRNA levels are downregulated by CRF-induced activation of protein kinase A, protein kinase C, and calcium signal transduction. These findings suggest that in the brain, both homologous and heterologous mechanisms regulate CRF receptor signaling.

6. REGULATION OF OTHER RECEPTOR SYSTEMS PARTICIPATING IN THE STRESS RESPONSE

6.1. Regulation of CRF-R1 Receptor Expression in the Hypothalamic PVN

Although CRF-R1 receptors have not been detected in the hypothalamic PVN under normal conditions, ICV CRF administration or stress elicits a significant increase in paraventricular CRF-R1 expression. This autoregulatory process may facilitate CRF activation of PVN CRF neurons during stress (*see* Fig. 5).

6.2. Regulation of ACTH Receptors in the Adrenal Cortex

To date, five melanocortin receptors (MC-Rs) have been cloned and sequenced. Evidence suggests that of these five receptors, only MC-2 and MC-5 receptors are important mediators of the stress response: The MC-5 receptor regulates exocrine secretion, whereas the MC-2 receptor, the classical ACTH receptor, regulates glucocorticoid steroidogenesis in adrenocortical fasciculata cells during stress. The MC-2 receptor couples to G_S proteins to signal via the cyclic AMP pathway. Surprisingly, prolonged exposure of mammalian adrenal cortex ACTH receptors to ACTH in vivo fails to elicit homologous receptor desensitization. Instead, ACTH-stimulated cyclic AMP accumulation increases in fasciculata cells exposed to nanomolar ACTH levels for 12–48 h. MC-2 receptor upregulation increases adrenocortical sensitivity to ACTH. MC-2 receptor mRNA expression increases in fasciculata cells or adrenocortical tumor cells exposed to ACTH because ACTH receptor transcription and/or mRNA stability increases. Another mechanism potentiating ACTH-stimulated cyclic AMP accumulation is ACTH-induced expression of the G protein subunit α_S. Further, chronic stress potentiates ACTH-stimulated cyclic AMP accumulation and corticosterone release in fasciculata cells, presumably because MC-2 receptors upregulate in response to the positive, trophic action of ACTH released during stress (*see* Fig. 5). Although adrenocortical sensitization to ACTH may serve to promote survival during prolonged stress, if sustained, this mechanism may induce pathophysiological states such as Cushing's disease and nodular adrenocortical hyperplasia.

6.3. Regulation of Corticotrope Vasopressin V1b Receptors

Chronic stress upregulates vasopressin V1b receptor expression in anterior pituitary corticotropes (*see* Fig. 5). Because chronic stress increases vasopressin expression in PVN CRF neurons, raising the level of vasopressin secreted into portal circulation, vasopressin may drive enhanced corticotrope responsiveness during adaptation to chronic stress. Furthermore, vasopressin potentiation of ACTH release is not constrained by glucocorticoid feedback inhibition because glucocorticoids, acting at corticotrope GRs, increase the efficiency of V1b receptor signaling via the phos-

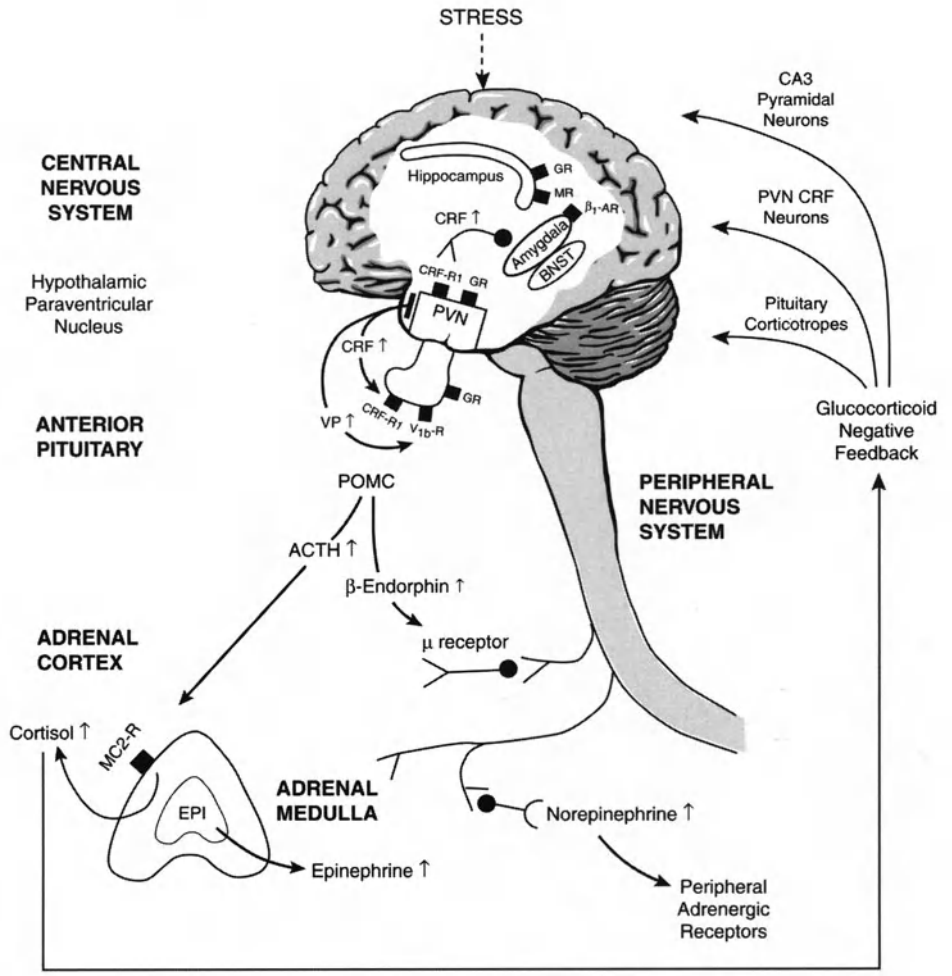


Fig. 5. Regulation of receptor systems in the hypothalamic-pituitary-adrenocortical axis and central nervous system during the stress response.

pholipase C pathway by upregulating Gαq expression. In vitro, vasopressin-stimulated ACTH release increases in anterior pituitary corticotropes exposed to 100 nM CRF for 6 h.

6.4. Regulation of Brain and Peripheral Adrenergic Receptors

During the process of homologous receptor desensitization, mechanisms mediated by GRK, β-arrestin, and RGS may also serve to counterregulate stress-induced adrenergic receptor activation. In frontal cortex collected from rats exposed to repeated stress, a significant decrease in isoproterenol-stimulated adenylate cyclase activity and β_1-adrenergic receptor downregulation was observed. Presumably, desensitization and downregulation of brain and peripheral adrenergic receptors occurs during chronic stress in response to GRK phosphorylation and GRK- and β-arrestin-mediated internalization (*see* Fig. 5).

6.5. Regulation of Opioid Peptide Receptors

Evidence suggests that stress-induced β-endorphin release produces analgesia by activating μ-opioid receptors (OP-3) (*see* Fig. 5). Because some μ-receptor agonists are incapable of eliciting GRK phosphorylation and β-arrestin sequestration, μ-opioid receptors fail to undergo desensitization. It remains to be determined, however, whether β-endorphin-induced antinocioceptive responses during stress are influenced by this phenomenon.

A novel opioid peptide, orphanin FQ, may also mediate behavioral responses to stress. Studies show that orphanin FQ inhibits stress- and opioid-induced antinocioception, stimulates feeding behavior, exerts

an anxiolytic effect on the behavior of animals subjected to open field and elevated plus maze tests, and reverses urocortin-induced anxiety-like behavior. Evidence suggests that during stress, orphanin FQ acts at a novel receptor site in limbic brain regions where CRF receptors are also expressed.

7. TRANSGENIC MOUSE MODELS OF HPA PATHOPHYSIOLOGY AND ABNORMAL CRF RECEPTOR FUNCTIONING

7.1. The CRF-Overexpressing Mouse

In 1992, the Vale lab created a brain CRF-overexpressing transgenic mouse by fusing the CRF gene with the murine metallothionein-1 gene promotor, a manipulation which eliminated glucocorticoid negative feedback-induced inhibition of CRF transgene expression. The CRF-overexpressing mouse exhibits the following neuroendocrine abnormalities:

1. Sustained hypersecretion of ACTH and glucocorticoids.
2. A Cushingnoid phenotype.
3. High levels of CRF mRNA expression in the hypothalamic PVN and BNST (sites which normally contain CRF cell bodies), and in the hypothalamic arcuate nucleus, granule cells of the hippocampal dentate gyrus, and cerebellar deep nuclei (brain regions which normally do not express CRF).

When subjected to open field and elevated plus maze testing by George Koob's lab, the CRF-overexpressing mouse exhibited anxiety-like behavior that was reversed by central administration of the CRF receptor antagonist α-helical-CRF-(9–41). Interestingly, immunohistochemical staining revealed that anterior pituitary ACTH cells do not proliferate in these transgenics. Moreover, brain CRF hypersecretion in the CRF-overexpressing mouse may fail to elicit the normal counterregulatory process of CRF receptor desensitization. These important transgenic mouse studies support the hypothesis that CRF hypersecretion in limbic neurocircuits causes anxiety.

7.2. The CRF Gene Knockout Mouse: A Model of Brain CRF Deficiency

Although the CRF homozygous knockout mouse exhibits selective atrophy of the zona fasciculata, its zona glomerulosa and adrenal cortex are normal. To prevent lung dysplasia, this mouse requires glucocorticoid treatment during the neonatal period. CRF-immunoreactive neurons and CRF mRNA expression in the hypothalamic PVN, central nucleus of the amygdala, and layers II and III of the cerebral cortex are undetectable in this mouse. Neuroendocrine testing of the CRF knockout mouse revealed the following abnormalities:

1. A marked decrease in basal secretion of corticosterone.
2. Deficient ACTH and corticosterone responses to acute stress.
3. A reduction in ACTH-stimulated corticosterone secretion.
4. A large reduction in insulin-stimulated epinephrine release.

The CRF homozygous knockout mouse's abnormally low sympatho-adrenomedullary response may be explained by the hypothesis that limbic and brainstem CRF neurons regulate peripheral catecholamine outflow during stress. Surprisingly, stimulation of ACTH secretion by CRF injection is greater in homozygous CRF-deficient mice than in wild-type controls. Moreover, because hypothalamic PVN vasopressin expression is low in these mice, their increased ACTH responsivity is attributable to only two mechanisms: (1) deficient glucocorticoid negative feedback inhibition of the HPA axis, possibly combined with (2) upregulation of anterior pituitary CRF receptor signaling. Unfortunately, no information has been published concerning the CRF knockout mouse's behavioral responses to open field, elevated plus maze, and startle reactivity testing.

7.3. Other HPA-Dysregulated Transgenic Mice

A transgenic mouse exhibiting deficient glucocorticoid negative feedback responsivity has been developed using a transgene with a neurofilament promotor to induce constitutive expression of Type II glucocorticoid receptor (GR) antisense RNA in brain neurons. The following neuroendocrine abnormalities have been detected in the GR knockdown mouse: (1) reduced GR expression (~60%) and reduced Type II GR binding (~30% to 40%) in the hypothalamus and cortex; (2) an exaggerated level of ACTH (but not corticosterone) secretion when exposed to anxiogenic test environments, and (3) diminished HPA axis sensitivity to dexamethasone inhibition (i.e., decreased glucocorticoid negative feedback). Other HPA processes

appear to be normal in GR knockdown mice, including anterior pituitary POMC expression, CRF-R1 mRNA expression, and CRF receptor binding in the anterior pituitary and brain. Decreased hypothalamic CRF release may account for the normalcy of these processes. Behaviorally, the GR knockdown mouse exhibits reduced anxiety-like behavior in the elevated plus maze test and increased latency during the forced swim test. These findings are consistent with data concerning the role of Type II GR in hippocampal-related learning and memory. Because antidepressants increase GR expression, most of the neuroendocrine and behavioral abnormalities exhibited by the GR knockdown mouse are reversible by antidepressant administration. Information provided by GR knockdown mouse experiments generated the hypothesis that brain glucorticoid receptor regulation contributes to the therapeutic action of antidepressants.

7.4. The CRF-BP-Overexpressing Mouse

A recently developed transgenic mouse that overexpresses the CRF binding protein (CRF-BP) in anterior pituitary corticotropes exhibits normal basal and stress-stimulated secretion of both ACTH and corticosterone. In contrast, in vitro studies show that CRF-stimulated ACTH release is attenuated when the amount of "free" CRF available to bind to corticotrope CRF receptors is decreased by high levels of CRF-BP. The most likely explanation for the failure of CRF-BP to inhibit anterior pituitary ACTH secretion in the CRF-BP-overexpressing mouse is the large increase in CRF mRNA and smaller increase in vasopressin mRNA present in the hypothalamic PVN of this transgenic. Increased vasopressin expression potentiates the action of CRF at corticotrope receptors. Further, high levels of CRF-BP in the anterior pituitary may block CRF receptor desensitization that would normally occur in response to increased hypothalamic CRF release.

7.5. CRF Receptor, Type 1 Knockout Mice

Recently, the Vale lab at the Salk Institute and the Koob lab at Scripps Research Institute jointly published an important paper describing a homozygous CRF-R1 knockout mouse that they had developed and tested. The Vale/Koob CRF-R1 knockout is genetically engineered to express a transgene of CRF-R1 that lacks the last 12 amino acids of the first extracellular loop of the CRF-R1 protein. Studies show that brain CRF-R2 expression is unaltered in the Vale/Koob CRF-R1 knockout mouse. HPA abnormalities exhibited by this knockout mouse include the following:

1. a marked reduction in basal glucocorticoid secretion accompanied by normal basal ACTH secretion,
2. severely diminished release of ACTH and corticosterone during acute restraint stress, and
3. a large increase in CRF expression and normal vasopressin expression in PVN neurons.

The Vale/Koob CRF-R1 knockout mouse exhibits significantly less anxiety-like behavior than wild-type controls when tested in the open field and elevated plus maze. Despite treatment with corticosterone to correct glucocorticoid hyposecretion, these knockouts continue to exhibit diminished anxiety-like responses during plus maze testing, evidence that the mechanism responsible for reducing anxiety-like behavior in this homozygous CRF-R1 knockout mouse is deficient brain CRF-R1 expression.

A research group led by Florian Holsboer and Wolfgang Wurst at the Max Planck Institute in Munich, Germany, recently developed homozygous and heterozygous CRF-R1 knockout mice. These transgenics express a mutant CRF receptor Type I which substitutes a neomycin reporter construct for amino acid sequences encoding TM5, TM6, and TM7, the locus of G_S coupling in the third intracellular loop. The homozygous CRF-R1 knockout mouse fails to exhibit increases in ACTH and corticosterone release in response to forced-swim stress and shows markedly less anxiety than wild-type controls in the elevated plus maze and open field tests. In contrast, stress-stimulated ACTH secretion tends to be somewhat more elevated in the heterozygous CRF-R1 knockout mouse than in wild-type controls. In vitro experiments show that although homozygous and heterozygous CRF-R1 knockout mice both exhibit reductions in CRF-stimulated cyclic AMP accumulation and ACTH release, the magnitude of the reduction is significantly greater in the homozygous mouse. Furthermore, although heterozygous knockouts exhibit a large reduction in anterior pituitary CRF receptors, corticotrope responsiveness remains normal. The following adaptational changes in HPA functioning may account for this phenomenon: (1) increased vasopression expression in parvocellular CRF neurons, and (2) upregulation of anterior pituitary vasopressin V1b receptors. These adaptional HPA responses develop in normal animals exposed to chronic stress.

8. CONCLUSION: THE ROLE OF CRF RECEPTOR ABNORMALITIES IN HUMAN PATHOPHYSIOLOGY

8.1. Cushing's Disease: A Neuroendocrine Model of Deficient CRF Receptor Desensitization

In Cushing's disease, corticotrope adenoma cells exhibiting abnormal CRF receptor signaling engender ACTH-dependent hypercortisolism. Although hormone hypersecretion can be produced by point mutations that generate constitutively active receptors—as is the case in certain thyroid adenomas—the CRF receptor, Type 1, that has been cloned and sequenced from a human corticotrope adenoma cDNA library contains no mutations. Similarly, vasopressin V1b receptors expressed in human corticotrope adenoma cells are also normal. Homologous CRF receptor desensitization fails to develop in human corticotrope adenoma cells exposed to CRF (1–100 nM) for periods ranging from 4 h–10 days. Instead, experiments show that ACTH secretion increases in these cells during CRF exposure. Studies also show that the level of CRF-R1 mRNA in human corticotrope adenoma cells is twofold higher than the level of CRF-R1 mRNA in normal corticotropes. Furthermore, compared to normal corticotrope cell membranes, human corticotrope adenoma cell membranes contain a significantly greater number of CRF-R1 receptors which may not internalize normally in response to CRF agonists. Although exposure to CRF significantly decreases CRF-R1 mRNA expression in normal anterior pituitary cells, human pituitary corticotrope adenoma cells and mouse AtT-20 pituitary corticotrope tumor cells show increased CRF-R1 mRNA expression when exposed to CRF. Furthermore, because CRF-BP expression decreases in human corticotrope adenoma cells, the concentration of free CRF in portal circulation increases, activating CRF receptors. Finally, pathological CRF receptor signaling in corticotrope adenoma cells is exacerbated by deficiencies in types of glucocorticoid negative feedback inhibition of ACTH secretion, including the absence of glucocorticoid-induced inhibition of POMC expression and glucocorticoid-induced downregulation of CRF receptors. Paradoxically, although the α and β isoforms of the glucocorticoid receptor contain no mutation, the glucocorticoid negative feedback mechanism is deficient in corticotrope adenoma cells. These findings suggest that underexpression or mutation of GRKs, β-arrestin, and/or RGS proteins blocks homologous desensitization and internalization of CRF receptors in human corticotrope adenoma cells.

8.2. Abnormal CRF Receptor Signaling in Alzheimer's Disease

The prevalence of CRF neurons and CRF receptors in neocortical and limbic regions of the brain suggests that CRF mediates cognitive and emotional processes. There is evidence that underexpression of CRF in the CNS contributes to cognitive impairment associated with Alzheimer's disease and other neuropsychiatric disorders. Studies show that Alzheimer's patients exhibit the following brain CRF abnormalities:

1. Decreased CRF expression upregulating CRF receptors in temporal, parietal, and occipital regions of the cerebral cortex.
2. CRF neuron loss in the amygdala.
3. Low cerebrospinal fluid levels of CRF.

Because CRF-BP expression is normal in the brains of Alzheimer's patients, increased CRF binding to CRF-BP has been hypothesized to decrease synaptic levels of free CRF (*see* Fig. 3), reducing CRF receptor activation in neocortical regions regulating cognition. Consequently, increasing CRF expression in neocortical and amygdalar presynaptic neurons during early stages of Alzheimer's dementia may improve cognition. The following forms of HPA dysregulation occur in Alzheimer's patients:

1. Increases numbers of CRF neurons and CRF mRNA expression in the hypothalamic PVN.
2. Decreased ACTH responsiveness to CRF injection.
3. Hypercortisolemia.

Two processes likely contribute to cognitive impairment in Alzheimer's patients are deficient central CRF neurotransmission and exaggerated hypothalamic CRF release resulting in glucocorticoid hypersecretion.

8.3. The Role of CRF Receptor Desensitization in Anxiety, Depression, and Stress Disorders

During stress, CRF neurotransmission in limbic neural networks requires rapid counterregulation via the process of homologous receptor desensitization to prevent CRF receptor overactivation. Recent studies show that the molecular pathophysiology of endocrine and cardiovascular diseases includes not only rare point mutations in G protein-coupled receptors, but also abnormalities in GRK expression which impair

GPCR functioning. For example, GRK overexpression has been shown to contribute to congestive heart failure and hypertension by generating excessive degrees of cardiac and vascular β-adrenergic receptor phosphorylation and desensitization. Abormal GRK expression may also play an important role in the pathophysiology of psychiatric illnesses. The level of CRF in cerebrospinal fluid of patients suffering from severe depression and posttraumatic stress disorder is abnormally elevated. Deficient CRF receptor desensitization resulting from GRK underexpression or mutation is one mechanism which may explain how brain CRF hypersecretion contributes to the genesis of anxiety, depression and stress disorders. Furthermore, deficient cellular expression of GRKs or a GRK mutation may mediate stress-induced precipitation of anxiety, depression, and mania. Studies show that patients suffering from bipolar disorder exhibit G protein abnormalities. Moreover, lithium has been shown to alter G protein expression and function, as well as phospholipase C signaling. Consequently, targetting novel pharmacotherapies that normalize GPCR desensitization by GRK and β-arrestin may prove to be clinically beneficial.

SELECTED READINGS

Aguilera G. Regulation of pituitary ACTH secretion during chronic stress. Frontiers in Neuroendocrinol 1994; 15:321.

Aguilera G, Kiss A, Hauger R, Tizabi Y. Regulation of the hypothalamic-pituitary-adrenal axis during stress: role of neuropeptides and neurotransmitters. In: Kvetnansky R, McCarty R, Axelrod J, eds. Stress: Neuroendocrine and Molecular Approaches. New York: Gordon and Breach Science, 1992: 365.

Aguilera G. Corticotropin releasing hormone, receptor regulation and the stress response. Trends Endocrinol Metab 1998; 9:329.

Arborelius L, Owens MJ, Plotsky PM, Nemeroff CB. The role of corticotropin-releasing factor in depression and anxiety disorders. J Endocrinol 1999; 160:1.

Chalmers DT, Lovenberg TW, DeSouza EB. Localization of novel corticotropin-releasing factor receptors (CRF_2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF1 receptor mRNA expression. J Neurosci 1995; 15:6340.

Chang C-P, Pearse RV, O'Connell S, Rosenfeld MG. Identification of a seven transmembrane helix receptor for corticotropin-releasing factor and sauvagine in mammalian brain. Neuron 1993; 11:1187.

Chen R, Lewis KA, Perrin MH, Vale WW. Expression cloning of a human CRF receptor. Proc Natl Acad Sci USA 1993; 90:8967.

Clark JL, Cammas FM. The ACTH receptor. Balliere's Clinical Endocrinol Metab 1996; 10:29.

Cone RD, Lu D, Koppula S, et al. The melanocortin receptors: agonists, antagonists, and the hormonal control of pigmentation. Recent Progress in Hormone Res 1996; 51:287.

Dautzenberg FM, Wille S, Lohmann R, Spiess J. Mapping of the ligand-selective domain of the *Xenopus laevis* corticotropin-releasing factor receptor 1: implications for the ligand-binding site. Proc Natl Acad Sci USA 1998; 95:4941.

Dautzenberg FM, Kilpatrick GJ, Wille S, Hauger RL. The ligand selective domains of corticotropin-releasing factor receptor type 1 and type 2 reside in different extracellular domains: generation of chimeric receptors with a novel ligand selective profile. J Neurochem, 1999; 73:821.

Dieterich KD, Lehnert H, DeSouza EB. Corticotropin-releasing factor receptors: an overview. Exp Clin Endocrinol Diabet 1997; 105:65.

Dunn AJ, Berridge CW. Physiological and behavioral responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety or stress responses? Brain Res Rev 1990; 15:71.

Gold PW, Wong M-L, Chrousos GP, Licinio J. Stress system abnormalities in melancholic and atypical depression: molecular, pathophysiological, and therapeutic implications. Molec Psychiat 1996; 1:257.

Gray JA. The Psychology of Fear and Stress, 2nd ed. Cambridge: Cambridge University Press, 1987.

Hauger RL, Dautzenberg FM, Flaccus A, Liepold T, Spiess J. Regulation of corticotropin-releasing factor receptor function in human Y-79 retinoblastoma cells: rapid and reversible homologous desensitization but prolonged recovery. J Neurochem 1997; 68:2308.

Herman JP, Cullinan WE. Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. Trends Neurosci 1997; 20:78.

Koob GF, Heinrichs SC, Pich EM, Menzaghi F, Baldwin H, Miczek K, Britten KT. The role of CRF in behavioral responses to stress. CIBA Found Symp 1993; 172:277.

Miyata M, Okada D, Hashimoto K, Kano M, Ito M. Corticotropin-releasing factor plays a permissive role in cerebellar long-term depression. Neuron 1999; 22:763.

Owens MJ, Nemeroff CB. Physiology and pharmacology of CRF. Pharmacol Rev 1991; 43:425.

Penn RB, Benovic JL. Mechanisms of receptor regulation. Handbook of Physiology: Section 7: Endocrinology, Voume I: Cellular Endocrinology, Conn PM (ed). New York: Oxford University Press, 1998; pp 125–164.

Pitcher, JA, Freedman, NJ, Lefkowitz RJ. G protein-coupled receptor kinases. Annu Rev Biochem 1998; 67:653.

Plotsky PM. Pathways to the secretion of adrenocorticotropin: a view from the portal. J Neuroendocrinol 1991; 3:1.

Smith GW, Aubry J-M, Dellu F, et al. Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. Neuron 1998; 1093–1102.

Stenzel-Poore MP, Heinrichs SC, Rivest S, Koob GF, Vale WW. Overproduction of corticotropin-releasing factor in transgenic mice: a genetic model of anxiogenic behavior. J Neurosci 1994; 14:2579.

Timpl P, Spanagel R, Sillaber I, Kresse A, Reul JMHM, Stalla GK, Blanquet V, Steckler T, Holsboer F, Wurst W. Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. Nature Genet 1998; 19:162.

Turnball AV, Rivier C: CRF and endocrine responses to stress: CRF receptors, binding protein, and related peptides. Proc Soc Exp Biol Med 1997; 215:1.

Vale W, Vaughan J, Perrin M. Corticotropin-releasing factor (CRF) family of ligands and their receptors. The Endocrinologist 1997; 7:3S.

16 Neuroendocrine Regulation of Sexual Behavior

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Contents

1. INTRODUCTION

Beginning with the pioneering work of Frank Beach and his colleagues, together with W.C. Young and his colleagues, the effects of sex-steroid hormones on the reproductive behaviors of experimental animals have comprised one of the most reliable sets of empirical phenomena in endocrinology as well as in neurobiology. Estrogenic compounds are known to foster female-typical sex behaviors in genetic females. In a wide variety of cases, administration of progestins following estrogenic priming greatly amplifies the behavioral response. In genetic males, circulating testosterone fosters courtship and copulatory behaviors using at least three routes:

1. Actions in the chemical form of testosterone itself.
2. Actions following reduction to dihydrotestosterone.
3. Actions following aromatization to estrogens.

All of these hormone/behavior phenomena are subject to certain rules that appear to apply across a wide range of vertebrate forms. First, the assertion of efficacy in a variety of vertebrate animals, sex-steroid hormones, working through specific neuronal groups, promote behaviors associated with mating. Second, for stimulating behavior as well as for facilitating pituitary hormone release, the rates of onset of hormone treatments, the durations, and other temporal features of hormone administrations are all important. In males, androgens must be circulating at high levels for long periods to be effective. In females, estrogens are never absent and their effects seem to build up, working through very fast membrane mechanisms as well as very slow nuclear mechanisms, to achieve their overall results. In contrast, a rapid increase in progesterone will facilitate both female reproductive behavior and the ovulatory release of luteinizing hormone (LH) from the pituitary in estrogen-primed animals. However, a longer duration of progesterone action without a sudden increase will inhibit both lordosis behavior and the ovulatory release of luteinizing hormone from the pituitary.

Not only the presence and the duration of hormone treatment, but also the order of appearance of hormones in the brain can be important for stimulating reproductive behavior. Progesterone by itself is not effective, but following a period of estrogen exposure

From: *Neuroendocrinology in Physiology and Medicine*, (P. M. Conn and M. E. Freeman, eds.),

of at least 24 h, although preferably 48 or 72 h, progesterone can greatly amplify the effect of estrogen on female-typical behaviors and LH release. Gene expression for the progesterone receptor is required for this effect. Gene knockout data, antisense DNA data, and progesterone receptor blocker data all agree on this point.

Finally, when the steroid-sex hormones are administered by various routes, their metabolites also can be important for triggering reproductive behavior. Testosterone can act in its own chemical form, but also can be converted to dihydrotesterone or by a different chemical reaction, to estradiol. The case of progesterone is similarly complicated. Progesterone can operate through the progesterone receptor, which goes from the cytoplasm of the nerve cell into its nucleus and affects gene expression. Metabolized, progesterone can operate in the brain by affecting transmission through cell-membrane receptors for an inhibitory transmitter, GABA.

Because of the specificity of estrogenic actions on behavior through neural mechanisms, and because of the relative simplicity of certain female-typical mating behavior responses, the greatest progress has been made using female-typical behaviors as endpoints. Relying heavily on this reference and on a voluminous literature covering this and closely related subjects we review first, the neural and genetic mechanisms underlying the production of normal female-typical sexual behavior (Section 2.), and then we treat mechanisms underlying three of the normal biological constraints upon sexual behavior (Section 3.).

2. PRODUCTION OF HORMONE-DEPENDENT SEXUAL BEHAVIOR

2.1. Neural Circuitry Responsible for Producing the Behavior

The neural circuit for female rat lordosis behavior (Fig. 1) has been presented in detail. Because the evidence establishing this circuit has been published *in extenso*, only a few of the main points will be illustrated here.

Lordosis is triggered by cutaneous stimulation on the flanks of female rats followed by pressure on the posterior rump, tail base, and perineum. This pressure, which is applied by the male rat during natural mating behavior, is necessary and sufficient for lordosis to occur. Such cutaneous stimulation as applied by the male rat, leading to lordosis, can cause a barrage of action potentials from most of the cutaneous mechanoreceptive unit types in the dorsal root ganglia of the female. However, among all primary sensory neurons, only pressure units and Type I units give sustained responses to a lordosis-triggering type of cutaneous pressure stimulation. These types of sensory units have requirements that most closely fit the pattern of stimulus requirements for lordosis behavior as a whole. In order to evoke lordosis behavior, summation across pressure units certainly occurs and summation with other unit types may also be involved. If any single chain of events has the central role in the behavior, however, it is that pressure on the crucial skin areas deforms a special class of cutaneous receptors called Ruffini endings, thereby activating pressure units. Determining precise stimulus requirements for the lordosis circuit to function was important because the specificity of the behavioral result depends not only on hormone action and gene transcription, but also on the exact nature of the sensory input.

Behaviorally important sensory information triggers massive discharges in neurons deep in the rat dorsal horn of the spinal cord. However, local spinal circuits by themselves are not sufficient for lordosis behavior. Instead, a long circuit comprising ascending sensory information and descending neuronal facilitation from supraspinal nerve cell groups are required for lordosis behavior. The critical ascending and descending pathways run in the anterolateral columns of the spinal cord. The behaviorally important targets of the ascending fibers in lordosis behavior circuitry are in the medullary reticular formation and the lateral vestibular nucleus (*see* Fig. 1). Some of these fibers also make it to the midbrain central gray. Ascending sensory terminations in the brain stem do not immediately control descending neurons in a simple and direct manner. That is, it appears that the subsequent descending facilitation of lordosis behavior by brain stem neurons is not simply the result of a spinal-brainstem-spinal reflex, but rather has a tonic nature which reflects, in part, durable estrogenic influences originating in the hypothalamus.

At the top of the circuitry that facilitates lordosis behavior are the nerve cells in and immediately surrounding the ventromedial nucleus of the hypothalamus. Lesions or pharmacologic blockage of these cells leads to a loss of lordosis behavior. Electrical stimulation of these cells leads to lordosis facilitation. No other lesion or electrical stimulation sites in the forebrain can account for the facilitation of lordosis behavior. Therefore, among all telencephalic and diencephalic sites, the main source of lordosis behav-

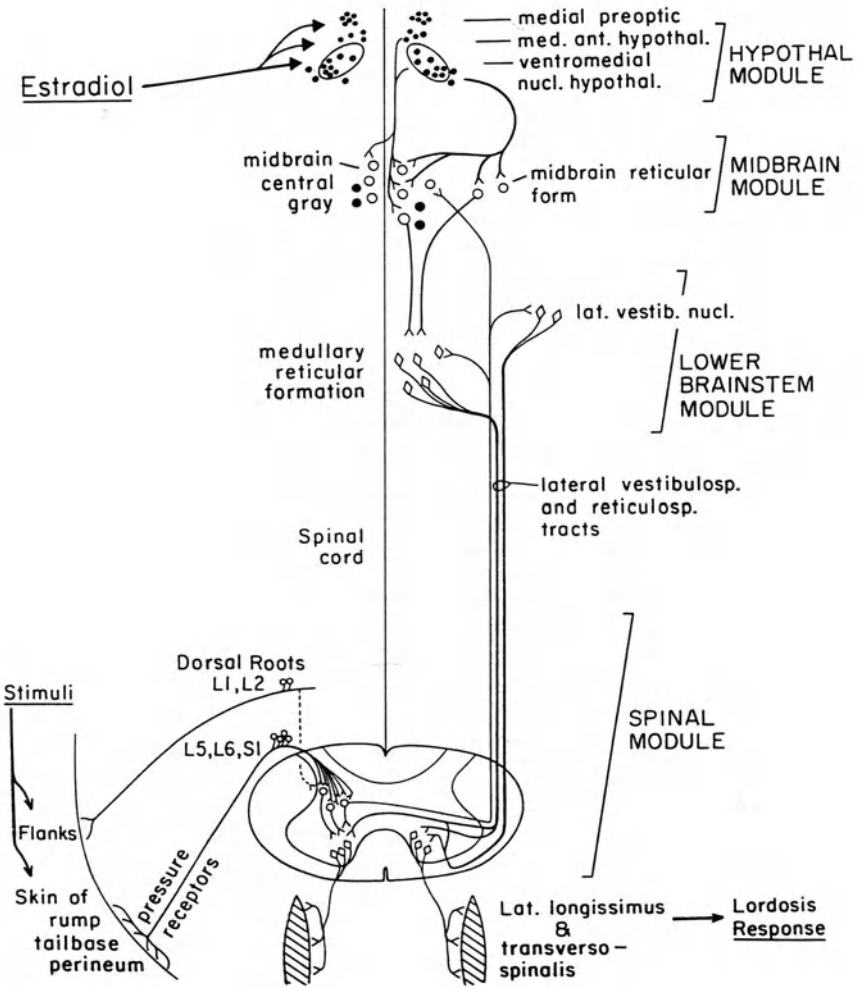

Fig. 1. Neural circuit responsible for producing lordosis behavior. From Pfaff et al. (1994) and Pfaff (1999).

ior facilitation must be ventromedial hypothalamic neurons.

The organization of axons descending from the hypothalamus has begun to be sorted out. Those related to lordosis behavior descend from the ventromedial hypothalamus either through a medial periventricular trajectory or through a lateral sweeping trajectory back to the midbrain reticular formation and periaqueductal gray. Those axons descending through a lateral sweeping trajectory make a larger quantitative contribution to lordosis. Neurons in the central gray send axons descending into the medullary reticular formation. The descending central gray signal activates medullary reticulospinal neurons as they synergize with lateral vestibulospinal neurons to control the deep back muscles that execute lordosis.

This midbrain central gray module is crucial. Electrical stimulation of the midbrain central gray will facilitate lordosis and central gray lesions disrupt it. The physiology of central gray neurons also allows us to understand how a strong somatosensory stimulus from the male, which ordinarily would be treated as noxious, can lead to a reproductive behavior. The same subregions of the central gray of the midbrain that are important for lordosis behavior, when activated, will lead to a decrease in pain. They cause stimulus-dependent analgesia. Thus, we can see how their activation by hormonally dependent inputs from the hypothalamus, as well as by perineal stimuli from the male, actually permits the lordosis response to occur.

The synergizing input from the lateral vestibular nucleus is also important, as lesions there will reduce lordosis in proportion to the number of vestibulospinal cells destroyed, and electrical stimulation of the lateral vestibular nucleus will facilitate lordosis. The lateral vestibulospinal tract from the lateral vestibular nucleus and the lateral reticulospinal tract from the

medullary reticular formation are the descending pathways that facilitate lordosis. They enhance the throughput from behaviorally adequate sensory stimulation to the deep back muscle motor neurons. These descending systems themselves and the muscle groups that execute lordosis behavior have physiological properties absolutely congruent with the requirements of lordosis behavior as a whole.

The deep back muscles, called lateral longissimus and transversospinalis, are attached dorsally to the spinal column so that when they contract, the spinal column will be bent "concave up." Consequently, these muscles are perfectly positioned to execute the vertebral dorsiflexion of lordosis. Thus, these muscles are responsible for the rump elevation, the most crucial component of lordosis behavior, which allows fertilization by the male. These muscles are anatomically connected to the skeleton so as to be physically competent to execute the lordosis response, and they are electrically active during the initiation of lordosis behavior. Bilateral electrical stimulation of the lateral longissimus or transversospinalis muscles produces vertebral dorsiflexion. Ablation of these muscles reduces lordosis strength.

The motor neurons for these muscles lie on the medial and ventral side of the ventral horn. They can be found at spinal levels receiving dorsal roots from thoracic level 12 through sacral level 1—that is, just anterior to, in and just posterior to the lumbar enlargement. As a result of the contraction of these muscles, lordosis behavior is displayed.

2.1.1. Modules in the Circuit

The modules in the lordosis behavior circuit, as designated in Fig. 1, match embryologically defined segments of the neuraxis (Fig. 2).

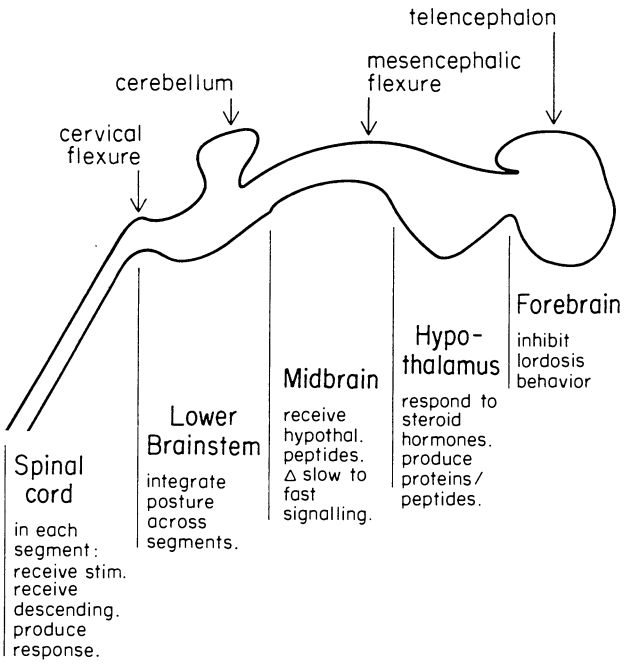

Fig. 2. The neural modules in the lordosis behavior circuitry match embryological divisions of the neuraxis. Also indicated are brief summaries of the main functions of each module with respect to female reproductive behavior. From Pfaff et al. (1994) and Pfaff (1999).

2.2. Genetic Mechanisms Mediating Effects of Hormones on Behavior

2.2.1. Hormone Effects on Gene Expression

Hormones influence genes, and those gene products influence behavior. Therefore, it is logical to conclude that part of the way in which sex-steroid hormones influence behavior is through genetic alterations. Different genes are turned on by estrogens in different neurons, and their respective gene products have different biochemical functions within those neurons.

Perhaps one of the best examples of this causal relation is the effect of estrogen on the gene for the progesterone receptor. Not only does estrogen administration induce the binding of radioactive progesterone in the hypothalamus, but also it causes an increase in the messenger RNA for the progesterone receptor. This effect occurs in females, but not males, and is restricted to brain regions related to reproductive behaviors. The effect of estrogen really is transcriptional, as shown by the use of neurotropic viral vectors for "in vivo promoter analysis," a technique in which the ability of the progesterone receptor gene promoter to respond is tested in normal neurons. In fact, estrogen induces the progesterone receptor in the cells in the hypothalamus needed for reproductive behavior. Because the progesterone receptor itself is a genetic transcription factor, these experiments represent the first example of the induction of a specific transcription factor key for the performance of a specific behavior. Both antisense DNA and genetic knockout technology show that gene expression for the progesterone receptor is, in its turn, necessary for normal hormone-driven reproductive behavior.

Likewise, estrogen induces expression of the gene for the opioid peptide enkephalin, whose messenger RNA levels are perfectly correlated with the performance of female rat reproductive behavior. In the ventromedial hypothalamus (VMH), enkephalin mRNA fluctuates during the normal estrous cycle,

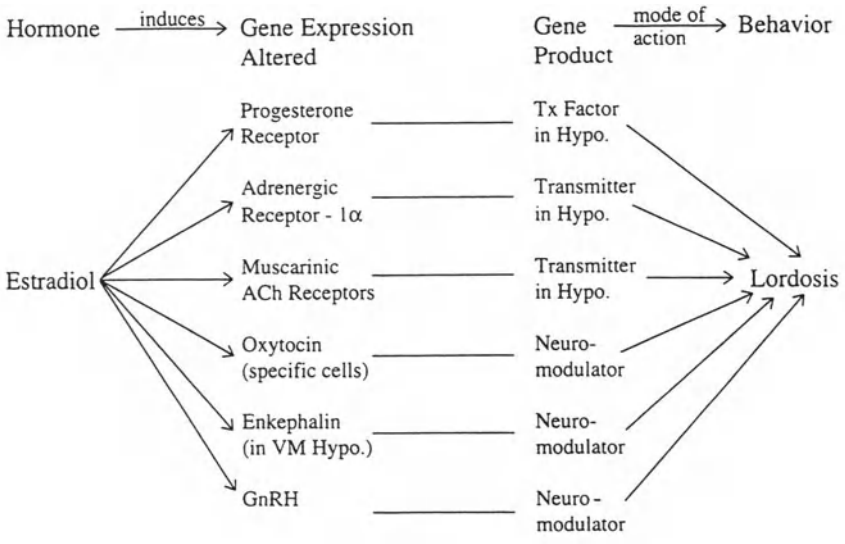

Fig. 3. Estrogen administration turns on certain genes in the hypothalamus and basal forebrain whose products, in turn, facilitate lordosis behavior. From Pfaff (1999).

suggesting that enkephalin gene expression is important for the performance of lordosis. Again, the use of a viral vector for in vivo promoter analysis shows not only that the enkephalin gene promoter directs expression of a reporter gene correctly in the brain but also that it is turned on by estrogen. Because the normal receptor for enkephalin, the delta opioid receptor, is also increased by estradiol, the enkephalin effect and the receptor effect should multiply each other. Again, the parallelisms between gene expression and behavior are striking: enkephalin induction occurs in females to a greater extent than in males and is strongest in the parts of the brain correlated with female reproductive behavior.

This genomic action of estrogen on enkephalin in ventromedial hypothalamic neurons appears to produce a partial analgesia (allowing the female to tolerate strong cutaneous and visceral stimuli from the male). VMH projections in the lordosis circuit (*see* Fig. 1) to ventral periaqueductal gray would be most important in this regard.

2.2.2. Gene Effects on Behavior

It is widely recognized that causal relations between genes and the behavior of higher animals will be difficult to discern for a variety of reasons. Among them are the pleiotropy of gene actions, redundancies among the functions of different genes, and variations in penetrance Fig. 4. Quantitative relations between genes and behaviors appear to be neither linear nor modular. With respect to reproductive behavior, genetic influences may be indirect, as from their effects on sexual differentiation, or they may be direct, as from their induction during adulthood in the neurons that execute sex behavior (see above).

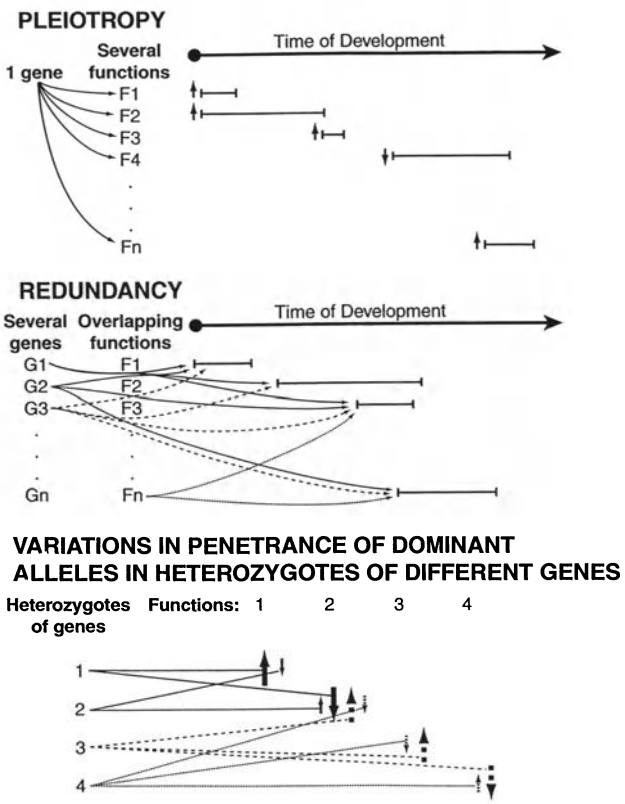

Fig. 4. Sources of difficulty in tracing causal relations from individual genes to behavioral functions. From Pfaff (1999).

Two successful approaches to demonstrating influences of specific genes on reproductive behavior have used mice with gene "knockouts" following homologous recombination and local CNS application of antisense DNA.

2.2.2.1. Gene Knockout Discoveries. Preparation of mice lacking the function of a normal, classical

	Incidence of female reproductive behavior	Incidence of female - female aggression
Wild type	normal	2/21 mice
Estrogen Receptor KnockOut	none	10/25 mice*

Fig. 5. Under circumstances where 100% of wild-type female mice show normal lordosis behavior, 0% of female mice whose classical estrogen receptor gene (ER-alpha) has been "knocked out" will show this behavior. From Ogawa et al. (1996).

estrogen receptor (ER-α) gene permitted a thorough assessment of the contribution of that gene to female mouse reproductive behavior. The answer was clear cut: estrogen receptor knockout (ERKO) female mice simply would not show lordosis (Fig. 5). There were at least three reasons for the absence of this behavior. First, ERKO females were treated as males by would-be stud males, with aggression following rapidly. Second, the ERKO females did not permit full mounts by stud males. Third, even if cutaneous stimulation as should lead to lordosis was applied forcefully, the response by ERKO females was reduced.

2.2.2.2. Antisense DNA Evidence. How might the behavioral importance of the induction of the progesterone receptor gene by estrogen in hypothalamic neurons be experimentally tested? Microinjected antisense DNA directed against progesterone receptor mRNA amongst ventromedial hypothalamic neurons and compared the behavioral results following microinjection of a DNA "scrambled sequence" control. Not only was lordosis behavior significantly reduced by the antisense DNA and not by the control DNA (Fig. 6), but also courtship behaviors, known to depend heavily on progesterone, were reduced to a mere 20% of their normal frequency. In this and many other antisense DNA experiments, the recovery, with time, of the behaviors affected indicated that the antisense effect was not because of a neuronal lesion.

2.3. Summary of Neural and Genetic Mechanisms for Producing Female-Typical Sexual Behavior

Estrogens circulating in the blood, enter the brain and are retained in a small number of neurons that have nuclear proteins that are estrogen receptors. As these proteins are transcription factors, estrogens bound to their receptors turn on a small number of genes whose protein products are important for female-typical behaviors. Consequently, a neural circuit that stretches all the way from the lumbar spinal cord to the basal forebrain is activated. When the female receives the appropriate cutaneous stimuli from the male, mating behavior ensues.

3. CONSTRAINTS UPON THE INITIATION OF MATING BEHAVIOR

Among the environmental factors in Fig. 7, some have received a great deal of experimental attention and have yielded some insight into the mechanisms involved. These include the constraining effects of cold temperature (*see* Section 3.1.), the restraining effects of limited food supply (*see* Section 3.2.) and the disruptive effects of stress (*see* Section 3.3.).

3.1. Cold Environmental Temperatures

In a variety of circumstances, mammalian reproduction is inhibited by environmental cold. It came to our attention that we could, theoretically, unite environmental thinking in this zoological tradition with modern molecular biology if we made use of the following reasoning: environmental cold would cause elevated levels of circulating thyroid hormones, which, in turn, would elevate the level of liganded thyroid hormone receptors in nerve cell nuclei, which, in turn, could interfere with estrogen receptor effects on female reproductive behavior. Thus, we review:

1. The molecular requirements for binding of estrogen receptor to DNA and subsequent action on the genome.
2. The molecular requirements for thyroid hormone receptor DNA binding and action; and, importantly.
3. The impact of thyroid hormones on estrogenic mechanisms at the molecular and behavioral level.

3.1.1. Molecular Requirements for Estrogen Receptor DNA Binding and Transcriptional Facilitation

For estrogens to facilitate reproductive behavior, hormonally induced gene expression and new protein synthesis are required. The estrogen receptor (ER) transcriptionally activates several genes, including many key genes involved in the process of reproduction. On binding the steroid hormone estrogen, the estrogen receptor binds stably as a dimer to an estrogen response element (ERE) within the promoters of certain target genes, whose consensus sequence, 5′ AGGTCANNNTGACCT 3′, is an inverted palindrome. This consensus is derived from the *Xenopus*

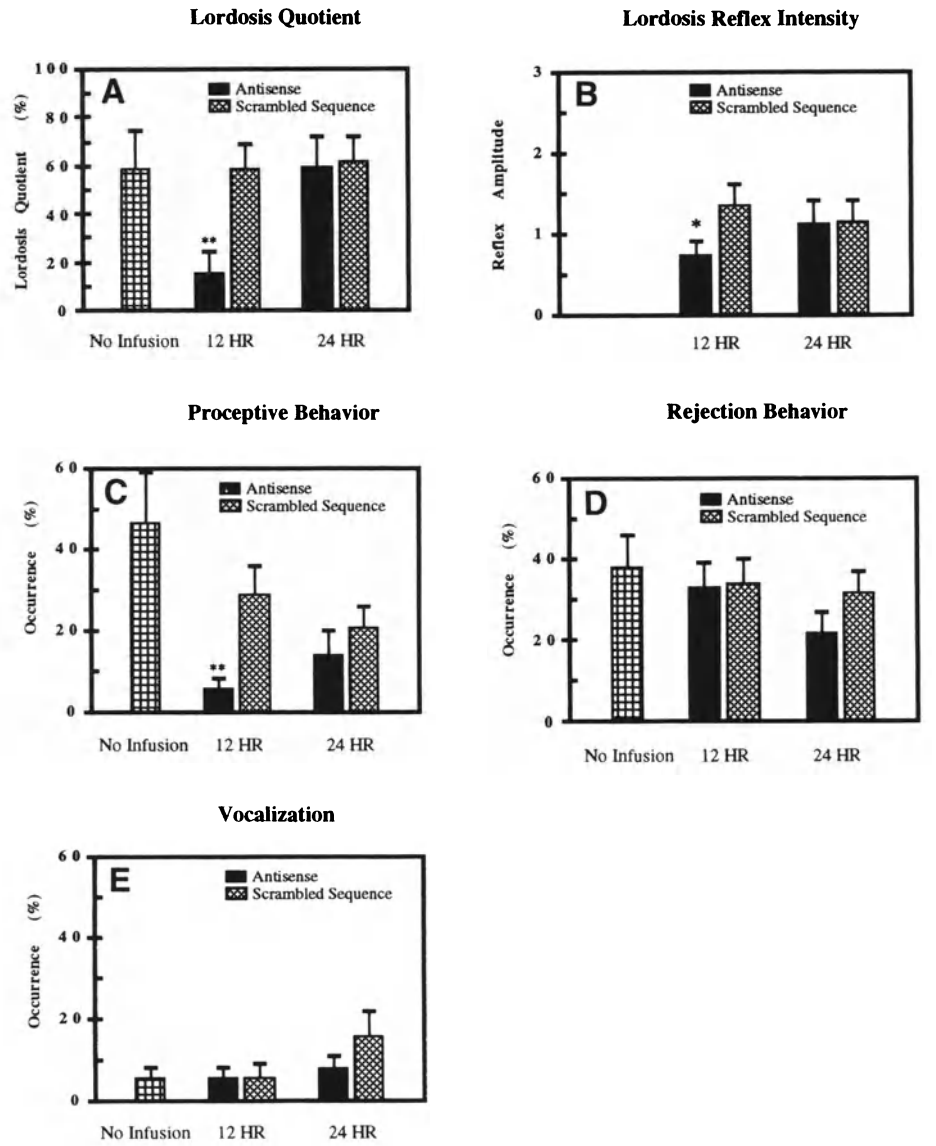

Fig. 6. Microinjection of antisense DNA directed against progesterone receptor (PR) mRNA amongst ventromedial hypothalamic neurons led to a significant decrease in lordosis behavior. Lordosis behavior recovered after 24 h. The scrambled sequence control DNA injection did not cause any behavioral change. (Panels **A** and **B**). Panel **C**: Antisense DNA against PR mRNA led to an even larger decrease in courtship (proceptive) behaviors. However, there were no significant changes in rejection behavior or vocalization. From Ogawa et al. (1996).

laevis vitellogenin (VTG) genes though many functionally relevant EREs differ considerably from the consensus.

Several groups have investigated the effect of ligand on the association of the ER with the consensus ERE. Kinetic analysis using the chicken VTG II ERE and the liganded human estrogen receptor revealed an association constant (Ka) of 0.1 x 10(9) M -1, and, at high receptor concentrations, the binding of the hormone ligand to the ER showed cooperativity. The same study also noted that the association constant for the unliganded receptor was five-fold lower than for the liganded receptor suggesting that there is greater stability on ligand binding. An ER containing a mutation in the hormone binding domain binds less tightly to an ERE than an intact ER, suggesting that hormones play a role in the formation of stable ER-ERE complexes. However, unliganded ER, as well as ER bound to estrogen or the mixed agonist-antagonist tamoxifen, bound ERE with the same affinity and protected the ERE in an identical manner.

Though there may be no conformational differ-

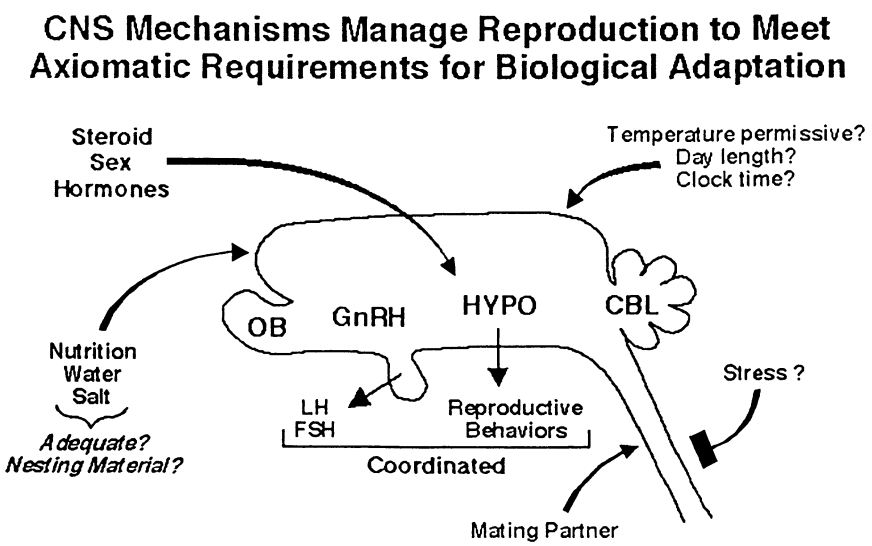

Fig. 7. In addition to the well-understood factors that drive female reproductive behavior (stimuli from the male and sex steroid hormones), several environmental factors constrain reproductive behavior in a manner which fills axiomatic biological requirements. Mechanisms for three of those factors are treated in this chapter: cold temperature, food restriction, and stress. From Pfaff (1999).

ences in the DNA binding domain of the ER when bound to estrogens and antiestrogens, immunological studies using monoclonal antibodies to different epitopes of the estrogen receptor indicate that there may be overall conformational changes, which would allow for differences in the formation of the transcription initiation complex between the estrogen liganded and the antiestrogen liganded receptor. Because unliganded estrogen receptor can dimerize and bind to EREs, it is possible that the role of estrogen may be to stabilize these complexes in vivo.

Several genes, including the well-studied amphibian and avian egg yolk genes, have more than one ERE in their promoters. Several studies using both estrogen and the antiestrogen, tamoxifen and its active metabolite, 4-hydroxy tamoxifen (4-OHT), have suggested that the presence of more than one hormone response element (HRE) facilitates cooperative binding of the liganded receptor to the ERE. What are the parameters that determine such cooperative binding? Genes like the vitellogenin family, which are very highly induced by estrogens and whose ERE sequence has led to the definition of a "consensus" ERE have a high percentage of AT base pairs in the regions 5′ and 3′ of the ERE. In the vitellogenin B1 gene of *Xenopus laevis*, there are two imperfect EREs at -302 and -334 upstream of the transcription start site. Deletion of either of the EREs, replacement of both the EREs with a single consensus ERE at -334, or addition of two consensus EREs at -359 made this promoter less responsive to estrogen than the natural promoter, suggesting that flanking sequences and natural promoter context was vital in the magnitude of induction by estrogen. In experiments involving the 15 bp perfect consensus ERE with the 3′ AT rich region, arranged singly or in tandem, cooperative binding occurred with estrogen-liganded ER (E2-ER) if the spacing between the center of the response elements was such that the estrogen response elements were on the same side of the DNA helix.

If binding of the ER to EREs is cooperative, is there synergism of gene activation because of multiple EREs in the promoters of target genes? Even when two EREs are placed at a distance as far as 2400 bp from the transcription start site, the activation of a reporter gene is synergistic as long as the distance between the consensus EREs is small, i.e., 23 bp. However, an ERE upstream can also synergize with an ERE downstream of the reporter gene. The distance of the ERE from the TATA box, and the center to center spacing between EREs, appear to be critical parameters such that the DNA between the EREs can presumably loop out and allow several EREs to contact each other effectively. Such a scenario would lead to synergism in gene activation. However, cooperative binding of the ER to the ERE is not a prerequisite for the cooperativity seen in the transcriptional activation of genes. In the chicken VTG II sequence, there is no cooperative binding observed in the binding of the ER to the ERE or progesterone receptor (PR) to the PRE though both elements synergise to activate transcription.

Why, then, are there so many EREs that deviate from the consensus? The consensus ERE sequence has been derived from the estrogen-induced vitellogenin genes in amphibians and birds. Most mammalian EREs deviate from this consensus, perhaps related to the degree of transcriptional activation of these genes by estrogen; most of them are less than the highly induced vitellogenin genes. The level of

induction of genes is presumably proportional to physiological need for the gene products.

In sum, a considerable amount of molecular work has defined mechanisms of estrogenic action mediated by the estrogen receptor. These experiments have laid the basis for molecular genetic studies related to mating behavior as it is fostered by estrogens and retarded by thyroid hormones.

3.1.2. Molecular Requirements for Thyroid-Hormone Receptor DNA Binding and Transcriptional Facilitation

The thyroid-hormone receptor (TR) is a nuclear receptor capable of binding to direct repeats of the ERE half site 5′ AGGTCA 3′ with 2 (DR2) or 4 (DR 4) intervening nucleotides. It can also bind the inverted palindrome, but without the gap of the intervening 3 nucleotides that characterize a "true" ERE. The thyroid response element (TRE) in the rat growth hormone gene is a 15 bp receptor binding site with a 3′ half-site similarity to the vitellogenin ERE; however, unlike the ERE, it has no intervening nucleotides. Introduction of a series of nucleotides from one to six in number into the growth hormone TRE to obtain "gapped" TREs reduced the binding affinity by less than a factor of five, suggesting that the TR recognizes determinants that are common to both EREs and TREs despite differences in spacing. Mutational analysis of the wildtype growth hormone TRE showed that most mutations, including those in the flanking sequences, resulted in decreased binding affinity of the TR. However, a limited number of mutations produced an increase in the binding affinity and if these mutations were to be simultaneously incorporated into the rat growth hormone TRE, a near perfect palindrome similar to the ERE would be obtained. Hence, Glass *et al.* suggested that the palindromic ERE sequence without the spacer is the consensus TRE and this binds the TR with greater affinity than the growth hormone TRE. Though the vitellogenin ERE differs from the TRE only in the addition of a spacer, the TR binds the ERE with lesser affinity than the nongapped TRE but with higher affinity than the rat growth hormone TRE.

Does binding affinity then correlate with the level of transcriptional activation from promoters containing such hormone response elements? Introduction of the growth hormone TRE and the consensus TRE with zero spacing upstream of the TK promoter showed that the magnitude of induction by thyroid hormone is approximately 2.7-fold with the growth hormone TRE and 4.1-fold with the consensus TRE. Similar to the increased activation that is observed with multiple copies of the ERE, multiple copies of the TRE also increase the induction of the target gene by the TR. Harbers *et al.* have investigated the nature of the spacer in the DR4 TRE, which is also bound with high affinity by the thyroid hormone/retinoid X receptor (TR/RXR) heterodimer. A pyrimidine nucleotide in position three of the spacer enhanced both TR/RXR binding and the transactivation of a reporter gene, suggesting that the nucleotide sequence of the spacer may also play a role in the binding and transactivation by nuclear hormone receptors.

3.1.3. Impact of Thyroid Hormones on Estrogenic Mechanisms at the Molecular and Behavioral Levels

Because thyroid hormone receptors can bind to DNA, which constitutes an ERE half-site, we hypothesized that cold environmental temperatures raising thyroid hormone levels could act through thyroid-receptor competitive DNA binding, to reduce estrogen receptor-dependent mating behavior. That is, thyroid hormones liganded to their receptors would bind to an ERE half-site thus disrupting formation of a transcriptionally productive ER homodimer and reducing estrogenic facilitation of the mating behavior circuit. Indeed, thyroid hormone coadministration with estrogens can reduce ER binding to DNA and subsequent ER-dependent transcription. As well, thyroid hormones interfere with estrogenic induction of oxytocin mRNA in the female rat brain and estrogenic induction of enkephalin mRNA. As should be clear from Fig. 3, such interference would reduce lordosis behavior. Behavioral studies confirmed that thyroid hormones, endogenous or exogenous, quantitatively reduced the estrogenic promotion of lordosis. This result held true in female rats and mice. Gene knockout experiments indicated that the thyroid-hormone receptor beta gene was most important in this regard.

3.2. Food Restriction Reduces Reproductive Behavior

As demonstrated by Frank Bronson and his colleagues during decades of research, restriction of food supplies can interrupt reproductive processes, especially when other important factors also are not optimal, for example, cold temperatures. The opportunities for chemical messengers to signal inadequate nutrition to the hypothalamus are manifold, as nothing less than thirteen physiological substances significantly altered by nutritional status have been identified as of July 1998. The indisputable effect of a decrease

in metabolic energy sources on lordosis behavior has been presented and reviewed by George Wade and his colleagues. Presumably, the biological reason for reduction of lordosis behavior is that, if the female does not have a food supply adequate for herself, she will not engage in behaviors which would eventuate in additional metabolic demands from offspring.

3.3. Stress Interferes with Reproductive Behavior

Situations of either acute or chronic stress suppress secretion of the pituitary gonadotropin, luteinizing hormone (LH), and, as a consequence, cause a decrease in reproductive competency. Stress leads to increased adrenal steroid secretion (corticosterone in rodents, cortisol in primates) and concomitant changes in other humoral and neural signals. As an example of acute stress effects, Briski showed that physical stress reduced serum LH levels. This phenomenon depended on the glucocorticoid receptor (GR) as the receptor antagonist RU486 reversed the effect. McGivern and Redei subjected adult rats to intermittent foot shock for 100 min. This stressor induced a suppression of serum LH within 20 min in ovariectomized (OVX) rats, whereas there was no effect on LH in animals both OVX and adrenolectomized (ADX). Adult male Wistar rats subjected to either acute or chronic immobilization stress also showed a significant decline in plasma LH levels. Again, RU486 blunted the inhibitory effects of acute and chronic immobilization stress on LH release. Chronic restraint stress (8 h/d for 10 d) and other long-term stressors led to a drastic decrease in LH titers that did not depend on the sustained elevation in adrenocorticotropin hormone (ACTH). These studies generally support a role for glucocorticoids as primary effectors in stress-induced inhibition of LH. In blocking the estrogen-induced ovulatory discharge of LH, stress interferes with the hormonal basis for female reproductive behavior during the normal estrous cycle.

Because endogenous opioid peptides (EOP) and corticotropin releasing factor (CRF) are activated during stress, Petraglia et al. (1986) used castrated male rats to investigate the roles of these peptides in the inhibition of LH secretion brought about by inescapable intermittent footshock. Their results implicated stimulation of β-endorphin and dynorphin systems via μ-1 or κ opiate receptors. CRF that was injected either icv or into the medial preoptic area (MPOA) likewise decreased plasma LH levels, suppressed hypothalamic gonadotropin releasing hormone (GnRH) release into the median eminence, but did not modify follicle-stimulating hormone (FSH) release in OVX rats. Rivest et al. (1993a) suggested that the effect of CRF might be because of the release of opioids from the MPOA, which activate μ-1 opiate receptors. Wardlaw et al. (1998) recently reported that adrenalectomy inhibits pro-opiomelanocortin (POMC) gene expression and peptide levels in the medial basal hypothalamus (MBH), whereas physiological levels of glucocorticoids reverse this effect, directly implicating glucocorticoids in regulation of EOP.

Immune challenges, which also stimulate the release of adrenal steroids, have been studied extensively by Rivier and Rivest in relation to their influence on the GnRH neuronal system and gonadotropin release. Acute injection of interleukin-1 (IL-1) into the brain (but not iv injection) significantly lowered plasma LH levels in gonadectomized male and female rats, interfered with GnRH release into the median eminence of proestrous females, and blocked cFos expression in GnRH neurons. IL-1 given icv also decreased GnRH gene expression in OVX rats. A subsequent study involved examination of the effect of chronic icv infusion of IL-1 on the estrous cycle. Exposure of female rats to IL-1 for 4-6 d disrupted the estrous cycle. This treatment significantly blunted gonadotropin secretion presumably because of reduced levels of the mRNAs encoding both GnRH and the gonadotropin subunits.

The available evidence does not support a role for CRF in the inhibitory effect of IL-1 on LH secretion, but rather participation of elevated glucocorticoid levels remains a viable possibility. Recently, Nappi and Rivest showed that exposure of female rats to bacterial endotoxin (LPS) for 3 h on proestrus reduced the number of GnRH neurons expressing cFos and the level of GnRH mRNA. Less GnRH released means less LH and FSH released, thus reducing ovarian hormonal support for mating behavior.

3.3.1. Clinical Importance

Clinical studies, as well, have indicated that adrenal disease and many types of stressors diminish reproductive function and fertility in affected individuals. For example, adult males with documented Cushing's disease exhibit significantly decreased basal levels of LH and FSH as well as responses to GnRH administration. Patients in remission from this disease showed a return of gonadotropin levels up to the normal range indicating that suppression of hypercortisolism in male Cushing's disease is able to reverse the hypogo-

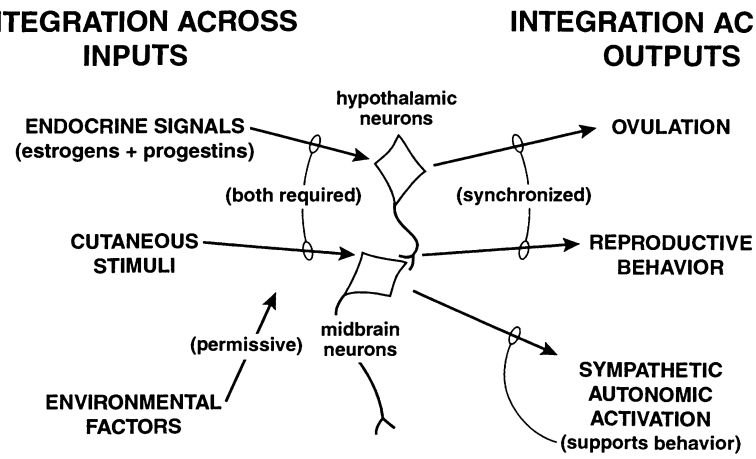

Fig. 8. Hypothalamic and midbrain neurons governing female reproductive behavior perform two remarkable types of neuronal integration. On the **input** side, endocrine signals facilitate the behavioral response to cutaneous stimuli from the male, constrained at the same time by limiting environmental factors. On the **output** side, the concerted effects of estrogens and progestins synchronize reproductive behavior with ovulation, supported by hypothalamic control of the sympathetic autonomic nervous system.

nadotropic hypogonadism. Dubey and Plant (1985) demonstrated that male rhesus monkeys subjected to prolonged exposure to hydrocortisone acetate (HCA) developed symptoms of Cushing's disease and a gradual decline in circulating LH and FSH to nondetectable levels. Following withdrawal of HCA, gonadotropin concentrations returned to normal. As an intermittent infusion of GnRH was able to restore circulating LH and FSH in HCA-inhibited animals, these authors concluded that the action of HCA on gonadotropin secretion in the rhesus monkey is mediated by inhibition of GnRH synthesis and/or release.

Studies have been carried out also in normal cyclic women and in those with functional hypothalamic amenorrhea (FHA). Saketos et al. (1993) investigated whether elevated cortisol affects the hypothalamic-pituitary-gonadal (HPG) axis in eumenorrheic women during the early follicular phase. Their results showed that cortisol can slow LH pulse frequency and, by inference, hypothalamic GnRH secretion, concluding that "cortisol alone may therefore play a role in the development of stress-associated menstrual disturbances." Furthermore, in women with FHA, both LH pulse frequency and total LH concentrations per 24 h were significantly lower than in normal women, and this decrease was associated with higher 24 h cortisol secretion in the women with FHA. Women who recovered from FHA had cortisol levels comparable to those of eumenorrheic women, underscoring the conclusion that FHA develops in response to stress-induced alterations in hypothalamic function. In women, the hormonal links to courtship and mating behaviors are less powerful than in lower animals, although the argument has been made that neural and molecular mechanisms for the most primitive aspects of sex drive have been conserved.

4. NEURAL INTEGRATION RELATED TO THE BEHAVIOR

4.1. At the Molecular Level

During most of the history of neurobiology, neuronal integration has been conceived as depending upon the complexity of synaptic connectivity and membrane biophysics. During the last thirty years, the tools of modern neurochemistry have allowed us a second approach to neuronal integration based on the pharmacology of neurotransmitters and neuropeptides.

Some of the data presented here, in Section 3.1. on thyroid hormone receptors, open up a new, third level of neuronal integration: interactions among transcription factors, which embody the combinatorial logic expected of sophisticated integrative mechanisms. In the case illustrated (TR vs ER) the interaction was competitive. In other cases, it might be synergistic. Explanations of behavior based on this new level of neuronal integration will be superimposed upon explanations based on the first two.

4.2. At the Physiological Level

Mechanisms of sexual behavior offer an unparalleled opportunity to unravel and demonstrate physiological integration both with respect to the inputs to forebrain neurons and the outputs managed by those neurons (Fig. 8). About the *inputs:* in this chapter, we have referred to (1) cutaneous sensory inputs from the male; (2) facilitating hormonal signals from the ovaries; and (3) environmental influences that can prevent reproductive behavior when they are not in their optimal range. Facts summarized here illustrate in some detail how the sensory and hormonal inputs

work and, in fact, how they are integrated. Some of the mechanisms underlying disruption because of cold temperature, food restriction, and stress are also noted.

Regarding the outputs: hypothalamic neurons manage the reproductive behavioral circuit emphasized here, the neuroendocrine signals to the anterior pituitary (reviewed elsewhere in this text) for the release of LH and FSH, and the autonomic nervous system adaptations, which support the muscular activity of reproductive behavior. Clearly, the synchronizing effects of estrogen priming followed by a rapid increase in circulating progesterone serve to integrate female reproductive behavior with ovulation. That is, the animal only engages in mating behavior when it is likely to lead successfully to a pregnancy. Further to this point, the facilitation of lordosis by GnRH synchronizes mating with ovulation. Finally, the status of the medial and posterior hypothalamus as the "head ganglion" of the sympathetic autonomic nervous system lays the basis for vascular changes preparative to the muscular activities of courtship and other reproductive behaviors.

SELECTED READINGS

Anolik JH, Klinge CM, Hilf R, Bambara RA. Cooperative binding of estrogen receptor to DNA depends on spacing of binding sites, flanking sequence and ligand. Biochemistry 1995; 4:2511.

Bailly A, Rauch C, Cato AC, Milgrom E. In two genes, synergism of steroid hormone action is not mediated by cooperative binding of receptors to adjacent sites. Mol Cell Endocrinol 1991; 82:313.

Becker JB, Breedlove SM, Crews D. Behavioral Endocrinology. Cambridge: The MIT Press, 1992.

Berga SL, Mortola JF, Girton L, Suh B, Laughlin G, Pham P, Yen SSC. Hypercortisolism in patients with functional hypothalamic amenorrhea. J Clin Endocrinol Metab 1988; 68:301–308.

Berga SL, Daniels TL, Giles DE. Women with functional amenorrhea but not other forms of anovulation display amplified cortisol concentrations. Fertil Steril 1997; 67:1024–1030.

Briski KP. Stimulatory vs. inhibitory effects of acute stress on plasma LH: differential effects of pretreatment with dexamethasone or the steroid receptor antagonist, RU 486. Pharmacol Biochem Behav 1996; 55:19–26.

Campfield AL, Smith FJ, Burn P. Strategies and potential molecular targets for obesity treatment. Science 1998; 280:1383–1387.

Chang T-C, Nardulli AM, Lew D, Shapiro DJ. The role of estrogen response elements in expression of the Xenopus laevis vitellogenin B1 gene. Mol Endocrinol 1992; 6:346.

Comuzzie AG, Allison DB. The search for human obesity genes. Science 1998; 80:1374–1377.

Demura R, Suzuki T, Nakamura S, Komatsu E, Odagiri E, Demura H. Effect of immobilization stress on testosterone and inhibin in male rats. J Androl 1989; 10:210–213.

Driscoll MD, Klinge CM, Hilf R, Bambara RA. Footprint analysis of estrogen receptor binding to adjacent estrogen response elements. J Steroid Biochem Molec Biol 1996; 58:45.

Dubey AK, Plant TM. A suppression of gonadotropin secretion by cortisol in castrated male rhesus monkeys (Macaca mulatta) mediated by the interruption of hypothalamic gonadotropin-releasing hormone release. Biol Reprod 1985; 33:423–431.

Glass CK, Holloway JM, Devary OV, Rosenfeld MG. The thyroid hormone receptor binds with opposite transcriptional effects to a common sequence motif in thyroid hormone and estrogen response element. Cell 1988; 54:313.

Harbers M, Wahlstrom GM, Vennstrom B. Transactivation by the thyroid hormone receptor is dependent on the spacer sequence in hormone response elements containing directly spaced half sites. Nuc Acids Res 1996; 24:2252.

Klinge CM, Peale FV, Hilf R, Bambara RA, Zain S. Cooperative estrogen receptor interaction with consensus or variant estrogen response elements in vivo. Cancer Res 1992; 52:1073.

Klinge CM, Bambara RA, Hilf R. Antiestrogen liganded estrogen receptor interaction with estrogen responsive element DNA in vitro. J Steroid Biochem Mol Biol 1992; 43:249.

Knobil E, Neill J. The Physiology of Reproduction (Second Edition.) New York: Raven, 1994.

Luton J-P, Thieblot P, Valcke J-C, Mahoudea JA, Bricaire H. Reversible gonadotropin deficiency in male Cushing's disease. J Clin Endocrinol Metab 1977; 45:488–495.

Meisel R, Sachs B. The Physiology of Reproduction: Male reproductive behavior. In Knobil and Neill, eds. (Vol. 2) op. cit., pp. 1–106.

Melamed M, Arnold SF, Notides AC, Sasson S. Kinetic analysis of the interaction of human estrogen receptor with an estrogen response element. J Steroid Biochem Mol Biol 1996; 57:153.

Nappi RE, Rivest S. Effect of immune and metabolic challenges on the luteinizing hormone-releasing hormone neuronal system in cycling female rats: an evaluation at the transcriptional level. Endocrinology 1997; 138:1374–1384.

Ogawa S, Olazabal UE, Parhar IS, Pfaff DW. Effects of intrahypothalamic administration of antisense DNA for progesterone receptor mRNA on reproductive behavior and progesterone receptor immunoreactivity in female rat. J Neurosci 1994; 14:1766–1774.

Ogawa S, Taylor J, Lubahn DB, Korach KS, Pfaff DW. Reversal of sex roles in genetic female mice by disruption of estrogen receptor gene. Neuroendocrinology 1996; 64:467–470.

Parker MG. Steroid hormone action. Oxford: IRL, 1993.

Petraglia F, Vale W, Rivier C. Opioids act centrally to modulate stress-induced decrease in luteinizing hormone in the rat. Endocrinology 1986; 119:2445–2450.

Pfaff DW. Drive: Neural and Molecular Mechanisms for Sexual Motivation. Cambridge: The MIT Press, 1999.

Pfaff DW. Introduction: Genetic influences on the nervous system and behavior. In Pfaff DW, Berrettini W, Joh T, Maxson S, eds. Genetic Influences on Neural and Behavioral Function. Boca Raton: CRC Press, 1999b.

Pfaff DW, McCarthy M, Schwartz-Giblin S, Kow LM. The Physiology of Reproduction: Female reproductive behavior. In: Knobil and Neill, 1994 Vol. 2. op. cit., 107–220.

Rivest S, Plotsky PM, Rivier C. CRF alters the infundibular LHRH secretory system from the medial preoptic area of female rats: possible involvement of opioid receptors. Neuroendocrinology 1993a; 57:236–246.

Rivest S, Rivier C. Interleukin-1 beta inhibits the endogenous expression of the early gene c-fos located within the nucleus of LH-RH neurons and interferes with hypothalamic LH-RH release during proestrus in the rat. Brain Res 1993b; 613: 132–142.

Rivest S, Lee S, Attardi B, Rivier C. The chronic intracerebroventricular infusion of interleukin-1 alters the activity of the hypothalamic-pituitary-gonadal axis of cycling rats. I. Effect on LHRH and gonadotropin biosynthesis and secretion. Endocrinology 1993c; 133:2424–2430.

Sathya G, Li W, Klinge CM, Anolik JH, Hilf R, Bambara RA. Effects of multiple estrogen responsive elements, their spacing and location on estrogen response of reporter genes. Mol Endocrinol 1997; 11:1994.

Saketos M, Sharma N, Santoro NF. Suppression of the hypothalamic-pituitary-ovarian axis in normal women by glucocorticoids. Biol Reprod 1993; 49:1270–1276.

Suh BY, Liu JH, Berga SL, Quigley ME, Laughlin GA, Yen SSC. Hypercortisolism in patients with functional hypothalamic-amenorrhea. J Clin Endocrinol Metab 1998; 66, 733–739.

Tache Y, Du Ruisseau P, Ducharme JR, Collu R. Pattern of adenohypophyseal hormone changes during chronic intermittent immobilization of the rat. Neuroendocrinology 1978; 22:325–336.

Tsai SY, Tsai MA, O'Malley BW. Cooperative binding of steroid hormone receptors contributes to transcriptional synergism at target enhancer elements. Cell 1989; 57:443.

Wade GN, Schneider JE. Metabolic fuels and reproduction in female mammals. Neurosci Biobehav 1992; 16:235–272.

Wade GN, Schneider JE, Li H-Y. Control of fertility by metabolic cues. Am J Physiol 1996; 270:E1–E19.

Wardlaw SL, McCarthy KC, Conwell IM. Glucocorticoid regulation of hypothalamic proopiomelanocortin. Neuroendocrinology 1998; 67:51–57.

Woods SC, Seeley RJ, Porte D, Schwartz MW. Signals that regulate food intake and energy homeostasis. Science. 1998; 280:1378–1383.

17 Neuroendocrine Regulation of Maternal Behavior

Robert S. Bridges, PhD and Elizabeth M. Byrnes, PhD

Contents

1. INTRODUCTION

Maternal behavior in mammals is controlled by a combination of biochemical, neural, and environmental factors. Understanding of the neuroendocrine events that regulate maternal behavior has increased significantly in recent years with the identification of roles for specific hormones and peptides and the partial elucidation of the neural network involved in regulating maternal care. In addition, much is now known regarding the importance of sensory feedback from offspring and the role of experience on these behaviors. The focus of this chapter concerns the neuroendocrine regulation of parental behavior in the mammal with a focus on research in the rat. Specifically, the neural substrate underlying the expression of maternal care will be reviewed together with the roles of various hormones and neurochemical agents within the context of normal development in the female. Sensory aspects of maternal responsiveness as well as the effects of gene knockouts will be discussed. Finally, the effects of parental experience on the biochemical regulation of this evolutionarily critical behavior is presented.

From: *Neuroendocrinology in Physiology and Medicine*, (P. M. Conn and M. E. Freeman, eds.),

2. BEHAVIORAL RESPONSES OF MOTHERS

Parturient animals display a set of behavioral responses over the course of lactation that help to ensure the growth and survival of their offspring. These behaviors can be divided into those which are directed toward the young and those which are not (*see* Table 1). Specific young-directed responses exhibited by mothers over the course of lactation vary according to the habitat and developmental needs of the young. For example, in rats these behaviors include retrieval of the young to the nest site, grouping the young together, crouching over the pups to provide warmth and the opportunity to suckle, anogenital licking to stimulate micturition and defecation, and tactile stimulation. In sheep, one of the larger animals that have received significant research attention, the primary behaviors that the mother displays includes tac-

Table 1
Compilation of behavioral responses displayed by maternal female mammals

Nesting Responses	Nest building
	Retrieval of the young to the nest
Nourishment	Nursing
	Placentophagia
Protection	Carrying the young
	Maternal aggression
Tactile Responses	Crouching/cuddling
	Grooming
	Licking

tile stimulation to facilitate bonding, vocal stimulation to foster communication, and access to the utter to permit the lamb to suckle. Likewise, in primates, young-directed responses would include holding and carrying the young, vocal and tactile communication, as well as nursing behavior. Nonyoung-directed maternal responses in these mothers include behaviors such as nest building, placentophagia, and defense of the young. Each of these maternal responses are quantifiable and have been used in numerous experimental studies to examine the proximate mechanisms underlying the expression of maternal behavior.

3. MATERNAL BEHAVIOR—A DEVELOPMENTAL PERSPECTIVE

The basic capacity to display maternal behavior is present throughout most of development in female mammals from prior to puberty and through adulthood. However, the amounts and kinds of stimuli required to elicit maternal care differ as a function of the animal's physiological and experiential state. The focus of this chapter will be on studies in the rat that provide an animal model for mammalian parenting. The early studies of Rosenblatt, as well as Cosnier and Couturier in the 1960s, established that behaviorally naive adult female and male rats would show parental responses if kept in constant contact with young pups (approx 3–10 d of age) for about one week. The latencies to display retrieval, grouping and crouching responses ranged from 5–8 d of age for both sexes, indicating that the neural substrate necessary to engage in parental behavior was present in both sexes and that the shortened latencies to display parental care at parturition were apparently the result of the physiological conditions associated with pregnancy. The average latency to display parental behavior in female rats during development is depicted in Fig. 1. Interestingly, the latency to display parental care is quite short during the postweaning-prepubertal period in both female and male rats with response latencies equal to 1–2 d. By 30 d of age, the latency increases to 5–7 d, a response latency generally retained until the female becomes pregnant. Once pregnant, the latency remains elevated at 5–6 d until just prior to parturition when rats exhibit a spontaneous onset of maternal care when presented with foster young. Female rats that have given birth and raised a litter continue to display short-latency maternal behavior when presented with young weeks after weaning or removal of the young. This retention of maternal responsiveness, i.e., maternal memory, appears to depend on mother–young interactions during the immediate postpartum period, but is not dependent upon establishment of nursing, because mothers whose young were removed after the completion of parturition, but did not nurse, still remembered how to be maternal 3–4 wk later.

During pregnancy and lactation most mothers display heightened levels of aggression toward strangers. Defense of the young, which is referred to as maternal aggression, appears to occur at two stages. First, during pregnancy dams display heightened levels of "pregnancy-induced" aggression. Next, parturient and lactating females display high levels of "postpartum aggression." In the rat postpartum, maternal aggression is highest at parturition and remains elevated through the first 2 wk of lactation, declining after day 14. These forms of defensive aggression are typically evoked by a male or female intruder. The level or intensity of aggression displayed by a maternal female in response to an intruder is a behavioral measure used to quantify this aspect of maternal behavior.

What is the basic underlying neural network responsible or involved in the regulation of these developmental shifts in maternal behavior in female mammals? Our basic understanding of the neural substrate underlying the expression of maternal care is reviewed below in order to provide a basis for discussions of the neuroendocrine factors that regulate maternal care.

4. NEUROANATOMY OF MATERNAL BEHAVIOR

Much of the recent work on the neuroanatomy of maternal behavior points to the central involvement of the medial preoptic area (MPOA), and to a lesser extent, the ventral bed nucleus of the stria terminalis (VBST). A schematic representation of key neural areas involved in the regulation of maternal behavior

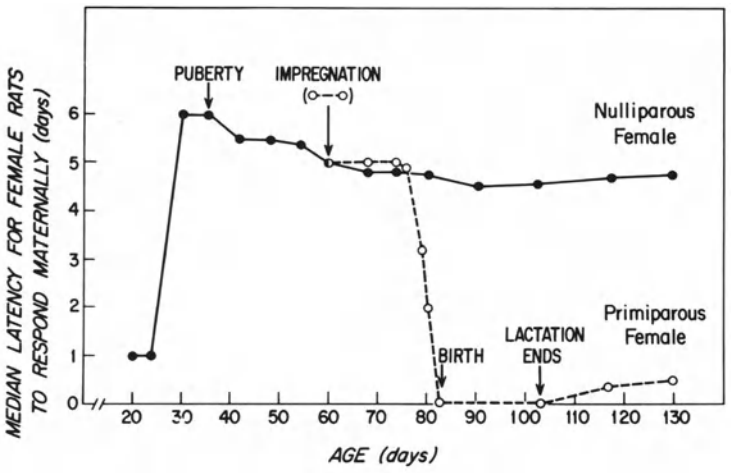

Fig. 1. A developmental profile of maternal responsiveness in female rats. Values represent the median number of days of pup exposure necessary to induce maternal behavior, that is retrieval, grouping and crouching during a 1-h test. From *Mammalian Parenting: Biochemical, Neurobiological, and Behavioral Determinants.* Norman A. Krasnegor and Robert S. Bridges, eds. 1990: Oxford University Press, Inc. (Reprinted by permission).

is shown in Fig. 2. Destruction of cell bodies within the MPOA or VBST severely disrupts the initiation as well as the maintenance of maternal behavior, in some cases eliminating maternal behavior in its entirety. Moreover, the application of either estradiol or prolactin to MPOA neurons results in the stimulation of maternal behavior in nulliparous females. Studies by Numan et al. have used tract-tracing techniques to unveil the connectivity of the MPOA and VBST. Initial studies were performed using anterograde tracers injected into these regions. Once the projection areas of these structures were determined, important double labeling studies were performed examining fos-induction during maternal behavior in conjunction with retrograde tracers injected into some of these MPOA/VBST efferent regions. It was found that a significant number of neurons activated during maternal responding (c-FOS labeled neurons) were double labeled when tracers were applied to particular areas. These regions included the ventral tegmental area (VTA), periaquaductal gray (PAG), ventromedial nucleus (VMN) of the hypothalamus, lateral septum (LS), and the retrorubral field (RRF). Whereas some of these structures were previously implicated in the regulation of maternal behavior, the double-labeling studies directly examined which connections of the MPOA and VBST may influence maternal behaviors.

As noted above, one region that is involved in the mediation of maternal behavior is the VTA. This nucleus, as well as the more caudal brainstem nuclei (PG and RRF), appear necessary for both the motivational and motoric aspects of maternal behavior. Indeed, a number of studies have demonstrated the potential involvement of tegmental neurons in the mediation of retrieval behavior in female rats. Destruction of the projections from the MPOA to

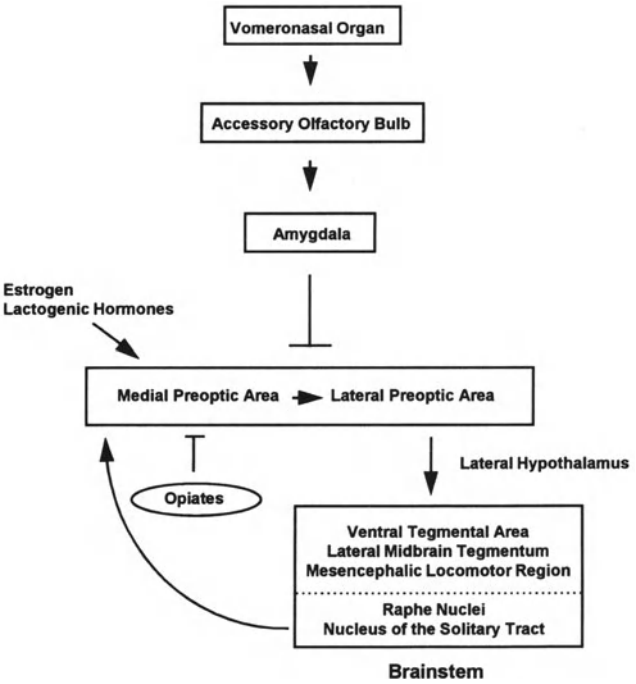

Fig. 2. Model depicting some of the neural circuitry that may underlie maternal behavior in rats. |— signifies inhibitory input. The areas in boxes represent brain nuclei. From *Mammalian Parenting: Biochemical, Neurobiological, and Behavioral Determinants.* Norman A. Krasnegor and Robert S. Bridges, eds. 1990: Oxford University Press, Inc. (Reprinted by permission).

the VTA by deafferentation results in a loss of pup retrieval behavior in primiparous females. Similar effects are observed using the neurotoxin 6-hydroxydopamine, which destroys DAergic neurons within the VTA. Some of these deficits, however, can be overcome by increasing the length of separation between pup removal and behavioral testing, indicating that these deficits do not result from decreased

motor behavior, but rather from decreased motivation. Lesions that destroy more caudal brainstem projection sites such as the PAG and RRF, however, do completely abolish retrieval. It, therefore, has been proposed that while the VTA may be associated with the motivational aspects of retrieval behavior, the more caudal brainstem nuclei may directly influence the motoric aspects of maternal behavior.

The VMN of the hypothalamus is another structure implicated in the control of maternal behavior. Interestingly, this region appears to be inhibitory to maternal responding. Studies employing excitotoxic lesioning of this nucleus decreased latencies to respond maternally in estrogen-primed, nulliparous female rats. It has been hypothesized that this nucleus may inhibit MPOA neurons in virgin females. The neuroendocrine alterations of late pregnancy are then believed to disinhibit the region allowing for the rapid development of maternal behavior upon parturition.

The VMN of the hypothalamus receives neural input from another potentially inhibitory site, the medial amygdala (mAMG). Lesions of the mAMG facilitate the development of maternal behavior. Likewise, destruction of the major efferent pathway of the mAMG, the stria terminalis, results in reduced latencies to display maternal behavior in females. Interestingly, the fibers of the stria terminalis terminate in both the MPOA and VMN. This presents the possibility that in inexperienced females the medial amygdala may inhibit maternal responding via two distinct processes. First, the mAMG could make directly inhibitory synaptic contact with MPOA neurons, decreasing neuronal activity within these cells and thereby preventing maternal behavior. Alternatively, the mAMG could make excitatory contacts on VMN neurons, such excitation could result in increased activation of inhibitory VMN to MPOA projections, producing decreased activity in the MPOA. It is possible that both scenarios are operating in the normal animals; indeed, redundancy in neural circuits is often present.

If the mAMG does function to inhibit maternal behavior in nulliparous females, then perhaps the next question to be asked is what determines the activity of the mAMG? The answer to this question has been provided by both anatomical and behavioral studies. Damage to olfactory systems leads to the rapid development of maternal behavior in inexperienced, nulliparous female rats. The olfactory system projects both directly and indirectly to the mAMG. The mAMG can then affect systems that are both faciliatory (MPOA) or inhibitory (VMN) to the induction of maternal behavior. In maternally experienced females, olfactory cues from the pups are no longer inhibitory and in fact may function to enhance maternal responding. The precise nature of the changes within the mAMG, which may correlate with this altered responding, remains to be determined.

Finally, the projections from the MPOA to the lateral septum suggests that maternal responding may influence structures associated with learning and memory. The lateral septum has long been associated with memory processes. More recent work suggests that this structure plays an important role in social recognition as well as other cognitive and emotional processes. Perhaps the activation of neurons projecting to the lateral septum during maternal responding induces a form of short- or long-term memory. As maternal experience results in profound long-term changes in the response of females to pups, it seems logical that any system involved in the coordination of maternal behavior must somehow result in memory of that experience. Indeed, some of the work on opioid systems have indicated that this neuropeptide may play a role in maternal memory. Future anatomical studies may reveal more connectivity between septal and hippocampal nuclei, providing further support for maternal memory function.

5. PHYSIOLOGICAL UNDERPINNINGS OF MATERNAL BEHAVIOR

5.1. Role of Hormones in Maternal Behavior

Shifts in secretory patterns of hormones and neurochemicals throughout development are thought to underlie the shifts in incidences and intensities of parental behavior in female mammals. A large amount of research has explored the role of hormones and various neurotransmitters in the regulation of parental care. Females showing pronounced shifts in latencies to display maternal behavior at the end of gestation initially led investigators to explore the relationships between the physiological changes of pregnancy and the expression of maternal behavior. The studies of Moltz et al. and Zarrow et al. in the 1970s were the first to indicate that the spontaneous onset of maternal behavior in the newly parturient rat was stimulated by a pregnancy-like hormone cocktail consisting of estradiol (E_2), progesterone (P), and prolactin (PRL).

Table 2
The average latencies of adult behaviorally inexperienced, female rats to respond maternally to donor pups following a variety of experimental manipulations.

Manipulation prior to pup exposure	*Latency in days to display maternal behavior*
Intact	5–7
Ovariectomized	5–7
Early pregnancy	6–8
Late pregnancy $_{(D17)}$	4–5 (parturition occurs)
HO	4–5
Ovariectomy + E_2	3–4
HO + E_2	1–2
Pregnancy Termination-HO$_{(D16)}$	Tested immediately: 2–3 Tested after 48 h: 1–2
Pregnancy Termination-HO$_{(D16)}$ + $E_{2\ (systemic\ or\ MPOA)}$	0–1
$E_{2\ (days\ 1\text{–}testing)}$ + $P_{\ (days\ 3\text{–}13)}$	1–2
Hypophysectomized + $E_{2\ (days\ 11\text{–}testing)}$ + $P_{(days\ 1\text{–}11)}$	5–7
Hypophysectomized + $E_{2\ (days\ 11\text{–}testing)}$ + $P_{(days\ 1\text{–}11)}$ + PRL	1–2

Abbreviations: D: Day of gestation; E2: estradiol; HO: hysterectomy and ovariectomy; MPOA: Medial Preoptic Area; P: progesterone.

The roles of these as well as other hormones have subsequently received significant research attention. The specific involvement of these hormones in the regulation of maternal behavior are presented below.

5.1.1. Estradiol

Estradiol plays a critical role in the hormonal stimulation of maternal behavior in almost all mammals studied to date. In such studies, treatment with P and PRL without E_2 fail to stimulate a rapid onset of maternal care. Strong evidence, which emphasized the importance of E_2 in stimulating short-latency maternal behavior, was provided by the studies using a hysterectomy-ovariectomy (HO) model in which both the uterus and the ovaries were removed to eliminate potential sites of sex-steroid uptake and production, respectively. It was shown that HO virgin rats injected with a high dose of estradiol benzoate (EB) displayed full maternal behavior (defined as retrieval, grouping and crouching within the standard 1 h test session) within 1–2 d. These latencies as well as average latencies induced by other commonly used experimental designs are presented in Table 2. In contrast, vehicle-treated females became maternal after about 5 d of pup exposure. Moreover, when behaviorally inexperienced, pregnant rats were hysterectomized on day 16 of gestation and first tested for maternal behavior 48 h later, a high percentage (>60%) displayed maternal behavior after only 24 h of exposure to foster pups. This high level of responsiveness appears to have resulted from the prolonged hormonal priming associated with pregnancy followed by the decline in P levels and the relatively unopposed E_2 exposure in the hysterectomized female (*see* Fig. 3 for the normal hormone patterns during pregnancy in rats). Maternal behavior, likewise, rapidly appears in estrogen-treated rats whose pregnancies are surgically terminated by a combination of HO on 16 day of pregnancy. HO on day 16 of pregnancy results in a rapid and demonstrable decline in E_2 and P which are produced by the ovaries and in placental hormones; pregnancy followed by HO appears to prime the female to the stimulatory effects of estrogen. It is worth reemphasizing that E_2 appears to be a key hormone in the induction of maternal behavior. Essentially, all reported hormone (P, PRL) as well as peptide (oxytocin, cholecystokinin) stimulatory effects on maternal behavior require exposure to E_2. However, it is unknown whether estrogen's actions occur directly on the brain independent of P and PRL to stimulate maternal behavior under pregnancy conditions or whether estrogen has a permissive action, i.e., allows P and/or PRL to stimulate the neural processes mediating the expression of parental care. It is possible that E_2 exerts both direct and permissive actions.

The medial preoptic area (MPOA) is one site of E_2 action in the stimulation of maternal behavior. This region of the brain is crucial for the expression of both the induction of maternal behavior at the end of pregnancy and in the maintenance of maternal care

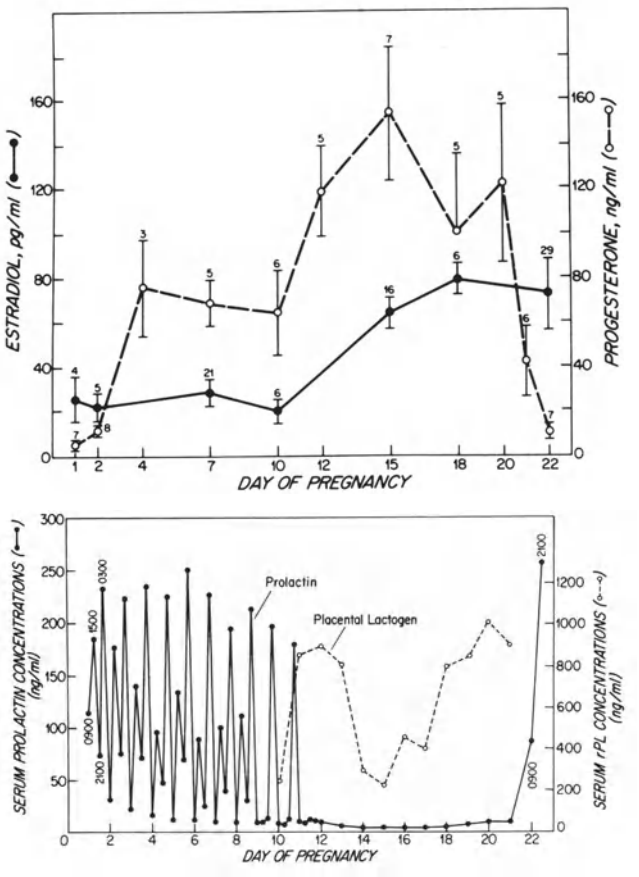

Fig. 3. Endocrine changes during pregnancy in rats. Profiles for the steroid hormones estradiol and progesterone are shown in the upper panel, while the levels and patterns of prolactin and rat placental lactogens are depicted in the lower panel. From *Mammalian Parenting: Biochemical, Neurobiological, and Behavioral Determinants.* Norman A. Krasnegor and Robert S. Bridges, eds. 1990: Oxford University Press, Inc. (Reprinted by permission).

during lactation in rats. Application of E_2 to the MPOA of 16-d-pregnant hysterectomized-ovariectomized rats stimulates an almost immediate onset of maternal behavior in these females. Quantification of nuclear estrogen receptors in various neural regions during pregnancy and after parturition have demonstrated that estrogen receptor (ER) concentrations in the MPOA are increased during pregnancy and postpartum. Injections of EB into day 16 pregnant rats that are HO also produced transient elevations in estrogen receptor concentrations. Recent findings have found modest increases in mRNA for the ER in the ventromedial nucleus (VMN) of the hypothalamus just prior to parturition. The actions of E_2 in the VMN in controlling maternal behavior are unestablished, although perturbation of this region in steroid-primed virgins appears to stimulate the induction of maternal care.

5.1.2. Progesterone

Progesterone is secreted in large amounts by the corpus lutea of the ovaries during most of gestation in the rat and is essential for pregnancy maintenance. P serum levels first increase on day 3 of pregnancy and reach an initial plateau at midpregnancy, then increase further during the second half of the 22-d gestation period before declining precipitously about 24–48 h prior to parturition (*see* Fig. 3). Two roles for P in the stimulation of maternal behavior have been proposed. First, the long-term exposure to P, together with E_2 during gestation, helps to prime the female to respond maternally at parturition. Latencies of female rats to respond maternally to foster young decrease progressively as a function of the length of gestation and the duration of steroid priming. However, this increased responsivity is only revealed after midgestation when pregnancy is terminated by procedures such as HO and is first statistically apparent around midgestation. P's second important function is to regulate the timing of maternal behavior at the end of pregnancy. If one maintains high-circulating P levels after HO on day 17 of pregnancy, the rapid onset of maternal behavior induced by P withdrawal is prevented. Similarly, P blocks the rapid expression of maternal behavior in estrogen-treated, nulliparous rats. Attempts to ascertain potential neural sites of P's inhibitory action have not been successful.

5.1.3. Prolactin and Lactogenic Hormones

Strong evidence supporting a role for PRL, a protein secreted by the anterior pituitary gland, and other lactogenic hormones produced by the placenta in the induction of maternal behavior has emerged during the past decade. A stimulatory role for PRL in the induction of maternal behavior has been established by employing an experimental model that uses a combination of steroid treatment and PRL administration. The initial studies utilized a hypophysectomy (no pituitary) model to delineate a role for endogenous PRL on the development of maternal behavior. These studies demonstrated that adult, hypophysectomized, nulliparous female rats failed to respond to the stimulatory effects of sequential exposure to P (days 1–11) and E_2 (days 11 to the completion of testing), whereas females with intact pituitary glands which secrete PRL became maternal in 1–2 d. Replacement of PRL in steroid-treated, hypophysectomized animals either by the placement of ectopic pituitary grafts under the

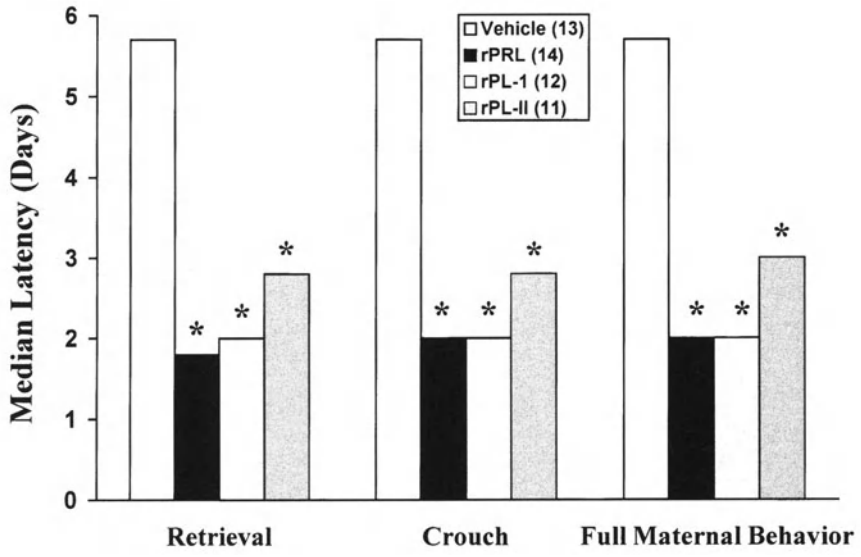

Fig. 4. The effects of bilateral MPOA infusions of rPRL, rPL-I, or rPL-II on the induction of maternal behavior in steroid-primed, behaviorally inexperienced, female rats. Numbers in parentheses are the *N*s for each group. *p<0.05 vs controls. Compilation of data modified from R.S. Bridges et al. Neuroendocrinology 1996; 64:57–64. Copyright © 1996 by S. Karger AG, Basel; and Bridges RS, Robertson MC, Shiu RPC, Sturgis JD, Henriquez BM, Mann PM. *Endocrinology* 1997; 138(2):756–763. Copyright © 1997 by The Endocrine Society. (Reprinted with permission.)

renal capsule that results in the secretion of PRL or injecting ovine (o) PRL resulted in the rapid onset of maternal behavior in oPRL-exposed animals.

More recent studies have used nonhypophysectomized, ovariectomized, steroid-treated nulliparous rats in which endogenous PRL was suppressed by twice daily injections of bromocriptine (CB-154), a dopamine agonist. Maternal latencies averaged about 4–5 d for steroid-primed rats treated with CB-154, whereas controls responded in 1–2 d. When steroid-primed rats were treated with both CB-154 plus exogenous PRL a rapid onset of maternal behavior occurred, again demonstrating a stimulatory role for PRL.

One site of PRL's behavioral action is the central nervous system (CNS). Intracerebroventricular (ICV) infusions of PRL or bilateral infusions of PRL into the MPOA of steroid-primed, bromocriptine-treated, nulliparous rats stimulates short-latency maternal behavior (*see* Fig. 4). The central actions of PRL in stimulating the onset of maternal behavior in adult female rats appear to be steroid-dependent. ICV infusions of PRL into nonsteroid-primed animals fail to affect the rate of onset of maternal behavior. Likewise, MPOA infusions of rat(r) PRL are ineffective in nonsteroid-treated females and in animals primed only with either P or E_2. Thus, the most effective hormone regimen includes the combination of steroids plus prolactin.

The actions of PRL on maternal behavior in rats are shared by other lactogenic molecules, including ovine growth hormone (oGH) and the rat placental lactogens (rPLs). When oGH is injected daily s.c. into steroid-primed, hypophysectomized rats, maternal behavior is stimulated. Likewise, bilateral infusions of rPL-I or rPL-II into the MPOA of steroid-primed, bromocriptine-treated, nulliparous rats result in a rapid onset of maternal behavior when compared with vehicle-infused controls (*see* Fig. 4). Latencies of experimental animals ranged from 1 to 2 d, whereas those of controls averaged about 6 d. These findings indicate that the normal rapid onset of maternal behavior at parturition is brought about by the prolonged exposure of the female to P, E_2, and lactogenic hormones (PRL, rPLs) during gestation. As shown in Fig. 3, pregnant rats are exposed to high titers of PRL during the first half of gestation and during the final days prior to parturition, whereas from midpregnancy onward, the female is exposed to high levels of placental lactogens. Lactogenic hormones appear to gain access to the brain in humans as well as rats presumably by binding to lactogenic receptors on cells of the choroid plexus which transport these molecules or their derivatives into the cerebrospinal fluid (CSF). Once in the CSF, these hormones can diffuse into neuropil, such as the MPOA, which is adjacent to the ventricles.

Biochemical measurements of mRNA for the PRL receptor in rat brains indicate that the expression of the long, but not short, form of the PRL receptor is increased by day 7 of pregnancy. Expression of mRNA for the long form of the PRL receptor continues to increase during midpregnancy, reaching peak levels during the second half of gestation and continuing to remain high throughout most of lactation. PRL itself, progesterone, and estrogen (through its stimulation of PRL) are all able to stimulate expression of the long form of the rat PRL receptor and likely contribute to the increased expression of mRNA for

the PRL receptor during pregnancy and lactation. Sites of increased expression of mRNA for the PRL receptor within the brain have begun to be identified. One site of increased expression is within the choroid plexus; similar changes in receptor expression occur in the MPOA during late pregnancy that may mediate PRL's central stimulation of maternal behavior.

The relative behavioral potencies of PRL and placental lactogens appear to be similar as indicated in Fig. 4. rPRL, rPL-I and rPL-II are equally effective when infused into the MPOA of steroid-primed rats. Varying the number of infusions from 1 to 5 doses at the beginning of behavioral testing does not differentiate between the stimulatory potencies of rPRL and rPL-I. It is unclear, however, whether these two hormones have equal access to the CSF. During late pregnancy, for example, rPL-II may have greater access to the CSF and brain, since immunoreactive PRL is not detectable in the CSF just prior to parturition when lactogenic activity (rPL-II) in the CSF is high. Thus, while the behavioral potencies of PRL and rPLs appear comparable when infused directly into the MPOA, under physiological conditions when these lactogenic hormones may compete for the same lactogenic receptors on epithelial cells of the choroid plexus, rPLs may actually have greater access to the brain and play a more critical role during the latter stages of pregnancy in stimulating the onset of maternal behavior. Communication between the developing conceptus through its secretion of placental lactogens provides an efficient mechanism to prime the expectant mother to become maternal.

5.2. Neurochemical Regulation of Maternal Behavior

The role of a number of neurochemicals in the regulation of maternal behavior have been explored in recent years. Among these are the neural oxytocinergic and dopaminergic systems, the endogenous opioids, as well as various other neuropeptides. In addition, the use of gene knockout studies have also increased the understanding of both neurotransmitter and neurohormone involvement in the development and maintenance of maternal behavior. In general, the neuropeptide, oxytocin (OXY), and the gut peptide, cholecystokinin (CCK), stimulate the onset of parental behavior, whereas corticotropin-releasing factor (CRF) disrupts the establishment of maternal care. In addition, β-endorphin (β-E) and endogenous opioids appear to have dual roles in the induction and maintenance of parental care. The actions of these peptides seem to be more acute and turn the behavior on or off and affect the response tendencies within a restricted time frame. This is in contrast to the actions of the hormones that act over a much longer period of time, e.g., days versus hours, to prime the maternal brain to respond positively to young. The specific involvement of these neuropeptides and neurotransmitters are discussed in greater detail below.

5.2.1. Oxytocin (OXY)

Oxytocin appears to play an important role in stimulating the onset of maternal behavior at parturition. ICV infusions of OXY facilitate maternal care in estrogen-primed females, but are ineffective in non-steroid-treated animals. The stimulatory actions of OXY were also demonstrated in EB-treated, day 16 pregnant HO rats. Central infusions of anti-OXY or the oxytocin antagonist $d(CH_2)_5$-8-ornithine-vasotocin delay the rapid onset of maternal behavior. The actions of OXY in the rat also appear to be dependent upon the test environment and the olfactory status of the female. OXY stimulates the onset of maternal behavior in animals tested in a novel cage, but fails to affect the behavior when the subject is tested in its home cage. OXY's action is most effective in olfactory impaired rats; ICV infusions of OXY stimulate maternal behavior in rats made anosmic with zinc sulfate, but not in EB-treated, control animals.

One site of OXY's stimulatory action is the paraventricular nucleus (PVN) of the hypothalamus. Lesions of the PVN prior to parturition disrupt the establishment of full maternal care in rats, but fail to disrupt ongoing maternal responsiveness. Another region of the CNS that appears to modulate OXY stimulation of maternal behavior is the bed nucleus of the stria terminalis (BNST). OXY receptor concentrations increase in the BNST prepartum and bilateral knife cuts that sever fibers passing into and out of this region block active maternal care in lactating rats. This region is an attractive candidate for OXY involvement in maternal behavior, because it receives input from the amygdala, a structure that regulates olfactory input associated with the establishment of maternal behavior.

The dependence of OXY's action on estrogen is a relatively unexplored phenomenon. Estradiol and progesterone appear to modulate OXY receptor concentrations in specific neural loci, including the ventromedial hypothalamus (VMH). While this region of the CNS is intimately involved in the hormonal control of feminine sexual behavior in rats, only recently has evidence emerged that the VMH may also be part of the neural network underlying the

control of maternal behavior. Infusions of the tachykinin, neuropeptide K, into the VMH delays the onset of maternal behavior in pregnancy-terminated rats. Moreover, neurotoxic lesions of this region in steroid-primed female rats stimulates a rapid onset of maternal behavior. Interestingly, OXY receptor concentrations increase in the VMH at parturition. Whether the VMH is a site of OXY-steroid interaction in stimulating the spontaneous onset of maternal care at parturition merits examination.

Finally, studies in rats and sheep with prior maternal experience have shown that the vaginal and cervical stimulation associated with delivery help potentiate the expression of maternal behavior, possibly by central activation of oxytocinergic systems. Thus, the sensory input received by the female during parturition may help to acutely tune the maternal responses of the mother to the newborn.

5.2.2. Dopamine

Examinations of the interaction of brain dopamine (DA) systems in the context of endocrine function have been the subject of numerous studies. It is known that hypothalamic DAergic neurons located in the arcuate nucleus and projecting to the median eminence (the tuberoinfundibular DA system: TIDA), play a major role in the inhibitory regulation of prolactin release from lactotrophs within the anterior pituitary. This effect is mediated by DA D2 receptors located on the cell surface of the lactotrophs. As previously discussed, increased PRL and/or placental lactogen release during pregnancy are important for the rapid induction of maternal behavior at parturition. Given these relationships, it is possible that increased TIDA activity during pregnancy may influence the development of maternal behavior. Support for dopaminergic involvement in maternal behavior is provided by studies using the DA D2 agonist bromocriptine. In both hamsters and rats, DA-mediated suppression of PRL release disrupts the onset of maternal behavior. This disruption can be overcome by treating the subject with PRL in conjunction with the bromocriptine. Thus, DAergic modulation of PRL release can effect the normal onset of maternal behavior.

In addition to the regulation of PRL release by hypothalamic DA, other brain DA systems may be important for the maintenance of maternal behavior. Several studies have demonstrated that destruction of ventral striatal DA neurons via the neurotoxin 6-hydroxydopamine (6-OHDA) leads to a decrease in pup retrieval, nest building, and licking behavior in postpartum, lactating female rats. These effects do not appear to be caused by impaired motor activity as these same rats will readily retrieve food pellets following brief food deprivation. The ventral striatal DA system appears to be important for the motivational aspects of maternal responding. Indeed, while animals treated with the DA antagonist haloperidol display disruption in pup retrieval behavior, this deficit can be overcome by increasing the amount of time the pups have been separated from the dam. Furthermore, brain microdialysis studies have demonstrated that mother rats reunited with pups after an overnight separation have significantly enhanced levels of ventral striatal DA. Thus, DA release in the ventral striatum appears to mediate some aspects of established maternal behavior.

The possible clinical importance of DA systems in maternal behavior has also been demonstrated in studies that examine the effects of drugs of abuse on maternal behavior. One such drug is the indirect DA agonist, cocaine, which in rodents can disrupt both the onset and maintenance of maternal behavior. It is possible that these effects are because of both decreased maternal motivation as well as an inhibition of PRL release.

5.2.3. β-Endorphins

Another well-characterized drug of abuse, morphine, can inhibit maternal behavior. Morphine, an opioid agonist, when administered either systemically or directly into the medial preoptic area (MPOA), severely disrupts both retrieval and nursing behaviors. These deficits can be overcome by simultaneous administration of the opioid antagonist, naloxone. Does this morphine-induced disruption indicate that endogenous opioids function to inhibit maternal behavior? To examine this question, more physiologically relevant, or endogenous, substances must be utilized. To this end, several studies have demonstrated that the endogenous opioid, β-endorphin, when infused directly into the MPOA, can disrupt maternal behavior. As shown in Fig. 5, this behavioral effect is mediated by the μ-opioid receptor subtype, because only central infusion of μ-agonists disrupted ongoing maternal behavior in lactating rats. Infusions of δ or κ opiate agonists at high doses fail to disrupt maternal behavior in lactating rats.

The nature of the opioidergic modulation of maternal behavior is not as simple as the previous studies may suggest. While increased activity at the μ-receptor within the MPOA may inhibit ongoing maternal behavior, opiates may stimulate the induction of

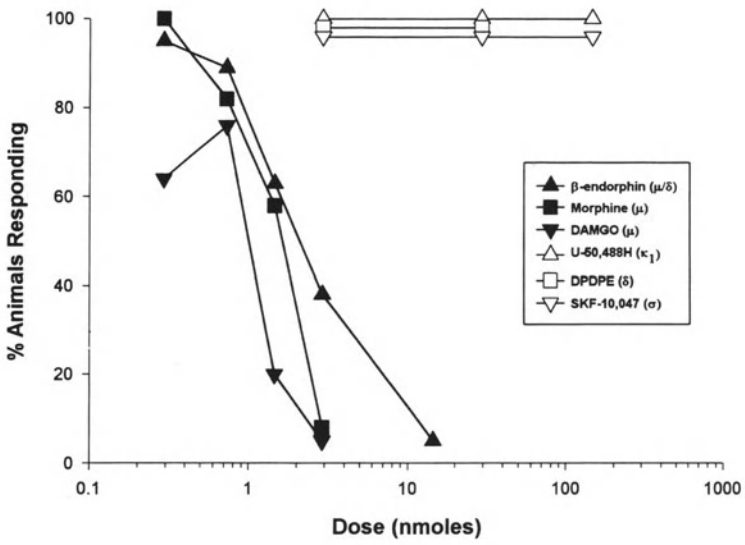

Fig. 5. Percentage of primiparous, lactating rats exhibiting full maternal behavior following intracerebroventricular infusions of β-endorphin, morphine, DAMGO, U-50488, DPDPE, and SKF-10,047. Receptor subtypes are indicated within the parentheses. Modified from Mann et al., *Neuroendocrinology* 1991; 53:487–492. Copyright © 1991 S. Karger AG, Basel. (Reprinted with permission.)

maternal behavior immediately pre- and postpartum. For example, infusion of morphine into the VTA shortens the latency of virgin rats to express maternal behavior. In addition, the opioid antagonist, naloxone, interferes with placentophagia. Thus, in contrast to the maintenance of maternal behavior, the induction of maternal behavior may be associated with increased levels of endogenous opioids. In support of the oppositional nature of the opioid effects, studies have shown that β-endorphin levels and opioid receptor binding are increased during pregnancy and parturition when the induction of maternal behavior occurs. These levels subsequently decline and remain low during the postpartum period, during which time the female is engaging in maternal behaviors.

5.2.4. Cholecystokinin (CCK)

The inhibitory effect of β-endorphin on established maternal behavior, has been shown to be antagonized by the gut peptide, CCK. Interestingly, CCK has been reported to facilitate maternal behavior. While these effects remain equivocal, the action of CCK as an endogenous opioid antagonist, its colocalization and agonistic effects on brain DA systems, as well as its influence on olfactory systems suggests that CCK may potentially be an influential component in the neurochemical regulation of maternal behavior.

5.2.5. Corticotropin-Releasing Factor (CRF)

CRF is a neuropeptide that stimulates ACTH release from the anterior pituitary gland. It functions in part by mediating the neuroendocrine axis during an organism's stress response. ICV infusions of CRF into nulliparous rats resulted in increased incidences of pup killing and lower incidences of maternal care. It is unclear from this initial work whether this CRF effect is dose dependent or absolute and what the normative CRF release patterns are during the peripartum period. Increased CRF secretion during this period would be expected, given the involvement of ACTH in lactation. It is possible that severe stress during this peripartum period might produce abnormally elevated patterns of CRF release which in turn may disrupt parental care and induce pup killing. Again, the role of this peptide and its interactions with the hormones of pregnancy and important peptides and neurotransmitters merits attention.

5.3. Knockouts

The relatively recent advent of targeted gene deletion makes possible an even greater understanding of the critical components involved in the establishment and maintenance of maternal behavior. Several studies in mice have selectively deleted genes and then examined the maternal responding of these "knockout mice." These studies include the disruption of particular hormones, receptors, peptides, neurotransmitters, and protooncogenes.

Within the past year or two, both the disruption of the PRL gene as well as disruption of the PRL receptor have been examined individually in knockout mice. The homozygous form of both of these knockouts are completely infertile. However, when tested for spontaneous maternal behavior with donor mouse pups, mice homozygous for the PRL gene showed no deficits. Because the extent of behavioral testing was limited in this study, further testing of these PRL deficient mice is warranted to delineate whether all aspects of maternal care are unaffected in these knock-

Table 3
Summary of the effects on the maternal behavior of nulliparous and primiparous female mice following specific gene deletion.

Gene Deleted	*Nulliparous*	*Primiparous*
prolactin	**Homozygotes**: Infertile; No Deficit in MB.	**Heterozygotes**: No deficit in MB.
prolactin receptor	**Heterozygotes**: Increased retrieval latencies as compared to wild type following presentation of donor pups. **Homozygotes**: Infertile; Increased retrieval latencies or complete absence of maternal responding following presentation of donor pups.	**Heterozygotes**: Delayed retrieval and crouching as compared to wild type.
Oxytocin	No effect on MB.	No effect on MB.
Norepinephrine	No retrieval following presentation of donor pups.	No retrieval; Normal nursing once pups were placed in nest.
Fos B	No retrieval following presentation of donor pups.	No retrieval, grouping or crouching—pup death.

Abbreviation: MB - maternal behavior.

out animals. The effects on maternal behavior in mice with altered PRL receptor expression, however, do reveal significant deficiencies in maternal responding. In addition to being infertile, nulliparous homozygous PRL receptor knockout mice demonstrate delayed latencies to retrieve donor pups and in some instances fail to retrieve donor pups all together. Furthermore, heterozygous PRL receptor knockout mice also show behavioral deficits. In nulliparous heterozygous females, retrieval latencies in the presence of donor pups, is delayed, as compared to wild type mice. Moreover, primiparous heterozygous females, display delayed retrieval and crouching responses toward their own pups. Thus, in mice PRL receptor deletion, but not deletion of PRL itself, results in significant deficiencies in the development of maternal behavior in both homozygous and heterozygous females. These results along with those of other knockout studies are summarized in Table 3.

Another peptide linked with the normal induction of maternal behavior, oxytocin, has also been examined using a knockout model. The results of these studies demonstrate that whereas oxytocin is critical for milk ejection, it is not essential for normal fertility, parturition or milk production. There is no apparent effect of oxytocin gene deletion on the onset of maternal behavior in parturient females. There does, however, seem to be a modest effect of this gene disruption on aggressive behavior, with homozygous knockouts displaying reduced intensity of aggression.

Examination of the effects of the neurotransmitter norepinephrine (NE) was determined by deleting the gene for dopamine beta-hydroxylase (DBH), an enzyme critical for the production of both NE and epinephrine. Mice with complete DBH depletion, and thus lacking NE, gave birth normally, but their offspring died several days after birth. Further investigation of this phenomenon determined that the pups were not nursing, although the knockout mice had normally developed mammary tissue. Pups were not retrieved and grouped in the nest, instead, they remained scattered around the cage. DBH knockout virgin females tested with donor pups also demonstrated the same failure to retrieve. Interestingly, whereas restoration of NE in either virgin or postpartum females had little effect on behavior, restoration of NE, immediately prior to parturition, restored retrieval behavior and prevented the death of the pups. This decrease in pup death was even greater when NE was restored both pre- and postpartum. Thus, there appears to be a critical time period during which NE is necessary to induce maternal behavior in parturient females.

In addition to examining neurotransmitters and neuropeptides, recent studies have also demonstrated the importance of immediate early genes (IEG) in the expression of maternal behavior. In females with the deletion for the IEG, FOS B, pregnancy and parturition proceed normally, however, 1 to 2 d postpartum all of the pups died. Similar to the results discussed

with regard to elimination of NE, the loss of pups born to knockout females appeared to result from the lack of retrieval, grouping, and crouching. No deficits were observed with regard to olfactory systems, spatial learning, or motor behavior. The knockout females would routinely approach their offspring and sniff them, but did not retrieve their pups and were never observed to crouch over them. In addition, this deficit in maternal nurturing was also observed in virgin females tested with donor pups. In accordance with the data from DBH knockouts, deficits resulting from deletion of the FOS B gene were not overcome by multiple pregnancies nor did housing a homozygous mutant female with a heterozygous female and her pups induce any learning of maternal behavior in the homozygous mutant female. These findings indicate that FOS B activity appears necessary for the expression of maternal behavior. Indeed, both FOS B and c-FOS induction within the MPOA, a structure critical for the expression of maternal behavior, has been observed following pup exposure. The similarity in deficits observed in both the FOS B and DBH deficient mice suggest the possibility that NE may be involved in the induction of the IEG FOS B.

The use of the knockout mouse model has, as detailed above, enhanced our understanding of the role of particular neurochemicals involved in maternal behavior. The results of these gene deletion studies, however, have to be carefully considered. As these deletions are performed early in development, it is possible that the CNS compensates for deficits, either by down- or upregulation of related systems. For example, disruption of the OXY gene results in decreased expression of vasopressin as well as increased expression of the endogenous opioid, dynorphin. Whether the behavioral changes, or the failure to see changes, is the result of the loss of OXY or rather is the result of compensatory changes in the mutant mouse remains to be determined. Future studies that can disrupt gene expression in the adult mouse will help clarify these issues. For now, however, the work in knockout mice presents interesting avenues to be explored regarding the role of individual genes and neurochemical systems involved in the induction and expression of maternal behavior.

6. SENSORY REGULATION OF MATERNAL BEHAVIOR

Several sensory modalities, including olfaction, audition and touch, are involved in the normal expression of maternal behavior. Auditory cues provided by pup vocalization as well as olfactory input, aid in locating pups that have strayed away from the nest. Once pups have been located, tactile input, especially from the perioral region, appears to be critical for retrieval back to the nest. In addition, tactile input from the ventral trunk, is important for the execution and maintenance of the crouching position associated with nursing. Interestingly, whereas all of these modalities are normally utilized during maternal behavior, no one system is absolutely necessary for the continuation of established maternal behavior. The functions of each of these sensory systems with regard to maternal behavior have been explored, both in terms of disruption of ongoing maternal behavior and their effects on the development of maternal behavior.

6.1. Olfaction

Olfaction appears to play an important role in the expression of maternal behavior in most mammals. While olfaction aids in pup location, an intact olfactory system may not be crucial for the expression of maternal behavior. Indeed, much of the research on olfaction and maternal behavior has focused on the inhibitory nature of olfactory cues in virgin rats. Virgin females when presented with a conspecific pup will actively avoid the pup often retreating to the opposite side of the cage and even abandoning the nest within which a pup has been placed. It is thought that this active avoidance is indicative of the fear-evoking quality of pups in females with no prior maternal experience. Furthermore, it has been postulated that the odor of the pups is of primary importance in the mediation of this avoidance behavior. This hypothesis has been supported by the data obtained in studies of virgin females who have undergone varied transections of the olfactory system. In a critical study by Fleming et al. various lesions were made within the olfactory system of nulliparous females, and the effect of such manipulations on the development of maternal behavior were then assessed. The findings of this study are shown in Fig. 6. To summarize, in virgin females, transection of either the vomeronasal nerves or the main olfactory bulbs, results in significantly reduced latencies to respond maternally to donor pups. While control females typically respond to donors within approximately 6 d, females deprived of pup-mediated olfactory cues respond within 2 d. Interestingly, females with the most extensive damage to the olfactory system (transection of both the main and vomeronasal olfactory afferents) display the shortest latencies. Based upon these findings it seems

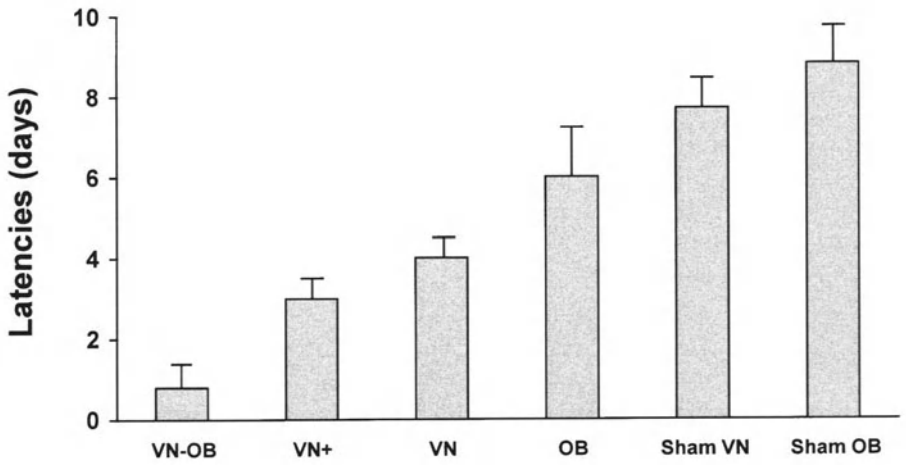

Fig. 6. Mean latency to become maternal (in days ± SEM) in virgin female rats that received cuts to the vomeronasal nerves (VN), main olfactory bulbs (OB), or both systems (VN+ and VN-OB). Group VN-OB sustained more damage to the main olfactory bulbs than did group VN+. Groups sham-OB and sham-VN are controls. Differences between groups VN and sham-VN, VN+ and sham-VN, VN-OB and the combined control groups (sham-VN and sham-OB) and VN+ or VN-OB and both OB and VN were significant ($p<0.05$). Differences between groups OB and sham-OB were not significant. Modified from Fleming A, Vaccarino F, Tambosso L, Chee P. Vomeronasal and olfactory system modulation of maternal behavior in the rat. *Science* 203:372–374, Copyright © 1979 by the Amer. Assoc. Advance. Sci.

probable that pup odors may indeed be aversive to virgin females. The longer latencies to become maternal in virgin females with intact olfactory systems may therefore be due to the necessity to overcome the fear-provoking aspects of pup odor. Finally, it has been hypothesized that the physiological changes at the time of parturition may serve to override the aversive quality of pup odors, allowing dams to become immediately maternal postpartum. The nature of the interaction between the hormonal and neurochemical changes of pregnancy and parturition and the changes in the olfactory system remains to be determined.

6.2. Vocalization

While pup vocalization is an integral component of pup-dam interactions, it appears to be unnecessary to both the development and maintenance of maternal behavior. Studies investigating the role of vocalization, have revealed that even anesthetized, and therefore, nonvocalizing pups are readily located and retrieved by the dam. Thus, while auditory cues may aid in pup localization, they are not crucial to either the development or maintenance of maternal responding.

6.3. Tactile Inputs

Several studies have demonstrated that afferent input from the perioral region is of primary importance in normal retrieval behaviors. Disruption of somatosensory input either by trigeminal denervation or injection of a local anesthetic into the perioral region, significantly increases pup retrieval latencies and can, in fact, abolish pup retrieval for several days. The severity and duration of these deficits is dependent upon the extent of denervation. Loss of tactile sensation from the upper lip and snout, only, produces deficits that disappear in about 24 h; more substantial denervation including upper and lower lip, snout, and chin produces deficits that persist for approx 72 h. Further investigations have revealed that such trigeminal denervations can severely disrupt parturition and the onset of maternal behavior as well as result in decreased maternal aggression.

In addition to perioral tactile inputs, somatosensory information from the ventral truck also provides important feedback to the mother. Specifically, these afferents are involved in the induction of the crouching posture associated with lactation. Loss of inputs from the ventral truck decreases the amount and duration of crouching in the presence of pups. Furthermore, like the loss of perioral inputs, decreased somatosensory information from the ventral trunk leads to a reduction in maternal aggression. Thus, tactile information from both the trunk and snout are important for the mediation of pup retrieval and nursing, as well as the intensity of maternal aggression.

7. NEUROCHEMICAL ELEMENTS AND EXPERIENTIAL ASPECTS OF MATERNAL CARE

The dependency and involvement of hormones, peptides, and biogenic amines in parental behavior change as a function of the reproductive experience. Most female mammals that have previously given birth appear to retain the ability to display short-latency maternal care or more intensive maternal behavior regardless of the female's hormonal status. Specifically, rats undergoing a second pregnancy respond maternally within a day during midgestation. In contrast, females at the same stage of their initial pregnancy require 5–7 d of exposure to foster young before maternal care emerges. Hypophysectomy after parturition, likewise, appears to have minimal effects on ongoing maternal care. Hormones, therefore, appear to prepare the female to respond maternally at the end of the initial pregnancy after which experiential and neurochemical factors are sufficient to regulate expression of most aspects of this behavior.

The establishment of the long-term retention of maternal care or "maternal memory" requires only a short period of maternal-young interaction postpartum. If primiparous rats are allowed to interact with their young throughout parturition prior to pup removal, four weeks later these females will display a rapid onset of maternal care toward foster young. In contrast, if each pup is removed immediately after its birth preventing maternal care from being expressed, response latencies remain prolonged and at virgin response latencies a month later. The establishment of maternal "memory" involves some form of genomic activation, because the development of this maternal memory can be blocked by treatment with the protein synthesis inhibitor, cycloheximide. There have also been reports of increased expression of the protooncogene c-FOS in the MPOA of maternal virgins. C-FOS appears to be a marker of neuronal gene transcription and may be associated with neuronal activity involved in controlling the expression of maternal care. It would be of interest to determine whether c-FOS activity increases or is modified in the MPOA and other neuronal populations when "maternal memory" is activated. For example, is there a specific region of the CNS where this memory is stored as reflected in alterations in c-FOS activity? It would also be valuable to know whether c-FOS activity increases in parental males, since the biochemical and neurochemical control of paternal behavior in males are virtually unexplored.

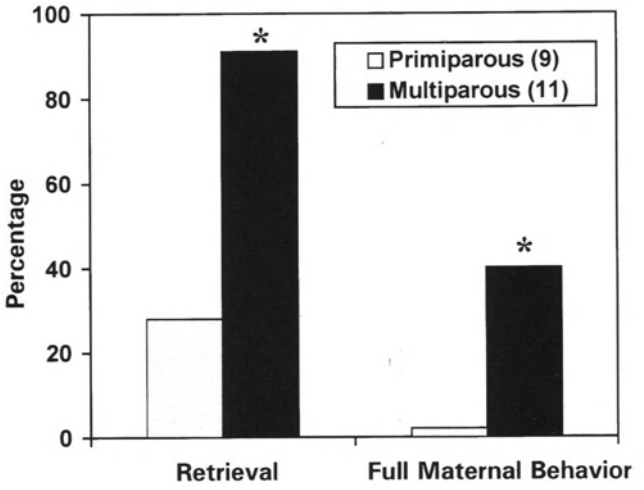

Fig. 7. Percentage of primiparous and multiparous, lactating rats exhibiting full maternal behavior and retrieval of at least one pup to the nest following infusions of β-endorphin into the MPOA. Postpartum multiparous and age-matched primiparous, lactating rats were infused with either physiological saline (0.4 μL/side; day 5) or 0.29 nmol β-endorphin (day 6) 30 min after pup removal. Thirty minutes following the infusions, females were tested for maternal behavior. All animals were fully maternal after saline infusions on day 5. Numbers of animals in each group are in parentheses. * Significantly different ($p<0.05$) from corresponding multiparous groups. Modified from Mann PE, Bridges RS. *Brain Res.* 1992; 580:241–248. Copyright © 1992 Elsevier Science Publishers B.V. (Reprinted with permission.)

One important factor that influences the reestablishment of parental care is the effect of repeated reproductive experience on maternal care. In rats, as in many other mammals, maternal behavior improves with maternal experience. That is, muliparous females are "better" mothers than are primiparous females. Interestingly, it appears that the neural sensitivity to peptidergic regulation of maternal behavior shifts as a function of parity. Specifically, as shown in Fig. 7, multiparous lactating rats are less sensitive to the behavioral actions of centrally administered β-endorphin. Bilateral MPOA infusions of β-endorphin, for example, block ongoing maternal behavior in primiparous rats much more effectively than in age-matched, multiparous rats. Furthermore, increases in opiate receptor densities in the MPOA are found in lactating, multiparous rats. In addition to changes in the opioid system as a function of reproductive experience, dopaminergic alterations have also been observed. Increased levels of DA metabolites (DOPAC and HVA) are present in multigravid (>1 pregnancy) rats in both the median eminence and the striatum compared with levels found in primigravid animals. Such

alterations may account for both the decreased PRL levels observed and in the decreased ability of dopaminergic antagonists to stimulate PRL secretion in nonlactating parous women and rats. One other set of recent studies suggests that oxytocin may also modulate certain aspects of the reemergence of maternal care. The interactions of the opiate and dopaminergic systems with other neurochemical systems, especially OXY, needs to be explored.

Together, these series of studies indicate that whereas hormonal regulation of maternal care lessens after the initial parturition, central biochemical modulation of the behavior continues, accompanied by changes in the sensitivity of these neurochemical systems. It is possible, for example, that the control of maternal behavior after parturition shifts from a systemic endocrine to some form of central endocrine-like regulation. Although unproven, the expression of ongoing maternal care may be influenced after birth more by central lactogenic elements. It is conceivable that central lactogen-producing neurons and receptors become upregulated once maternal behavior is established and thereby help regulate ongoing maternal care.

8. SUMMARY

Maternal behavior in the rodent is characterized by a set of stereotypical responses, activated immediately prepartum and requiring the convergence of a number of endocrine, neurochemical, and environmental factors. In reviewing the critical components of maternal behavior, several facts became clear. First, in pregnant females, the balance of hormones, particularly estrogen, progesterone, prolactin, and the placental lactogens, is necessary for the development of maternal behavior. Moreover, nonpregnant females can be rapidly induced to behave maternally by treating them with hormone regimens composed of steroids and lactogens. Once maternal behavior has become established, the role of these hormones is diminished. The maintenance of maternal behavior is mediated by a wide variety of neurochemical substances including neuropeptides, such as β-endorphin, and catecholamines, including dopamine. In addition, sensory inputs processed through the olfactory system facilitate efficient responding towards pups in postpartum females. All of these factors appear to affect, either directly or indirectly, the function of the MPOA, a brain region crucial for both the development and maintenance of maternal behavior. Finally, once maternal behavior has been established, long-term alterations in neurochemical functions occur that may help account for the more intense level of maternal behavior displayed by experienced mothers. The development of maternal behavior provides an example of neuroendocrine-induced plasticity in the rodent brain, a plasticity that can lead to significant and seemingly permanent alterations in brain and behavior.

SELECTED READINGS

Bridges RS. Endocrine regulation of parental behavior in rodents. In: Krasengor NA, Bridges RS, eds., Mammalian Parenting: Biochemical, Neurobiological, and Behavioral Determinants. New York: Oxford University Press, 1990; 93–117.

Fleming AS, Sarker J. Experience-hormone interactions and maternal behavior in rats. Physiol Behav 1990; 47:1165–1173.

Insel TR. Regional changes in brain oxytocin receptors postpartum: Time-course and relationship to maternal behaviour. J Neuroendocrinol 1990; 2:539–545.

Krasnegor NA, Bridges RS, ed. Mammalian Parenting: Biochemical, Neurobiological, and Behavioral Determinants. New York: Oxford University Press, 1990.

Morgan HD, Fleming AS, Stern JM. Somatosensory control of the onset and retention of maternal responsiveness in primiparous Sprague-Dawley rats. Physiol Behav 1992; 51:541–555.

Numan M. Maternal behavior. In: Knobil E, Neill JD, eds. Physiology of Reproduction, Vol. 2, 2nd ed. New York: Raven 1994; 221–302.

Numan M, Sheehan TP. Neuroanatomical circuitry for mammalian maternal behavior. Annals NY Acad Sci 1997; 807:101–125.

Pedersen CA. Oxytocin control of maternal behavior. Annals NY Acad Sci 1997; 807:126–145.

Rosenblatt JS, Snowden CT, eds. Parental Care: Evolution. Mechanisms, and Adaptive Significance. San Diego, CA: Academic, 1996.

Rosenblatt JS, Wagner CK, Morrell JI. Hormonal priming and triggering of maternal behavior in the rat with special reference to the relations between estrogen receptor binding and ER mRNA in specific brain regions. Psychoneuroendocrinology 1994; 19:543–552.

18 Neuroendocrine Regulation of Fluid Intake and Homeostasis

Joseph G. Verbalis, MD and Edward M. Stricker, PHD

CONTENTS

INTRODUCTION

Body fluids provide the matrix in which the biochemical reactions that comprise cellular metabolism occur. The concentration of substrates in the cellular fluid is a major factor that determines the rate at which these reactions occur. Furthermore, all body tissues depend on the circulation of blood to provide the nutrients needed to support cellular metabolism and to carry away metabolites for excretion. Thus, the maintenance of concentrations of solutes, or osmolalities (*osmotic homeostasis*), and the regulation of volumes of the various body fluid compartments (*volume homeostasis*) are critical functions for normal physiology in all animals.

When the regulation of body fluid osmolality or volume is threatened, various physiological and behavioral responses are stimulated that adaptively serve to maintain or restore the basal state. For example, during water deprivation, animals conserve body water so that they do not exacerbate the induced dehydration, and consume water to replace the fluid they have lost. Similarly, following hemorrhage, loss of blood plasma stimulates conservation of water and sodium in urine as well as ingestion of water and NaCl. Renal water and sodium retention are accomplished through actions of the antidiuretic hormone arginine vasopressin (AVP) and the antinatriuretic hormone aldosterone, as described in Chapter 14, whereas water and NaCl ingestion are motivated by the sensations of *thirst* and *salt appetite*, which will be the main topics of this chapter. All of these complementary responses are mediated and coordinated by the brain, and this chapter will describe the various mechanisms by which the needs for fluid homeostasis are detected and integrated by the central nervous system (CNS) in order to activate appropriate physiological and behavioral responses.

From: *Neuroendocrinology in Physiology and Medicine*, (P. M. Conn and M. E. Freeman, eds.),

1.1. Review of Body Fluid Physiology

Before discussing the neural regulation of thirst and salt appetite, it is important to first briefly review several key aspects of body fluid physiology in order to provide a context in which to consider these regulated functions in greater detail.

1.1.1. COMPARTMENTALIZATION OF BODY FLUIDS

Water is the largest constituent of the body, constituting 55–65% of body weight in animals and humans, varying largely in relation to the amount of body fat.

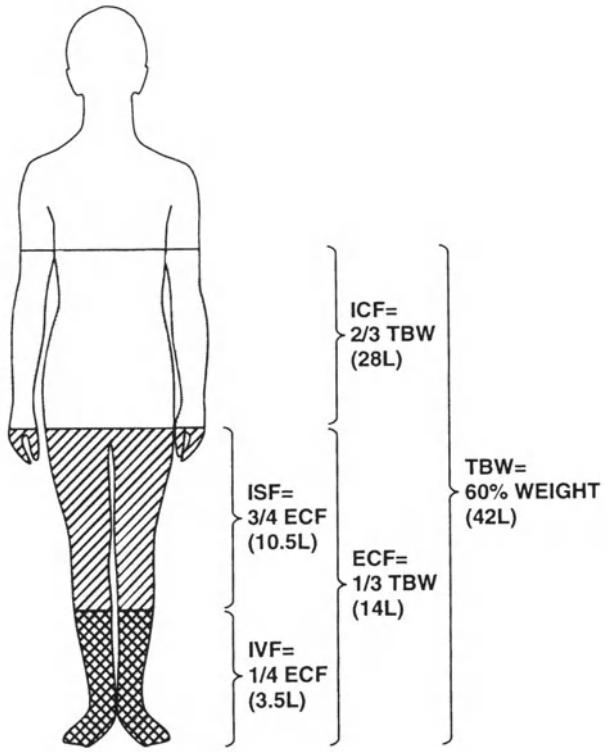

Fig. 1. Schematic representation of body fluid compartments in man. The shaded areas depict the approximate size of each compartment as a function of body weight. The figures indicate the relative sizes of the various fluid compartments and the approximate absolute volumes of the compartments (in liters) in a 70 kg adult. Abbreviations: TBW = total body water; ICF = intracellular fluid; ECF = extracellular fluid; ISF = interstitial fluid; IVF = intravascular fluid. Reproduced from Verbalis (1997).

Total body water is distributed between the *intracellular fluid (ICF)* and the *extracellular fluid (ECF) compartments*, with 55–65% in the former and 35–45% in the latter. The ECF can be further subdivided into the *interstitial fluid* surrounding the cells and the plasma volume within blood vessels. The *intravascular fluid* averages 7–8% of total body water, or approximately 1/5 of the ECF. Fig. 1 summarizes the estimated body fluid spaces of an average-weight adult human.

The body fluid compartments differ not only in their volumes, but also in the solutes that are dissolved in them. Specifically, membrane-bound Na^+/K^+ pumps maintain Na^+ primarily outside the cells whereas K^+ is largely found inside cells. However, the osmotic pressure, which reflects the concentrations of all solutes in a fluid compartment, is always equivalent in the ECF and ICF because most biological membranes are freely permeable to water. Thus, water flows across the membranes by *osmosis* from a relatively dilute compartment into one with a higher solute concentration, until a steady state is reached in which the osmotic pressures have equalized on both sides of the cell membrane.

1.1.2. Maintenance of Blood Volume and Pressure

Of all the body fluid compartments, the intravascular fluid volume is of special importance because it must always be sufficient to allow the circulation of blood throughout the body. The distribution of fluid between the intravascular and interstitial fluid compartments is determined by a balance between the *hydrostatic pressure* of the blood, which is maintained by cardiac output and arteriolar vasoconstriction, and the opposing osmotic pressure contributed by plasma proteins. Although those proteins contribute only 1–2% to overall plasma osmolality, the permeability of capillary membranes to such large molecules is low and, therefore, they exert a pressure differential (approximately 15–20 mm Hg), which is called the *colloid osmotic (oncotic) pressure*, that tends to pull interstitial fluid into the circulation. Fig. 2 summarizes the forces governing transcapillary fluid transfer between the two extracellular compartments, which was first described by the English physiologist Starling (1896) at the turn of the century. Note that according to this arrangement, the interstitial fluid in effect acts as a reservoir for plasma. Thus, hemorrhage-induced decreases in blood, i.e., hydrostatic, pressure shift the Starling equilibrium of forces so that more interstitial fluid is absorbed into the circulation, thereby helping to restore plasma volume. The reverse occurs when saline is added to the blood, because plasma proteins are diluted and the oncotic pressure they provide therefore diminishes, thereby allowing a portion of the extra fluid to flow into the interstitial space.

The maintenance of blood pressure is supported by two other mechanisms intrinsic to the cardiovascular system. First, although the arteries that receive the cardiac output of blood must be thick-walled in order to preserve blood pressure, the veins are thin-walled and distensible vessels. Thus, after a moderate hemorrhage, the veins collapse around the remaining blood whereas the arteries cannot and they remain full. Consequently, arterial blood pressure is not compromised because the blood is redistributed so that the deficit occurs primarily on the venous side of the circulation. Conversely, fluid accumulates in the veins when blood volume is expanded, again without much effect on arterial blood pressure. This is called the *capaci-*

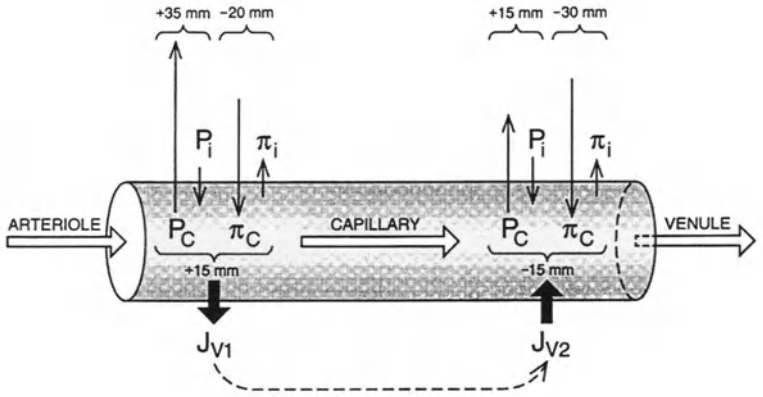

Fig. 2. Starling equilibrium of forces governing transcapillary fluid transfer. At the arteriolar end of the capillary, the difference between the intravascular hydrostatic pressure (P_C) and the interstitial hydrostatic pressure (P_i) exceeds the opposite difference between the intravascular oncotic pressure (π_C) and the interstitial oncotic pressure (π_i); the resultant pressure gradient drives capillary fluid into the interstitial space (solid arrow, J_{V1}). As fluid leaves the capillary, P_C decreases owing to fluid loss and π_C increases owing to hemoconcentration. Consequently, at the venous end of the capillary interstitial fluid is pulled back into the vascular space (solid arrow, J_{V2}). The numerical values indicate approximate net pressure differences (in mm Hg) between the intravascular and interstitial spaces. Note that any fluid accumulating in the interstitial space is ultimately returned to the blood via the lymphatic system (not shown). Reproduced from Verbalis (1997).

tance or the *compliance* of the vascular system. Second, the filtration of blood through the glomeruli of the kidneys is determined in large part by the renal arterial blood pressure. Thus, a drop in blood pressure reduces glomerular filtration rate (GFR) and lowers urinary excretion whereas a rise in blood pressure elevates GFR and promotes urinary fluid loss *(pressure diuresis)*. This normal function of the kidneys is so efficient that the development of hypertension, whatever its etiological basis, generally implicates renal dysfunction as an additional contributing factor because the kidneys failed to adjust to the elevated blood pressure by increasing fluid and solute excretion in the urine.

1.1.3. Role of Neural Mechanisms in Fluid Homeostasis

Fluid homeostasis is directed at achieving stability in the osmolality of body fluids and maintenance of the plasma volume. Osmotic homeostasis is important because it prevents large osmotic shifts of water into and out of cells, which would interfere with normal cell function, while volume homeostasis is important because it allows the normal circulatory functions upon which all tissue functions depend. Such homeostatic regulations are promoted by several mechanisms intrinsic to the physiology of body fluids and the cardiovascular system. For example, changes in the osmolality of ECF are buffered by the rapid osmotic movement of water across cellular membranes. Similarly, acute changes in plasma volume are soon modulated by the movement of fluid across capillary membranes according to the Starling equilibrium, by venous compliance, and by compensatory alterations in GFR. Nevertheless, in many situations the perturbations in body fluid osmolality and/or blood volume may be so large that additional mechanisms must be recruited to maintain or restore homeostasis. These other responses largely involve the central neuroendocrine control of renal water and sodium excretion through the actions of specific hormones secreted for that purpose, and the central neural control of water and NaCl consumption motivated by thirst and salt appetite, respectively.

1.2. Regulated vs Unregulated Fluid Metabolism

Body fluid metabolism represents a balance between the intake and excretion of both water and solutes. Each side of this balance equation can be considered to consist of a *regulated* (or "need-induced") and an *unregulated* (or "need-free") component, the magnitudes of which can vary quite markedly under different physiological and pathophysiological conditions. For example, the unregulated component of water intake consists of the intrinsic water content of ingested foods, the consumption of beverages primarily for reasons of palatability or desired secondary effects (e.g., caffeine), or for social or habitual reasons (e.g., alcoholic beverages), whereas the regulated component of water intake con-

sists of fluids consumed in response to a perceived sensation of thirst. Similarly, the unregulated component of water excretion occurs via insensible water losses from a variety of sources (cutaneous losses from sweating, evaporative losses in exhaled air, gastrointestinal losses) as well as the obligate amount of water that the kidneys must excrete to eliminate solutes generated by body metabolism, whereas the regulated component of water excretion is comprised of the renal excretion of free water in excess of the obligate amount necessary to excrete metabolic solutes. As for water metabolism, it is possible to define regulated and unregulated components of both Na^+ intake and Na^+ excretion as well.

In effect, the regulated components are those that act to maintain fluid balance by compensating for whatever perturbations result from unregulated water or sodium losses or gains. Although this chapter will focus on the neuroendocrine mechanisms underlying the regulated aspects of fluid ingestion, it is clear that the unregulated components also play a major role in overall body fluid homeostasis and cannot be ignored.

2. OSMOTIC HOMEOSTASIS

As summarized in the previous section, the two major variables that are regulated to maintain body fluid homeostasis are the composition and the volume of the various body fluid compartments. This section will review the osmotic composition of body fluids, which is largely a function of the amount of body water, while the following section will address the volume of the extracellular fluid, which is largely a function of the amount of body sodium. Although such a separation represents an oversimplification of complicated interactions between water and sodium metabolism, it is nonetheless of practical utility in understanding how body fluids are regulated.

2.1. Total and Effective Osmolality

2.1.1. Definitions

Osmolality is an expression of concentration, that is, a ratio of the total amount of solute dissolved in a given weight of water:

$$\frac{\text{solute (osmoles)}}{\text{water (kilograms)}}.$$

Plasma osmolality can be measured directly (via determination of freezing point depression or vapor pressure, since each of these are colligative properties of the number of free solute particles in a given volume of plasma), or estimated as:

$$P_{osm}\ (\text{mOsm/kg } H_2O) = 2 \times \text{plasma } [Na^+]\ (\text{mEq/L}) + \text{glucose (mg/dL)}/18 + \text{BUN (mg/dL)}/2.8.$$

Both methods produce comparable results under most conditions, as will simply doubling the plasma sodium concentration ($[Na^+]$) because sodium and its accompanying anions are by far the predominant solutes present in plasma. However, the total osmolality of plasma is not always equivalent to the *effective* osmolality (sometimes referred to as the *tonicity* of the plasma), because the latter is a function of the relative solute permeability properties of the membranes separating the two compartments. Solutes that are impermeable to cell membranes (Na^+, mannitol) are restricted to the ECF compartment and are *effective solutes*, because they create osmotic pressure gradients across cell membranes leading to osmotic movement of water from the ICF to the ECF compartments. Solutes that are permeable to cell membranes (urea, ethanol, methanol) are *ineffective solutes*, because they do not create osmotic pressure gradients across cell membranes and therefore are not associated with such water shifts. Glucose is a unique solute, because at normal physiologic plasma concentrations, it is taken up by cells via active transport mechanisms and therefore acts as an ineffective solute, but under conditions of impaired cellular uptake (e.g., insulin deficiency), it becomes an effective extracellular solute.

The importance of this distinction between total and effective osmolality lies with the fact that only the effective solutes in plasma are determinants of whether significant hyperosmolality or hypoosmolality is present. An example of this is uremia: an animal or human with a urea concentration that has increased by 30 mEq/L will have a corresponding 30 mOsm/kg H_2O elevation in plasma osmolality, but the effective osmolality will remain normal when the increased urea is proportionally distributed across both the ECF and ICF. In contrast, when plasma $[Na^+]$ increases by 15 mEq/L the plasma osmolality will also increase by 30 mOsm/kg H_2O, since the increased cation must be balanced by an equivalent increase in plasma anions, but in this case the effective osmolality will also be elevated by 30 mOsm/kg H_2O since the Na^+ and accompanying anions will largely remain restricted to the ECF because of the relative impermeability of cell membranes to Na^+ and other univalent

ions. Thus, elevations of solutes such as urea, unlike elevations in plasma [Na^+], do not cause cellular dehydration, and consequently do not activate mechanisms that defend body fluid homeostasis by acting to increase body water stores.

2.1.2. Responses to Perturbations of Osmolality

Dehydration and the consequent need for more water occurs whenever the ratio between effective solutes and water is elevated, whether by a decrease in the denominator or by an increase in the numerator. Both changes occur quite often: a decrease in body water results from water deprivation or the loss of dilute fluids to accomplish evaporative cooling, whereas an increase in solute load results from the consumption of NaCl or food. The water loss associated with dehydration is distributed across both the ECF and the ICF in proportion to their relative size, so that the osmolality of the fluid in the two compartments remains in equilibrium. In contrast, the increase in plasma osmolality that results from a NaCl load results only in cellular dehydration, because the water leaving cells by osmosis has the net effect of expanding the ECF volume. Thus, a NaCl load represents a more abrupt but less complex treatment than water deprivation for stimulating thirst and AVP secretion, the two main osmoregulatory responses of the brain. Osmotic regulation of AVP secretion has already been described in Chapter 14; this section will concentrate on the complementary and equally important regulation of thirst and fluid intake during perturbations in body osmolality.

2.2. Osmotic Regulation of Thirst

Thirst can be defined as a strong motivation to seek, to obtain, and to consume water in response to deficits in body fluids. Like AVP secretion, thirst can be stimulated in animals and man either by intracellular dehydration caused by increases in the effective osmolality of the ECF, or by intravascular hypovolemia caused by losses of ECF. Controlled studies in animals have consistently reported thresholds for osmotically induced drinking ranging from 1 to 4% increases in plasma osmolality above basal levels, and analogous studies in humans using quantitative estimates of subjective symptoms of thirst have confirmed that increases in plasma osmolality of similar magnitudes are necessary to produce an unequivocal sensation described as "thirst." Also like AVP secretion, water intake increases linearly in proportion to induced increases in the effective osmolality of ECF. The dilution of body fluids by ingested water obviously complements the retention of water that occurs during AVP-induced antidiuresis, and both responses occur concurrently when drinking water is available. However, there may be marked individual differences in whether dehydrated subjects choose to respond to their needs for water promptly by drinking, or more slowly by increasing renal water conservation. Regardless, the two responses are flexibly linked so that one can increase when the other is constrained.

In contrast to intracellular dehydration, the threshold for producing hypovolemic, or extracellular, thirst is significantly greater in both animals and man. Studies in several species have shown that sustained decreases in plasma volume or blood pressure of at least 4–8%, and in some species 10–15%, are necessary to consistently stimulate drinking. In man, it has been particularly difficult to demonstrate any effects of mild to moderate hypovolemia to stimulate thirst independently of osmotic changes occurring with dehydration. This blunted sensitivity to changes in extracellular fluid volume or blood pressure in humans probably represents an adaptation that occurred as a result of the erect posture of primates, which predisposes them to wider fluctuations in blood and atrial-filling pressures as a result of orthostatic pooling of blood in the lower body; stimulation of thirst (and AVP secretion) by such transient postural changes in blood pressure might lead to overdrinking and inappropriate antidiuresis in situations where the ECF volume was actually normal, but only transiently maldistributed.

Although osmotic changes are clearly more effective stimulants of thirst than are volume changes in animals and man, it is not clear whether relatively small changes in plasma osmolality are responsible for most daily fluid intakes. Most humans consume the majority of their ingested water as a result of unregulated components of fluid intake, and generally ingest volumes in excess of what can be considered to be the actual "need." Consistent with this observation is the fact that under most conditions plasma osmolalities in man remain within 1–2% of basal levels, and these relatively small changes in plasma osmolality are below the threshold levels that have been found to stimulate thirst in most individuals. This suggests that despite the obvious importance of thirst during pathological situations of hyperosmolality and hypovolemia, under normal conditions, water homeostasis is accomplished more by AVP-regulated water excretion than by regulated water intake.

2.3. Contribution of Natriuresis to Osmotic Homeostasis

During conditions of hyperosmolality, it is adaptive not only to drink and to conserve water in urine (thereby increasing the denominator in the ratio that represents body fluid osmolality), but also to excrete the NaCl load and not to consume additional solutes (thereby decreasing the numerator of that ratio). It is well known that several endogenous natriuretic agents promote urinary sodium loss after an administered NaCl load or a period of imposed water deprivation. One such agent is the hormone *atrial natriuretic peptide (ANP)*, which is synthesized in the cardiac atria and released when increased intravascular volume causes distention of the atria. Another is the other neurohypophyseal hormone *oxytocin (OT)*. Like AVP, OT is synthesized in hypothalamic neurons in the *supraoptic nuclei (SON)* and *paraventricular nuclei (PVN)* and secreted from the posterior pituitary in proportion to induced hyperosmolality; in rats the kidney is as sensitive to the natriuretic effects of OT as it is to the natriuretic effects of ANP, though this has not been as well studied in man.

Salt loads are also known to decrease the intake of additional solutes, whether in the form of NaCl solution or food, an effect that complements the stimulation of thirst and the pituitary secretion of AVP and OT. However, because destruction of the *organum vasculosum of the lamina terminalis (OVLT)* eliminates the latter effects, but not the dehydration-induced reduction in the intake of NaCl or food, other osmoreceptors not located in the basal forebrain must be responsible for mediating this inhibition of NaCl and food intake. One possible site for such cells is the liver, which has been suspected of having osmoreceptor functions since hepatic cells are well situated to detect the osmolality of ingested food and to modulate its intake accordingly, though this remains to be proven.

2.4. Osmotic Homeostasis During Overhydration

Most discussions of osmotic homeostasis emphasize the osmotic stimulation of thirst and AVP secretion, which acts to conserve body water during periods of dehydration. However, it is important to remember that appropriate osmoregulation is required not only under conditions of dehydration, but also during acute periods of overhydration and hypoosmolality. This can result when fluids are consumed in excess of water needs, not because of thirst, but because of their palatability or the chemical substances they contain (e.g., caffeine, alcohol). Because no storage form for water resembles the sequestration of fuel energy as triglycerides in adipose tissue, excess water consumed for whatever reason is not stored for later use but is quickly excreted in urine. The major mechanism responsible for this diuresis when plasma osmolality falls below normal levels is inhibition of pituitary AVP secretion (see Chapter 14). However, as with dehydration, analogous behavioral contributions to osmoregulation during osmotic dilution occur as well in the form of inhibition of further water intake. Although ingestion of additional solute as food and NaCl might be expected to increase plasma osmolality as well, this does not appear to occur under conditions of overhydration and hypoosmolality.

2.5. Osmoreceptor Cells and Afferent Pathways

All body cells lose water by osmosis when the effective osmolality of ECF is increased. Thus, the cells that provoke AVP secretion and thirst do not have unique osmosensitive properties, as retinal receptor cells do for photic stimulation. Instead, the unique feature of osmoreceptor cells is thought to be their neural circuitry, which activates the central systems for AVP secretion and thirst when these cells are dehydrated.

Destruction of the osmoreceptor neurons should eliminate the detection of increased plasma osmolality and the AVP secretion and thirst responses that are elicited by dehydration. Ample research has now confirmed that this is the consequence of certain brain lesions, and such studies therefore have been useful in revealing the location of the osmoreceptor cells. Most such studies have implicated cells located in the OVLT and areas of the adjacent anterior hypothalamus near the anterior wall of the third cerebral ventricle (Fig. 3), because surgical destruction of that brain area abolishes the AVP secretion and thirst responses to hyperosmolality, but not their responses to other stimuli such as hypovolemia. The same conclusion was drawn from clinical observations of human subjects with focal brain tumors that destroyed the region around the OVLT who were unable to osmoregulate when water was deprived or they were given a NaCl load. Note that these results stand in contrast to the effects produced by destruction of the magnocellular neurons of the SON and PVN, which eliminate dehydration-induced AVP secretion, but not thirst, clearly indicating that osmotically stimulated thirst is generated proximal to these hypothalamic nuclei.

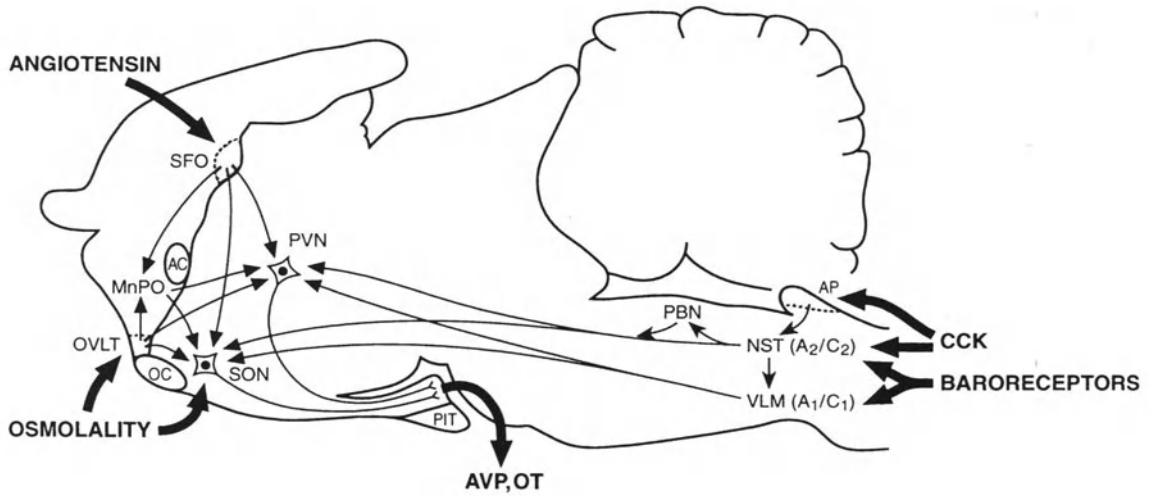

Fig. 3. Diagrammatic summary of pathways mediating AVP and OT secretion in response to various stimuli. Each of these stimuli except CCK activates thirst as well as AVP and/or OT secretion. The pathways responsible for AVP and OT secretion involve both excitatory and inhibitory projections to the SON and PVN of the hypothalamus whereas the pathways mediating thirst are less well characterized to date (Abbreviations: AC—anterior commissure; AP—area postrema; AVP—arginine vasopressin; CCK—cholecystokinin; MnPO—median preoptic nucleus; NST—nucleus of the solitary tract; OC—optic chiasm; OT—oxytocin; OVLT—organum vasculosum of the lamina terminalis; PBN—parabrachial nucleus; PIT—anterior pituitary; PVN—paraventricular nucleus; SFO—subfornical organ; SON—supraoptic nucleus; VLM—ventrolateral medulla. Modified from Verbalis (1995).

The location of osmoreceptor cells in the OVLT is consistent with the pioneering investigations of Jewell and Verney, in which hyperosmotic solutions were infused into various parts of the dog brain; injections into blood vessels perfusing the anterior hypothalamus were uniquely able to stimulate AVP secretion. The OVLT and surrounding areas of the anterior hypothalamus have also been implicated by recent studies in rats using immunocytochemical techniques to detect early gene products such as cFos, which serve as markers of cell activation, following systemic injections of hypertonic NaCl solution. Dense staining in the OVLT (and in the AVP-secreting cells in the hypothalamus) confirms that these areas are strongly stimulated by the induced dehydration.

Some of the neural pathways connecting the OVLT and other circumventricular organs with the magnocellular AVP-secreting cells in the hypothalamic SON and PVN have been identified (*see* Chapter 14), whereas the neural circuits in the forebrain that control thirst following osmoreceptor activation are still largely unknown. Although the data from lesion studies in both animals and man strongly support the concept of a single group of osmoreceptive neurons that control both AVP secretion and thirst (*see* Fig. 4), this has not yet been definitively confirmed and the possibility of separate but parallel pathways for these complementary functions remains. Early reports had identified the lateral hypothalamus as a "thirst center" because its destruction in rats was found to eliminate the drinking response to increased osmolality of body fluids, but not the associated AVP secretion. However, later investigations showed that the critical damage was not to hypothalamic cells but to dopamine-containing fibers of passage, and that the induced disruption of behavior was not specific to drinking but reflected a general inability of the brain-damaged animals to initiate all movements.

2.7. Relation Between Thirst and AVP Secretion

A synthesis of what is presently known about the regulation of thirst and AVP secretion in man leads to a relatively simple but elegant system to maintain water balance. Under normal physiological conditions, the sensitivity of the osmoregulatory system for AVP secretion accounts for maintenance of plasma osmolality within narrow limits by adjusting renal water excretion to small changes in osmolality. Stimulated thirst does not represent a major regulatory mechanism under these conditions, and unregulated fluid ingestion supplies adequate water in excess of true "need," which is then excreted in relation to osmoregulated pituitary AVP secretion. However, when unregulated water intake cannot adequately supply body needs in the presence of plasma AVP levels

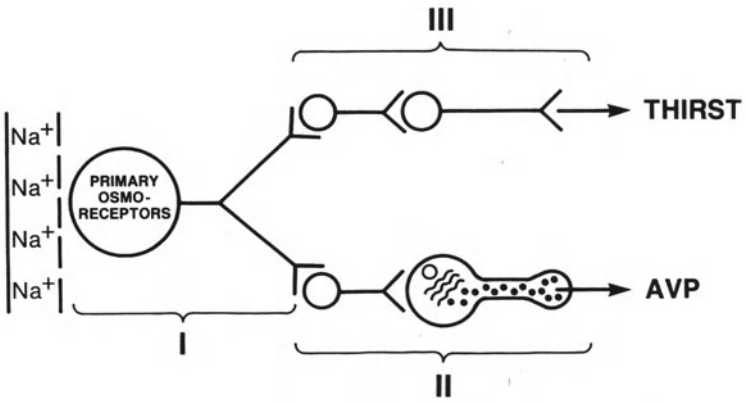

Fig. 4. Schematic representation of a model in which the same osmoreceptive cells regulate both thirst and AVP secretion. Destruction or dysfunction of the osmoreceptors themselves (I) would cause parallel changes in thirst and AVP secretion, but more distal dysfunction of either magnocellular neurons (II) or thirst pathways (III) would cause selective abnormalities of AVP secretion or drinking. Note that dysfunction at any point could potentially result in either excessive stimulation or inhibition of the final response. Reproduced from Verbalis (1990).

sufficient to produce maximal antidiuresis, then plasma osmolality rises to levels that stimulate thirst and produce water intake proportional to the elevation of osmolality above this threshold. In such a system, thirst essentially represents a backup mechanism called into play when pituitary and renal mechanisms prove insufficient to maintain plasma osmolality within a few percent of basal levels. This arrangement has the advantage of freeing animals and man from frequent episodes of thirst. This would require a diversion of activities toward behavior oriented to seeking water when water deficiency is sufficiently mild to be compensated for by renal water conservation, but would stimulate water ingestion once water deficiency reaches potentially harmful levels. Stimulation of AVP secretion at plasma osmolalities below the threshold for subjective thirst acts to maintain an excess of body water sufficient to eliminate the need to drink whenever slight elevations in plasma osmolality occur. This system of differential effective thresholds for thirst and AVP secretion therefore nicely complements many studies that have demonstrated excess unregulated, or "need-free," drinking in both man and animals.

Despite the intrinsic appeal of this theory and a substantial body of data in support of it, some recent studies have found that when human subjects are allowed to differentiate between basal degrees of thirst rather than assuming that thirst is absent under conditions of *ad libitum* fluid intake, then the thresholds for thirst and AVP secretion are quite similar. This raises an important question concerning neural regulation of water ingestion, namely, whether central pathways that stimulate fluid intake are activated in response to small changes in osmolality even before the subjective sensation of thirst occurs. Arguing against this interpretation is the observation that basal plasma osmolality in man and animals is maintained at levels associated with easily measurable plasma AVP concentrations and moderately concentrated urine, indicating that the threshold for AVP secretion must be below the threshold for stimulation of thirst. Although increases in basal plasma osmolality of only 1–4% are necessary for stimulation of thirst, the threshold for AVP secretion must lie below basal plasma osmolality because basal urine osmolalities in man and animals under conditions of *ad libitum* fluid intake are typically concentrated above isotonicity rather than maximally dilute. Consequently, although the magnitude of the difference in effective osmotic set points for thirst and AVP secretion remains debatable and clearly is subject to substantial individual variability, most experimental results to date support the concept of a higher effective osmotic threshold for thirst than for AVP secretion in man (*see* Fig. 5).

2.8. Summary

Osmoregulation in animals and man is accomplished by a combination of physiological responses to dehydration resulting in antidiuresis, and sometimes natriuresis, together with the behavioral response of water intake. Osmoreceptor cells critical for the mediation of these functions are located in the basal forebrain around the anterior wall of the hypothalamus. Those neurons are responsive to very small increases in plasma osmolality, and the effector systems they control also are very effective in rapidly correcting any increase in plasma osmolality that may have developed. These various responses are closely coordinated by virtue of the fact that the same group of neurons in the brain, when activated by hyperosmolality, stimulate different effector systems with complementary functions. Similarly, inhibition of these neurons during conditions of excess hydration suppresses these effector systems, thereby allowing excretion of free water and preventing the development of hypoosmolality. Despite this finely regulated

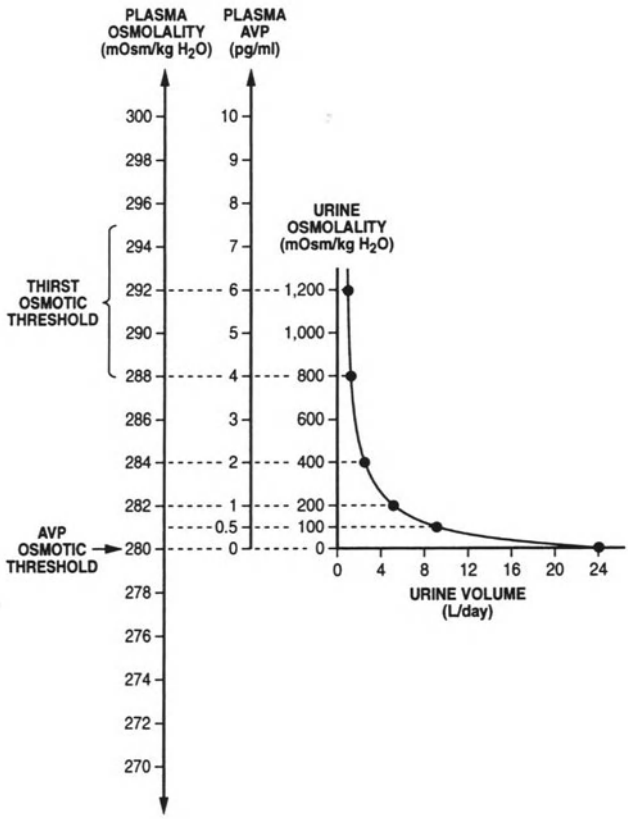

Fig. 5. Schematic representation of normal physiological relationships among plasma osmolality, plasma AVP concentrations, urine osmolality, and urine volume in man. Note the osmotic threshold for thirst is set 8–15 mOsm/kg H_2O higher than the threshold for AVP secretion so that osmoregulation within 1–2% of basal body osmolality is accomplished through AVP effects on kidney water excretion rather than by thirst-stimulated water intake. Reproduced from Verbalis (1997).

system for increasing body water in response to hyperosmolality, during normal day-to-day conditions body water homeostasis appears to be maintained primarily by *ad libitum,* or unregulated, fluid intake in association with AVP-regulated changes in urine flow, most of which occurs before the threshold is reached for osmotically stimulated, or regulated, thirst. But when these mechanisms become inadequate to maintain body fluid homeostasis, then thirst-induced regulated fluid intake becomes the predominant defense mechanism for the prevention of dehydration. Similarly, excess-free unregulated water intake can generally be adequately compensated for by suppression of AVP secretion rather than by suppression of regulated thirst, and only when such AVP suppression is impaired does thirst inhibition then become a major defense mechanism for prevention of overhydration.

3. VOLUME HOMEOSTASIS

Like osmotic dehydration, a loss of blood volume (*hypovolemia*) or blood pressure (*hypotension*) stimulates several adaptive compensatory responses appropriate to the body's need for additional dilute saline solution to restore circulatory blood volume. In considering the regulation of body fluid volume, it is important to remember that most biological membranes are freely permeable to water, so water will distribute along osmotic gradients between ICF and ECF fluid until the osmotic pressure is equivalent between these two compartments. Their relative volumes will therefore be a function of the number of free solute particles on either side of cell membranes. In mammals, the activity of ATP-dependent membrane Na^+-K^+ pumps maintains K^+ primarily within cells and Na^+ primarily outside cells. As a result of this active partitioning of Na^+ and K^+ across cell membranes, the volume of the ICF is largely a function of the amount of body K^+ along with other intracellular inorganic and organic solutes. Correspondingly, the volume of the ECF is predominantly determined by the amount of body Na^+ along with its attendant anions (Cl^- and HCO_3^-), since non-Na^+ cell membrane-impermeable solutes comprise only a small proportion (<5%) of all ECF solutes. Consequently, volume homeostasis hinges critically upon sodium metabolism.

3.1. Blood Volume, Blood Pressure, and Effective Arterial Blood Volume

As summarized in the Introduction, approximately 35-45% of total body water (TBW) resides in the ECF (*see* Fig. 1). From the arguments presented above, it follows that the ratio of ECF volume to ICF volume should be roughly equal to the ratio of exchangeable Na^+:K^+, which in adult man has been found to be 41%:59%. The distribution of fluid between intravascular and interstitial compartments of the ECF is determined by a balance between hydrostatic pressure and colloid osmotic (oncotic) pressure (*see* Fig. 2). The amount of intravascular ECF, or plasma volume, can be quantified more accurately using ^{125}I-labeled albumin or ^{51}Cr-tagged red blood cells, and averages 7–8% of TBW in adults; thus, approximately one-fifth of the ECF resides intravascularly.

3.1.1. Baroreceptor Function

In view of the importance of maintaining ECF and plasma volume at optimal levels, it is also necessary to understand how ECF volume is actually sensed.

A loss of blood volume is first detected by stretch receptors in the great veins entering the right ventricle of the heart (*low-pressure baroreceptors*), and provides an afferent vagal signal to the *nucleus tractus solitarius (NTS)* in the brain stem. Still larger decreases in blood volume also may lower arterial blood pressure, which reduces the stretch of receptors in the walls of distensible arterioles in the carotid sinus and aortic arch (*high-pressure baroreceptors*). That information similarly is communicated to the NTS and integrated there with neural messages from the low-pressure, venous side of the circulation. Note that these sensory neurons are not actually *baroreceptors* (literally, pressure receptors), although they are commonly referred to as such. The neural link between the NTS in the brain stem and the hypothalamus is known to include a prominent projection from the noradrenergic neurons arising from the A_1 catecholaminergic cell group in the ventrolateral medulla.

In addition to these classical baroreceptive pathways, experimental data have demonstrated that renal perfusion pressure, brain sodium receptors, and even compositional changes in the interstitial fluid can all modulate renal sodium excretion in response to manipulations that alter blood volume under some circumstances. Attempting to decipher the relative contributions of these various ECF volume sensing mechanisms during different physiological and pathophysiological conditions has been difficult, with seemingly conflicting reports. This undoubtedly reflects the fact that multiple systems are responsible for monitoring this important parameter; such redundancy of control mechanisms is commonplace in the nervous system, and ensures that vital functions are preserved even after malfunction of some of the regulatory components.

3.1.2. Effective Arterial Blood Volume

Regardless of which mechanisms actually monitor ECF volume, it is clear that it is not the absolute volume that is recognized, but rather the volume relative to the potential capacity of the intravascular system, which is often called the *effective ECF volume*. Although this remains a controversial concept, which has defied quantitative characterization, there is little doubt about the importance of effective ECF volume for volume homeostasis. Examples of marked alterations of renal function despite the absence of any changes in absolute ECF volume are numerous (e.g., cold-induced diuresis as a result of peripheral vasoconstriction thereby reducing the capacitance of the vascular system; orthostatic antidiuresis and antinatriuresis as a result of lower extremity pooling of blood; the diuresis and natriuresis accompanying water immersion as a result of increased negative intrathoracic pressure causing atrial distension, etc.). Evidence over the last decade has supported the concept that the critical component of the "effective" ECF volume that represents the primary determinant of renal Na^+ retention is the *effective arterial blood volume (EABV)*, which represents only approximately 15% of the total blood volume or about 0.5–0.7 L of the total body water of a 70-kg man. This is the body fluid compartment that is essential for maintenance of a sufficient volume in the arterial system to sustain systemic blood pressure at levels adequate to perfuse the vital organs.

Although all baroreceptors are basically mechanoreceptors, they alter their electrical output according to the degree of stretch of the arterial wall, which is a function of the arterial pressure (both mean arterial pressure as well as dynamic changes in pulse pressure) and of the intrinsic tensile properties of the arterial wall. Although the latter can change with age and disease processes, under normal conditions the major determinant of arterial baroreceptor activity is the pressure in the large vessels of the arterial circulation, primarily the aorta and carotid arteries. The arterial pressure is in turn primarily determined by two factors, cardiac output and peripheral arterial resistance. Similarly, renal perfusion pressure determines the activity of the baroreceptors of the renal afferent arterioles, which control renin secretion from the juxtaglomerular apparatus. Acute decreases in either variable are interpreted as a decreased EABV, and appropriate compensatory mechanisms are called into play, including activation of the *sympathetic nervous system* (both norepinephrine release from sympathetic nerve terminals and epinephrine release from the adrenal medulla), which increases peripheral arteriolar resistance and stimulates renin production by the kidney, suppression of brain stem vagal efferent output to the heart, which causes a reflex tachycardia, activation of the *renin-angiotensin system*, which promotes renal Na^+ conservation via angiotensin-stimulated aldosterone secretion, stimulation of AVP secretion, which promotes water conservation; and stimulation of water and sodium intake to replenish the dilute sodium solution, which is needed to restore the EABV. The remainder of this section will concentrate on the latter two behavioral components of this exceedingly complex set of responses enacted to preserve the integrity of the arterial blood supply to the body's vital organs.

3.2. Volemic Regulation of Thirst

The physiological contributions to volume regulation, including pituitary AVP secretion, have classically been studied in laboratory animals subjected to controlled degrees of blood loss. However, behaviors such as drinking are often compromised by the anemia and hypotension that result from extensive hemorrhage; consequently, a more slowly developing hypovolemia produced by subcutaneous injection of a colloidal solution often has been utilized instead in studies focusing on thirst and fluid intake. This treatment disrupts the Starling equilibrium at capillaries near the injection site because the extravascular colloid opposes the oncotic effect of intravascular plasma proteins, in consequence of which fluid is leached out of capillaries and remains in the interstitial space. Subsequently, ingested fluid also is drawn into the colloid-induced edema, so the total fluid volume that is required to correct the plasma volume deficit may be substantial. Water does not move out from the cells by osmosis, however, because the osmolality of the injected colloidal solutions actually is low relative to that in cells. Consequently, this method allows study of hypovolemia stimulated responses without simultaneous activation of osmotic pathways via cellular dehydration.

3.2.1. Hypovolemia-Stimulated Water Intake

In addition to its stimulating effects on AVP secretion and the activation of the renin-angiotensin system, with the consequent antidiuresis and vasoconstriction they produce, colloid treatment has long been known to increase water intake in rats. Like AVP secretion, the water intake elicited by hypovolemia increases in linear relation to the induced plasma volume deficit above a threshold of 5–10%. Also similar to AVP regulation, hypovolemia-stimulated thirst is not as nearly as sensitive to small changes in blood volume compared to osmotically stimulated thirst (*see* previous Section 2.2.), but it interacts additively with the stimulus of thirst produced by an administered NaCl load.

3.2.2. Role of Angiotensin II in Hypovolemic Thirst

Among the many consequences of volume depletion is the secretion of *renin* from the kidneys, which is mediated in part by the sympathetic neural input to the beta-adrenergic receptors on renin-secreting cells. Renin is an enzyme that catalyzes a cascade of biochemical steps resulting in the formation of *angiotensin II (AII)* in the blood. AII is the most potent vasoconstrictor agent that the body produces, and, like hypovolemia, it stimulates AVP secretion and thirst. It does so by acting in the brain at the *subfornical organ (SFO)*, a small structure located in the dorsal portion of the third cerebral ventricle. Importantly, this circumventricular organ lacks a blood-brain barrier, and the AT_1 AII receptors it contains can therefore detect very small increases in the blood levels of AII. Neural pathways from the SFO to the SON and PVN in the hypothalamus are responsible for mediating AVP secretion and thirst, and it is interesting to note that they also appear to use AII as a neurotransmitter. It also is of interest that a branch of this pathway goes to the OVLT, and might thereby provide an opportunity for the integration of hypovolemia with osmotic stimuli.

The overall stimulus of thirst during hypovolemia appears to be the same as the signal for the AVP secretion that occurs concurrently; that is, a combination of neural afferent messages from cardiovascular baroreceptors and endocrine stimulation provided by AII, and either signal could stimulate water intake in the absence of the other. For example, water intake by hypovolemic rats is not diminished by destruction of the NTS with resultant loss of neural input from cardiovascular baroreceptors, nor is it eliminated by the loss of stimulation by AII resulting from either bilateral removal of the kidneys or surgical destruction of the SFO. It remains to be determined whether colloid treatment still would elicit thirst in rats subjected to both nephrectomy and NTS lesions, but the presumption is that it would not.

Intravenous infusion of AII provides a strong stimulus of thirst in rats and most other animals studied in the laboratory (although, curiously, it is not very effective in human subjects), and it adds to the thirst stimulated by an osmotic load when the two treatments are combined. Despite these pronounced dipsogenic effects that are unique to this peptide, considerable controversy remains about whether AII functions normally as a physiological stimulus of thirst. The basis of the controversy is the finding that the doses of AII required to stimulate significant water intake produce blood levels of AII that are well above physiological ranges. Because such doses also increase arterial blood pressure, it has been argued that this hypertension is an inhibitory stimulus of thirst that limits the induced water intake; in other words, AII may provide a mixed stimulus of thirst, whereby a strong inhibitory component blunts the impact of its excitatory component. Although this is a plausible hypothesis, no independent evidence has yet demon-

strated that an increase in arterial blood pressure actually reduces water intake in any model of thirst, so this matter remains unsettled to date.

3.2.3. Osmotic Inhibition of Thirst and AVP Secretion

Hypovolemic rats need isotonic saline to repair their plasma volume deficits, not water alone. In fact, when they drink only water in response to colloid-induced hypovolemia, about two-thirds of the ingested volume moves into cells by osmosis and much of what remains extracellular is captured by the colloid-induced edema. Thus, the water consumed does not remain in the vascular compartment whose volume deficit provided the stimulus of thirst, and so the deficit persists. This is in contrast to the simpler single-loop negative feedback control of osmoregulatory thirst, whereby dehydration of osmoreceptor cells causes thirst and ingested water is retained and repairs the dehydration thus eliminating the stimulus of thirst.

The water ingested by hypovolemic rats actually aggravates the situation by causing a second serious challenge to body fluid homeostasis, namely, an osmotic dilution of the plasma. Furthermore, the animals cannot eliminate this self-administered water load because the hypovolemia reduces GFR and thereby diminishes urinary excretion independent of AVP. Under circumstances in which water is the only drinking fluid available, it is therefore appropriate that colloid-treated hypovolemic rats soon stop drinking in order to limit the secondary problem of osmotic dilution. Experimental results have indeed demonstrated that they actually do stop drinking water despite persistent hypovolemia, and that the stimulus for the inhibition of thirst is the osmotic dilution of body fluids. It is a very potent stimulus of inhibition, in that merely 4–7% dilution is sufficient to stop drinking motivated by a 30–40% loss of plasma volume. These results indicate that the osmoregulatory system is dominant over the volume regulatory system in the control of thirst under some conditions. The same is true of AVP secretion in colloid-treated rats: osmotic dilution inhibits AVP secretion even during severe hypovolema. Therefore, this adds further support to the tight linkage between AVP secretion and thirst.

3.4. Volemic Regulation of Sodium Appetite and Metabolism

To correct their volume deficits, hypovolemic animals need to consume and retain water and NaCl, not just water alone. Appropriate to that need, colloid-treated rats also drink NaCl solution and conserve sodium in their urine.

3.4.1. Renal Sodium Conservation

Renal sodium retention is mediated largely by aldosterone secreted from the adrenal cortex, although sodium conservation also occurs in association with decreases in the glomerular filtration rate. The CNS does not innervate the adrenal cortex, as it does the adrenal medulla, but a neural influence on aldosterone secretion is provided indirectly because AII is a very potent stimulus of aldosterone secretion, and renin secretion from the kidneys during hypovolemia is stimulated in part by the sympathetic nervous system. The secretion of aldosterone is also stimulated by *adrenocorticotrophic hormone (ACTH)*, which is secreted from the anterior lobe of the pituitary gland in response to *corticotrophin-releasing hormone (CRH)* released from the PVN by stimulation from cardiovascular baroreceptors. Yet another potent stimulus of aldosterone secretion is the increased plasma levels of potassium, which can develop as a consequence of the reduced GFR and the associated decrease in urinary potassium excretion. These three independent stimuli for aldosterone secretion are additive in their effects. It is significant that aldosterone can totally eliminate sodium from excreted urine, unlike the renal effects of AVP, which simply diminish urinary water loss to some level of maximal antidiuresis.

3.4.2. Salt Appetite

The only solute for which any specific appetite has been clearly demonstrated in mammals is sodium. This is generally expressed as an appetite for the chloride salt of sodium, so it is called *salt appetite*. Because of the importance of Na^+ for ensuring maintenance of the ECF volume, which in turn directly supports blood volume and pressure, its uniqueness insofar as meriting a specific mechanism for regulated intake seems appropriate. As might be expected, given the prominent activation of the renin-angiotensin system by hypovolemia, there is strong evidence that this system also plays a role in the salt appetite seen under these conditions. In addition to being a potent dipsogen, AII potently stimulates salt appetite in a dose-related manner in various models of stimulated sodium ingestion in animals. Even more intriguingly, *mineralocorticoid* administration potentiates AII-stimulated salt appetite, leading to the suggestion that these two hormones of the renin-angiotensin system, which both increase in concentration under conditions of hypovolemia may have a synergistic effect in the

brain to stimulate sodium ingestion. However, these effects cannot explain the finding that he marked NaCl intake induced in rats by colloid treatment usually is delayed relative to the appearance of thirst. More specifically, water intake is observed to increase within an hour or two after colloid treatment whereas the intake of NaCl solution does not increase until almost 5 h later. Investigations have indicated that this delay does not result from the gradual appearance of an excitatory stimulus of salt appetite, but rather from the gradual disappearance of an inhibitory stimulus for NaCl intake. Of note, during this delay hypovolemic rats drink water and thereby cause an osmotic dilution of body fluids, which is known to inhibit their thirst and the pituitary secretion of AVP and OT.

3.4.3. Central OT Inhibition of Salt Appetite

Examination of several other models of salt appetite in rats indicated that there was a strong inverse relation between NaCl intake and plasma levels of OT (but not AVP) in rats, suggesting that circulating OT might represent an inhibitory stimulus of salt appetite. However, intravenous infusion of OT in physiological doses does not decrease stimulated NaCl intake in hypovolemic rats, nor does systemic infusion of an OT receptor antagonist increase their NaCl intake. Instead, studies have supported the concept that coincident with the secretion of OT from magnocellular neurons, OT is released from parvocellular neurons projecting centrally from the PVN to other parts of the brain. This allows the possibility that plasma OT is simply a peripheral marker of the central secretion of OT that actually is partly responsible for mediating the inhibition of salt appetite in rats. Other studies have more directly supported this concept. For example, infusion of OT into the cerebrospinal fluid of rats inhibited the NaCl intake that was induced by colloid treatment, but did not affect the induced water intake. Similarly, salt appetite in hypovolemic rats was eliminated by systemic injection of naloxone, an opioid receptor antagonist that disinhibits OT secretion, and this effect was blocked by the prior injection of an OT receptor antagonist directly into the cerebrospinal fluid. Conversely, NaCl ingestion in response to hypovolemia or AII in rats was potentiated by several diverse treatments, all of which inhibit OT secretion; in addition to osmotic dilution, they include systemic injection of ethanol, pretreatment maintenance on sodium-deficient diet instead of the sodium-rich standard laboratory diet, and administration of mineralocorticoid hormones such as aldosterone.

The experimental observations suggesting that salt intake is inhibited by central oxytocinergic neurons resembles other findings leading to the hypothesis that central OT neurons also might participate in the inhibition of food intake. Both proposals operate according to the same principle, namely, that OT neurons play an important role in osmoregulation by inhibiting the intake of osmoles in order to avoid hyperosmolality. Thus, osmotic dehydration appropriately limits both food and salt intake. Importantly, from this perspective, food is a source of osmoles, not just of calories, NaCl solution is a source of calorie-free osmoles, and central OT-mediated inhibition of osmolar intake complements the natriuretic effects of neurohypophyseal OT to accomplish osmoregulation.

3.4.4. ANP Inhibition of Salt Appetite

Because the potential role of OT in osmoregulation has become apparent only during the last decade, the emergence of other peptide hormones as important factors in the homeostasis of body fluids can be anticipated. One likely candidate in this regard is *atrial natriuretic peptide (ANP)* and other members of this family. Like OT, ANP promotes sodium loss in urine by acting within the kidneys. Also like OT, ANP and related peptides are additionally secreted by neurons within the brain, and when administered directly into the cerebrospinal fluid ANP inhibits an induced salt appetite in rats. Moreover, destruction of ANP receptors in the brain has been found to eliminate the inhibition of salt appetite caused by a NaCl load. Further work will be needed to examine the role of ANP-containing neurons in the central control of salt intake, and their relation to the various other systems that participate in the regulation of water and NaCl intake by the brain.

3.4.5. Integration of Excitatory and Inhibitory Controls of Salt Appetite During Hypovolemia

The coordination of thirst and salt appetite stimulated by colloid treatment in rats can be conceptualized as a dual control system in the brain, with both excitatory and inhibitory components acting together to regulate fluid consumption (*see* Fig. 6). The combination of hypovolemia and AII stimulates thirst, but provides a mixed stimulus of salt appetite. Hence, animals drink water at first but, by doing so, the water they ingest and retain causes a dilution of body fluids, and the osmotic dilution ultimately becomes large enough to inhibit thirst. However, the dilution also

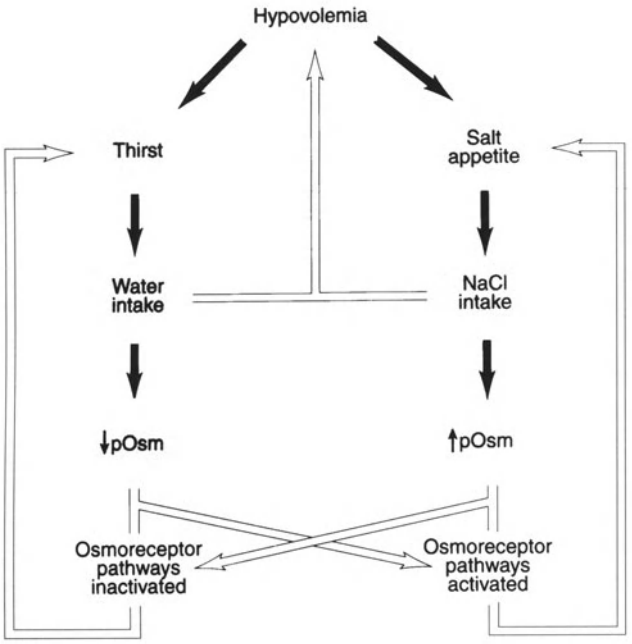

Fig. 6. Schematic representation of the physiological mechanisms controlling thirst and salt appetite during hypovolemia; solid arrows indicate stimulation and open arrows indicate inhibition. Hypovolemic rats are stimulated to drink water and concentrated saline solution (black arrows) by neural signals from cardiovascular baroreceptors to the brain stem and blood-borne Angiotensin II acting in the brain. The rats alternately drink the two fluids depending on the current plasma osmolality, and they ultimately consume sufficient fluid at an isotonic concentration to repair the volume deficit (shaded arrow). However, the water and salt intakes are limited when animals have access to only one of the drinking fluids, owing to activation of the respective inhibitory osmoregulatory pathways. Conversely, neither inhibitory pathway is activated when the rats drink isotonic saline solution, and consequently intakes proceed unabated in response to the neural and endocrine signals of hypovolemia. Modified from Stricker and Verbalis (1988).

has the effect of reducing central OT, and possibly ANP, secretion, which disinhibits salt appetite whereupon the rats begin to drink concentrated NaCl solution. That intake raises their plasma osmolality, however, and thereby removes the dilution-induced inhibition of thirst and OT secretion, so rats then stop drinking saline and resume drinking water. When they do, osmotic dilution again develops, thirst is again inhibited, and salt appetite is again disinhibited. Thus, the hypovolemic animals alternate their intakes of the two fluids, stimulated by neural and endocrine signals of hypovolemia, while maintaining body fluid osmolality near a point of isotonicity. Water and sodium are conserved in urine until their plasma volume deficits are repaired, at which point the stimuli for all the adaptive physiological and behavioral responses disappear and body fluid homeostasis is restored. The various responses are integrated because the same neural and endocrine signals of hypovolemia act to stimulate the interrelated responses of thirst, salt appetite, and the secretion of AVP and aldosterone.

3.5. Summary

Body-fluid homeostasis is accomplished by separate but related regulations of fluid osmolality and plasma volume. This osmo- and volume regulation is directed at controlling the intake and urinary excretion of water and sodium, the principal constituent of the body and the major electrolyte. Neural and endocrine signals, acting together with circulating substrates, combine to stimulate both the behavioral and the physiological responses. For example, stimulation of water intake and AVP secretion is provided by plasma substrates (osmoles) detected by cerebral osmoreceptors, a blood-borne hormone (AII) detected by cerebral AII receptors, and neural sensory signals generated by cardiovascular baroreceptors and projecting into the brain stem. Salt appetite is stimulated by these same neural and endocrine signals, but also is influenced by the concurrent inhibition they provide as well as by the inhibitory effects of plasma osmolality. Finally, aldosterone secretion is also stimulated by AII, by another hormone (ACTH), and by another substrate (potassium), but not directly by the nervous system. Thus, AII is a common participant in all aspects of body fluid homeostasis, whereas the other variables have more specific effects. The interrelatedness of these various stimuli ensures that the behavioral and physiological responses occur simultaneously, and their redundancy allows the vital regulatory processes they influence to occur even when one or another stimulus is lost owing to injury or disease. More generally, studies of water and NaCl ingestion provide insights into how peptide and steroid hormones interact with neural signals to control motivated behavior.

4. INTEGRATION OF OSMOTIC AND VOLUME HOMEOSTASIS

The two previous sections have described the regulation of osmotic and volume homeostasis separately. However, understanding the physiology of body fluids requires knowledge of how these regulatory pro-

cesses interact to achieve overall homeostasis. Because water balance is more finely regulated by changes in osmolality whereas sodium balance is regulated to a greater degree by changes in effective ECF volume, disorders of osmotic homeostasis are generally caused by abnormalities of water balance, and disorders of volume homeostasis largely result from abnormalities of sodium balance. It is apparent, however, that multiple interactions between osmotic and volume homeostasis can and do occur in individual disorders. When requirements for water and sodium intake and excretion are complementary, both homeostatic systems act in concert to regulate body fluids. However, in other situations the requirements for osmotic and volume homeostasis can conflict, and these cases are particularly useful for understanding the overall integration of the many different controls of body fluid homeostasis. A complete description of how such conflicts are resolved is beyond the scope of this chapter, but several individual examples will be discussed in the next section. Nonetheless, an important general principal has emerged over the last several decades of work in this area and should be stressed: *Interactions between the various components of osmotic and volume homeostasis are exceedingly complex and entail modulations of individual responses of both systems depending on the nature of the disorder, the relative severities of the perturbations to body osmolality versus body fluid volume, the chronicity of the perturbations, and the effectiveness of other compensatory mechanisms. Past statements such as "maintenance of body volume always overrides preservation of osmolality" may be relatively true under conditions of extreme hypovolemia, but are overly simplistic and serve no purpose for understanding the complex regulation of body fluid homeostasis across the wide range of perturbations known to occur in both animals and man.*

5. DISORDERS OF BODY FLUID HOMEOSTASIS

Disorders of body fluid homeostasis can result either from disturbances in the physiological mechanisms that control conservation and excretion of water and solutes, or from disturbances in the behavioral mechanisms that control the intake of water and solutes. As discussed in the previous sections, each of these systems attempts to compensate for physiological or pathophysiological perturbations in the others, however, often at the expense of extremes of activation of these regulatory systems and at other times unsuccessfully. This section will not attempt to discuss all disorders of body fluid homeostasis, but rather will highlight several in which primary or secondary abnormalities of fluid and solute intake are prominent.

5.1. Disorders Body Osmolality

5.1.1. Diabetes Insipidus

The crucial role played by the neurohypophysis in controlling the volume of water in the body is best illustrated by the disease of *diabetes insipidus (DI)*, in which secretion of AVP is impaired or absent, or kidney AVP receptors fail to respond normally to circulating AVP (*see* Chapter 14). This disorder was named diabetes insipidus, or insipid urine, by the early Greeks to distinguish it from *diabetes mellitus*, or sweet urine, caused when blood glucose concentrations are elevated abnormally and glucose spills into the urine. Because AVP is the only known antidiuretic substance in the body, in its absence the kidney is unable to maximally concentrate the urine and thereby conserve water. The result is continued excretion of copious amounts of a very dilute watery urine. In severe cases where the ability to excrete AVP is completely lost, this can result in the excretion of up to 25 L of urine each day. Such patients urinate almost hourly, which renders the completion of even simple tasks and activities of daily living, including sleeping, exceedingly difficult. Because renal mechanisms for sodium conservation are unimpaired, there is no accompanying sodium deficiency.

In addition to the substantial disruption of normal lifestyle caused by DI, such patients are at risk for dehydration and hyperosmolality. Volume depletion is initially minimized by osmotic shifts of water from the ICF to the more osmotically concentrated ECF, but eventually such patients will become dehydrated if they fail to replace their urinary fluid losses with ingested water. Luckily, thirst remains intact in most patients with DI, because the lesions that destroy the magnocellular neurons in the SON and PVN that synthesize AVP, or the posterior pituitary that contains the nerve terminals of these neurons, generally leave intact the osmoreceptors in the anterior hypothalamus as well as the higher brain centers that control thirst. Consequently, extreme thirst is one of the hallmarks of this disease, leading to the characteristic symptoms of *polydipsia* (excessive drinking) and *polyuria* (excessive urination). If, however, patients with DI become unable to drink or if drinking water

is unavailable, then the unreplaced urinary water losses can quickly lead to dehydration and death in the absence of medical intervention.

DI can result from either inadequate AVP secretion (central or neurogenic) or inadequate renal response to AVP (nephrogenic). Central DI is caused by a variety of acquired or congenital anatomic lesions that disrupt the hypothalamic-posterior pituitary axis, including pituitary surgery, tumors, trauma, hemorrhage, thrombosis, infarction, or granulomatous disease. Severe nephrogenic DI is most commonly congenital because of defects in the gene for the AVP V_2 receptor (X-linked recessive pattern of inheritance) or in the gene for the aquaporin-2 water channel (autosomal recessive pattern of inheritance), but relief of chronic urinary obstruction or therapy with drugs such as lithium can cause an acquired form sufficient enough to warrant treatment. Short-lived nephrogenic DI can result from hypokalemia or hypercalcemia, but the mild concentrating defect generally does not by itself cause hypertonicity and responds to correction of the underlying disorder.

The treatment of DI is similar to the treatment of other endocrine deficiency disorders, and consists of replacing the deficient hormone, in this case AVP. However, because the half-life of AVP in the circulation is quite short, which allows mammals to have minute-to-minute control of their urine output, it is more convenient to treat patients with longer acting synthetic analogues of AVP. Appropriate use of these agents can restore urinary concentrating ability to such patients thus allowing them to lead a normal life. Proof that the polydipsia of DI is secondary to the renal water losses is that water intake rapidly normalizes with institution of AVP therapy, to the marked relief of the patient.

5.1.2. Osmoreceptor Dysfunction

In very rare cases of DI, lesions of the anterior region of the hypothalamus, including the OVLT, destroy the osmoreceptors rather than the AVP-secreting neurons themselves. In contrast to typical DI patients, such individuals are not polydipsic because these osmoreceptors control thirst as well as AVP secretion. Their fluid intake is actually decreased rather than increased, which leads to the development of severe dehydration, generally manifested by chronic hyperosmolality. Because of this, initial reports in humans described this syndrome as "essential hypernatremia," and subsequent studies used the term "adipsic hypernatremia" in recognition of the profound thirst deficits found in most of the patients. It now makes more sense to group all of these syndromes as *disorders of osmoreceptor function*. Most of the cases reported to date have represented various degrees of osmoreceptor destruction associated with different brain lesions (tumors, granulomatous diseases, aneurysms), which occur more anteriorly in the hypothalamus, consistent with the anterior hypothalamic location of the primary osmoreceptor cells. Although one might think that such patients would die rapidly because of severe volume depletion, expression of their DI is limited by the fact that paradoxically they can secrete AVP in response to volume stimuli originating in the peripheral baroreceptors and relayed to the hypothalamus via brainstem afferents, which remain intact in most such patients (Fig. 3). Consequently, when their unreplaced water losses are sufficient to cause hypovolemia and/or hypotension, AVP secretion is stimulated thereby preventing excessive fluid losses beyond this point. Interestingly, thirst is generally not stimulated at the same time, indicating the absolute necessity of the anterior hypothalamus for all types of thirst, whether osmotic or volemic in origin.

5.1.3. Primary Polydipsia

Excessive water intake in the absence of any deficiencies of AVP secretion or AVP actions on kidney AVP receptors is called *primary polydipsia*. Several different etiologies for this have been described. Primary polydipsia is usually a result of psychiatric disease. Such patients ingest large amounts of fluids for a variety of reasons, such as to rid themselves of imagined toxins, but generally not because of physiological sensations of thirst; this is referred to as *psychogenic polydipsia*. A smaller subset of patients with primary polydipsia have a true disorder of thirst regulation. This can be owing to irritative rather than destructive lesions of the anterior hypothalamus, or idiopathically by a downward resetting of the osmotic threshold for stimulated thirst below the osmotic threshold for AVP secretion (*dipsogenic diabetes insipidus*). Because of these possibilities, all patients with primary polydipsia must have an MRI of their brain. Theoretically, patients with primary polydipsia can be identified by withholding all drinking fluids for a period of time to see if they are able to concentrate their urine. However, if fluid intakes have been very large, polydipsic patients frequently cannot achieve much antidiuresis because they have washed out the renal concentrating gradient that allows elabo-

ration of a concentrated urine in the presence of AVP. Consequently, the most reliable diagnosis is made by measuring plasma AVP levels after a controlled period of water deprivation in such cases.

5.2. Disorders Body Volume

5.2.1. Adrenal Insufficiency

Fifty years ago, a four-year-old boy was brought by his parents to the Johns Hopkins University Hospital, Baltimore, MD, with the peculiar symptom of having an uncommon interest in ingesting NaCl. The child heavily salted all his food, even juice, and often consumed salt directly from its container. His parents tried unsuccessfully to keep him from consuming salt. They suspected that such NaCl intake was not healthy, they knew it was not normal, and they brought him to the hospital in the hope that his strange behavior could be understood and stopped. The physicians were able to prevent the boy from having access to NaCl, but they were shocked when he died a few days later. An autopsy revealed that the child had bilateral tumors in his adrenal glands. In retrospect, he had *Addison's disease* with insufficient ability to secrete cortisol and aldosterone causing him to lose sodium excessively in his urine. Like patients with DI, who excrete a copious dilute urine and drink comparable volumes of water in compensation, this boy was ingesting salt as an adaptive compensation to his recurrent need for sodium. He died when that response was prevented.

Rats similarly are well known to increase consumption of NaCl after surgical removal of their adrenal glands, in amounts proportional to the sodium loss in urine. The biological stimulus of this salt appetite appears to be a combination of the excitation resulting from elevated circulating levels of angiotensin II and possibly reduced activity in the central oxytocinergic inhibitory system as well. Although some human patients with Addison's disease aslo have a pronunced salt appetite, as decribed above, the actual incidence of the symptom of *salt craving* in this disorder is only approx 15–20% in most series. This is likely a manifestation of significant species differences in the expression of this adaptive behavior.

5.2.2. Hypovolemia

The multiple physiological and behavioral adaptive responses to hypovolemia in animals have been discussed in great detail in the previous sections. Obviously, hypovolemia occurs frequently in man as well as a result of a wide variety of clinical disorders such as hemorrhage, diarrhea, excessive sweating, etc. For the most part, the same protective mechanisms are called into play in defense of body fluid homeostasis. The one exception to this, however, appears to be the expression of a salt appetite. Despite abundant evidence in many different species demonstrating a salt appetite that is proportionately related to Na^+ losses, the only pathological condition in which a specific stimulated sodium appetite has been unequivocally observed in humans is Addison's disease, which was discussed in the previous section. Particularly striking is the apparent absence of salt appetite during a variety of other disorders causing severe Na^+ and ECF volume depletion in humans (patients with hemorrhagic blood loss, diuretic-induced hypovolemia, or hypotension of any etiology become thirsty when intravascular deficits are marked, but almost never express a pronounced desire for salty foods or fluids). Yet, as with thirst, the possibility of subclinical activation of neural mechanisms stimulating salt intake without a conscious subjective sensation of salt "hunger" must be entertained. However, this possibility cannot be supported either, because many such patients actually become hyponatremic as a result of continued ingestion of only water or osmotically dilute fluids in response to their volume depletion. It is also interesting to note that athletes must be instructed to ingest sodium as NaCl tablets or electrolyte solutions during periods of sodium losses from profuse sweating because they fail to develop a salt appetite, which would be protective under these circumstances. As a corollary to the infrequency of stimulated salt appetite in man, there is also no evidence to support inhibition of sodium intake under conditions of Na^+ and ECF excess, as demonstrated by the difficulty in maintaining even moderate degrees of sodium restriction in patients with edema-forming diseases such as congestive heart failure.

The absence of a prominent sodium appetite in man is not unique, however, and appears to be characteristic of carnivores in general. This has been hypothesized to reflect an evolutionary adaptation in response to the naturally occurring high sodium intake of flesh-eating animals, as opposed to the low salt intake of herbivores, possibly accounting for the preservation of specific mechanisms to seek sodium in the latter group. Nonetheless, the fact that pathological conditions such as adrenal insufficiency are associated with sodium appetite even in carnivores indicates basic regulatory mechanisms for body fluid homeostasis are common to all mammalian species, but their

relative importance and utilization can vary markedly as a result of prior evolutionary pressures.

5.2.3. Hyperaldosteronism

Hyperaldosteronism is a classic example of primary sodium retention with subsequent ECF volume expansion. High plasma mineralocorticoid activity can result from an adrenal tumor or hyperplasia (primary hyperaldosteronism) or from pharmacological therapy with synthetic mineralocorticoids. Regardless of the etiology, the net effect is sodium retention and potassium excretion. Thus, as in patients with the syndrome of inappropriate antidiuretic hormone secretion (SIADH), hyperactivity of a normal regulatory system can lead to an abnormality of body fluid homeostasis.

Primary Na^+ expansion should activate compensatory mechanisms to produce a negative Na^+ balance to return body Na^+ towards normal, and these consist almost exclusively of mechanisms to enhance kidney Na^+ excretion. Because aldosterone-induced sodium retention occurs in the distal nephron, the ability of other more proximal mechanisms to induce sufficient sodium excretion to produce a negative sodium balance and correct the sodium overload is limited. However, there are limits to the degree of volume expansion that is tolerated. Just as a pressure-mediated renal escape from antidiuresis occurs when water loading becomes excessive in patients with fluid overload secondary to inappropriate AVP secretion, a similar "escape" from the antinatriuretic effects of aldosterone occurs in response to the chronic volume expansion in this disorder.

A potential conflict arises with regard to water balance because of opposite needs for maintenance of osmotic versus volume homeostasis in this case. The increased osmolality should stimulate thirst and AVP secretion to produce a positive water balance and thereby blunt further increases in osmolality, whereas volume expansion should inhibit thirst and enhance water excretion. The net effect of these stimuli on AVP secretion is complex, and depends on the chronicity as well as the severity of the sodium excess. However, patients with primary hyperaldosteronism are persistently polydipsic, suggesting that hypervolemia is not a very potent inhibitor of thirst. One might expect that another homeostatic mechanism in chronic hyperaldosteronism would be the inhibition of sodium intake. However, this does not occur, and patients with hyperaldosteronism require sodium restriction because they do not spontaneously decrease their salt ingestion. These observations provide further evidence against close regulation of sodium intake in man under most physiological and pathological circumstances, other than the intriguing occurrence of a stimulated salt appetite in some cases of Addison's disease.

SELECTED READINGS

Andersson B. Regulation of water intake. Physiol Rev 1978; 58:582–603.

Baylis PH, Thompson CJ. Osmoregulation of vasopressin secretion and thirst in health and disease. Clin Endocrinol 1988; 29:549–76.

Booth DB, Ramsay DJ. Thirst—Physiological and Psychological Aspects. Berlin, Germany: Springer-Verlag, 1991.

Denton D. The Hunger for Salt: An Anthropological, Physiological and Medical Analysis. Berlin, Germany: Springer-Verlag, 1982.

Fanestil DD, Moore FD. Compartmentalization of body water. In: Navins RG, ed. Clinical Disorders of Fluid and Electrolyte Metabolism. New York: McGraw-Hill, 1994:3–20.

Fitzsimons JT. The Physiology of Thirst and Sodium Appetite. Cambridge, England: Cambridge University Press, 1979.

Johnson AK, Buggy J. Periventricular preoptic-hypothalamus is vital for thirst and normal water economy. Amer J Physiol 1978; 234:R122–R125.

Oldfield BJ, Badoer E, Hards DK, McKinley MJ. Fos production in retrogradely labeled neurons of the lamina terminalis following intravenous infusion of either hypertonic saline or angiotensin II. Neurosci 1994; 60:255–262.

Ramsay DJ, Thrasher TN. Thirst and water balance. In: Stricker EM, ed. Handbook of Behavioral Neurobiology, vol. 10, Neurobiology of Food and Fluid Intake. New York: Plenum, 1990, 353–386.

Schrier RW. Body fluid volume regulation in health and disease: a unifying hypothesis. Ann Int Med 1990; 113:155–159.

Stricker EM. Thirst and sodium appetite after colloid treatment in rats. J Comparative Physiol Psychol 1981; 95:1–25.

Stricker EM, Verbalis JG. Sodium appetite. In: Stricker EM, ed. Handbook of Behavioral Neurobiology, vol. 10, Neurobiology of Food and Fluid Intake. New York: Plenum, 1990, 387–419.

Verbalis JG, Stricker EM. Water intake and body fluids. In: Bloom FE, Landis SC, Roberts JL, Squire LR, Zigmond MJ, eds. Fundamental Neuroscience. New York: Academic, 1999; 1111–1126.

Verbalis JG. Body water and osmolality; Body sodium and extracellular fluid volume. In: Wilkinson B, Jamison R, eds. Nephrology. London, England: Chapman & Hall, 1997, 89–101.

Verbalis JG, Blackburn RE, Olson BR, Stricker EM. Central oxytocin inhibition of food and salt ingestion: a mechanism for intake regulation of solute homeostasis. Regulatory Peptides 1993; 45:149–154.

19 Control of Food Intake

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Contents

1. INTRODUCTION

There are many factors that influence what and how much an individual eats at any one time, as well as the pattern of food intake that is maintained over time and determines body weight and composition. The control of food intake is governed both by internal mechanisms, which regulate hunger and satiety, and external factors which regulate food availability and the desire to eat. This chapter will focus on the internal physiological cues that coordinate to regulate both short-term modulation of meal initiation, meal size, and meal termination, as well as long-term modulation of energy consumption, energy expenditure, and maintenance of body fuel stores. Where possible, emphasis will be placed on mechanisms that are known to govern food intake in humans and nonhuman primates.

It is also important to acknowledge that external cues governing food intake can play very important roles in determining how much food is consumed, the types of food consumed, and the pattern of food intake. Ultimately, food intake is profoundly shaped by ecological constraints, such that an individual's food consumption will be strongly influenced by the total amount of food available and their societal status with regards to obtaining a portion of the food for their own consumption. Societal influences govern not only an individual's ability to obtain food, but also the timing of meal intake, the food choices available for consumption, and the quantity of food that is likely to be consumed in any given meal. The quality of potential food sources, including whether the sources contain poisonous substances, the taste, smell, texture, and visual presentation of the food are all important in governing what food will be selected and how much will be consumed.

Previous experiences with a particular food can play an important role in determining whether an individual will choose to consume it again. For example, consumption of a food that leads to nausea will have the short-term impact of stopping food consumption, but will also have the long-term impact of establishing an aversion for that particular food, such that the likelihood of that individual choosing to consume that particular food again is greatly diminished. Conversely, association of a food with a pleasant or rewarding experience will enhance the probability

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of further consumption of that food item. Internal demands for particular nutrients can also play a role in governing the choice and pattern of food intake, with special physiological mechanisms established to stimulate consumption of salt, fluid, essential minerals, and specific nutrients, in particular protein.

2. PERIPHERAL SIGNALS TRANSMIT METABOLIC INFORMATION TO THE BRAIN

2.1. What Kinds of Signals Transmit Information about Energy Availability from the Body to the Brain?

A number of peripheral factors such as temperature, nutrients, as well as neural and hormonal signals emanating from the periphery regulate food intake by providing information related to peripheral energy status to the central nervous system (CNS) (Fig. 1). With regard to temperature, both external temperature cues, as well as increases in body temperature resulting from the thermic effect of food, have been implicated in the regulation of food intake. The neural system sensing temperature is not well defined, but it is known to be within the hypothalamic region of brain. In general, an increase in the temperature of blood perfusing the hypothalamus is associated with an inhibition of feeding behavior. Evidence that humorally transported substances, such as nutrients or hormones, are involved in the control of food intake came originally from experiments in parabiotic animals sharing a common fluid compartment, food consumption in one animal could influence food intake in the paired animal. It is now known that nutrients traveling via the bloodstream can act directly either at the level of the CNS or at chemoreceptors in the gastrointestinal tract, portal vein, and liver. Hormones that modulate feeding behavior are produced by classical endocrine organs, including the adrenal glands, the thyroid and the endocrine pancreas, and by endocrine cells within other body tissue compartments, including the gastrointestinal tract and adipose tissue.

An important concept when considering the role of peripheral factors in the regulation of food intake, is whether an individual factor is involved in short- or long-term food intake regulation. A signal that is activated and contributes to satiety within a single meal is serving a very different function than one that regulates food intake and energy balance over an extended period of time to ensure that body weight

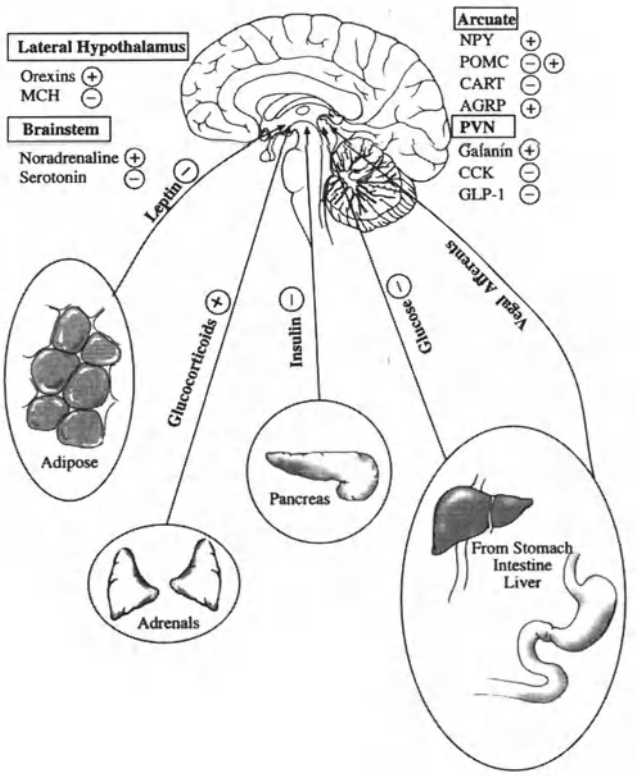

Fig. 1. Schematic diagram of CNS integration of signals modulating food intake and energy expenditure. Peripheral inputs to the brain, as well as the neural systems known to modulate food intake and energy expenditure, are indicated.

and adiposity remain relatively constant. Signals that play a role in the short-term regulation of food intake primarily serve to control meal size and short-term fuel availability (Fig. 2). In contrast, signals that play a role in the long-term regulation of food intake act primarily as determinants of body adiposity.

2.2. Nutrients and Nutrient Metabolites

2.2.1. Glucose

Marked changes in blood glucose levels can profoundly modulate short-term food intake, such that an abrupt lowering of blood glucose levels will trigger sensations of hunger, and infusion of glucose will acutely decrease food intake. It is likely that there is also a role for more subtle, physiological changes in blood glucose levels in the regulation of feeding behavior, but these have been more difficult to conclusively establish. However, such a role is supported by findings that small transient decreases in blood glucose concentrations precede spontaneous meal intake in both experimental animals and humans. Moreover, blocking these transient decreases in blood

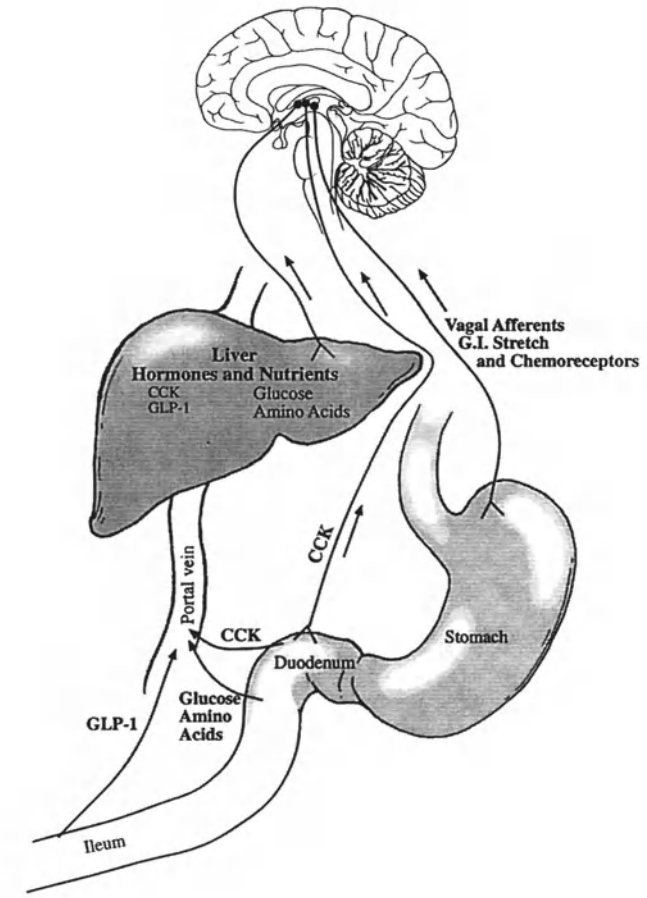

Fig. 2. Schematic diagram showing the signals that play an important role in the acute regulation of food intake during single meals.

glucose can prevent meal initiation, and artificially causing such transient decreases with a small injection of insulin can induce meal consumption.

It is likely that glucose regulates food intake by acting both at the CNS and at the level of peripheral body tissues. Populations of neurons within the ventromedial and lateral hypothalamus have been shown to change their firing rate in response to glucose application. These may be the neurons that are responsible for the effects of the nonmetabolizable glucose analog, 2-deoxy-D-glucose, to increase hunger sensations and food intake. These effects of 2-deoxy-D-glucose have been termed glucoprivic feeding. Although glucose receptors in other areas have been postulated to play a role in glucoprivic feeding, experiments showing that destruction of hypothalamic neurons by gold-thioglucose administration results in hyperphagia and blocks the effect of glucose to suppress food intake, provides strong evidence that hypothalamic glucose responsive neurons play a role in regulating feeding behavior. Glucose can also indirectly act as a signal to the brain centers controlling food intake, because the production of two major long-term modulators of energy balance, insulin and leptin, are regulated by glucose metabolism. Another indirect mechanism may be the stimulation by glucose of gastrointestinal satiety signals such as gastric inhibitory polypeptide (GIP) and glucagon-like peptide (GLP-1).

2.2.2. Protein (Amino Acids)

In contrast to glucose, there is considerably less evidence for dietary protein intake or the resulting increase of circulating amino acids as regulators of feeding. Dietary protein is known to have a satiating effect and low protein intake can lead to an increased appetite for protein-containing foods, however the mechanism underlying this action is not well understood. Administration of certain amino acids, most prominently, tryptophan, tyrosine, and phenylalanine, the precursors to serotonin and catecholamines, have been shown to suppress food intake in rodents as well as humans, with the ratio of these amino acids to other large neutral amino acids providing the critical signal regulating monoamine synthesis. In addition, it has been shown that imbalances of dietary amino acids can rapidly reduce food intake in rats via a central mechanism. Amino acids could influence food intake by acting directly on the CNS, or via chemoreceptors or other secondary sensors in the hepatoportal region. For example, absorbed amino acids may act indirectly to modulate food intake either by stimulating cholecystokinin (CCK) release, or in some cases by stimulating insulin secretion, which can, in turn, stimulate leptin production.

2.2.3. Fat (Fatty Acids)

Fat ingestion and an increase in circulating fatty acids can suppress food intake. Although there is evidence that transport mechanisms and enzymes for fatty acid oxidation are present in the brain, it is unclear how much the concomitant increases in ketonemia, resulting from the increased delivery of fatty acids to the liver, might contribute to the effect of lipid infusion on food intake, because ketones can be used as a metabolic substrate by the CNS and are known to inhibit feeding. Inhibition of fatty acid utilization with mecaptoacetate or methyl palmoxirate stimulates food intake in animals. This effect has been termed lipoprivic feeding and suggests a role for lipid metabolism in the regulation of feeding behavior.

Nonetheless, diets high in fat have an impaired ability relative to high-carbohydrate diets to stimulate leptin production, and could therefore lead to increased food intake and weight gain as part of a long-term regulation of food intake.

2.2.4. Other Metabolites (Lactate/Pyruvate/Ketones)

A number of other metabolic products can also affect food intake. For example, lactate and pyruvate and ketones, such as beta-hydroxybutyrate, all inhibit food intake. Because circulating lactate concentrations increase in proportion to the carbohydrate content of meals, and during glucose administration, circulating lactate could contribute to the effect of carbohydrate to inhibit short-term food intake. Moderate ketonemia occurring during short-term to moderate bouts of energy restriction probably does not have a marked effect on feeding behavior, because hunger ratings in humans can be markedly elevated concurrent with these moderate increases of ketones. However, during marked long-term energy deprivation (starvation) it is likely that severe ketonemia plays a role in inhibiting appetite.

2.3. Multiple Signals from the Gastrointestinal Tract Regulate Short-Term Food Intake

2.3.1. Volume

The activation of stretch and mechano-receptors in the gastrointestinal tract, triggering afferent neural signals to the CNS, has a key role in meal termination and is therefore an important signal regulating short-term energy intake. However, if caloric density is increased, smaller, more-frequent meals are eaten such that a similar amount of energy will be consumed. In this case, volume detection does not play a primary role in regulating short-term food intake. Thus, volume detection is not believed to be involved in the long-term regulation of energy balance and body adiposity. Signals that act to inhibit or delay gastric emptying such as the presence of fat into the duodenum or the release of a number of gastrointestinal peptides (gastrin, secretin, CCK, GIP, and GLP-1) will lead indirectly to satiety and decreased meal size via activation of gastric volume receptors. In addition to mechanoreceptors, gastrointestinal chemoreceptors which respond to nutrients may also modulate food intake by activating vagal afferent pathways.

2.3.2. Gastrointestinal Peptides

A number of gastrointestinal peptides or hormones are involved in the regulation of food intake. An interesting aspect of the gastrointestinal peptides is that a number of these are also produced in areas of the CNS regulating feeding behavior. For example, GLP-1 from endocrine cells in the gastrointestinal tract, has peripheral actions to reduce food intake, whereas GLP-1 produced by a population of hypothalamic neurons has a similar anorexic action in the CNS. Thus, the same peptide, produced by two different tissue types, can have a role to regulate food intake, as an endocrine agent in the periphery and as a neurotransmitter in the CNS. A similar dual role in the peripheral and central regulation of food intake is fulfilled by CCK (see below).

2.3.2.1. Cholecystokinin (CCK). CCK was the first-recognized and has been the best-studied gastrointestinal peptide involved in the regulation of food intake. CCK is released from endocrine cells in the duodenum in response to the presence of fat and breakdown products of protein (amino acids and peptides) in the proximal intestine. Peripheral administration of exogenous CCK decreases meal size in rats, nonhuman primates, and humans. Peripheral CCK acts through CCK-A type receptors, and blockade of CCK-A receptors can postpone satiety. CCK probably acts at several peripheral sites, including chemoreceptors in the pylorus and liver, which relay vagal afferent signals to the CNS. However, it is likely that not all of the satiating effects are transmitted via chemoreceptors, because CCK also has potent effects to inhibit gastric emptying, which can lead to an activation of gastric stretch receptors.

CCK is also widely distributed in the CNS, and there is evidence that CCK of neuronal origin is released in the hypothalamus of several species, including primates, in response to meals. Moreover, intraventricular administration of CCK inhibits food intake more effectively than peripheral CCK administration in nonhuman primates, demonstrating that central, as well as peripheral, CCK is involved in food intake regulation in primate species. This effect of central CCK to inhibit food intake is also mediated via CCK-A receptors, because CCK-A specific agonists reduce food intake in baboons.

It is important to consider that although CCK reduces meal size, chronic peripheral infusion of CCK does not produce a long-term reduction of food intake or weight loss. When CCK is administered repeatedly with each meal to decrease meal size, meal frequency

increases such that energy intake and body weight remain unchanged. Thus, CCK is a short-term regulator of meal size and intrameal satiety, and its actions are opposed by the actions of the long-term regulators of energy balance (insulin and leptin) that respond to decreased energy intake. It does appear, however, that an impairment of CCK signaling in both the periphery and the CNS has marked physiological consequences because the Otsuka Long-Evans Tokushima fatty rat with defective CCK-A receptors develops hyperphagia and obesity.

2.3.2.2. Glucagon-Like Peptide (GLP-1). GLP-1 is produced by the endocrine L-cells in the ileum. GLP-1 is secreted in response to nutrients in the intestine; a response that may be at least partially mediated via parasympathetic vagal activation. Endogenous GLP-1 contributes to the insulin responses to intragastric glucose in baboons. Peripheral (intravenous) administration of GLP-1 produces sensations of satiety in humans. Although it is likely that this effect, at least in part, is mediated by inhibition of gastric emptying, a role for GLP-1 to inhibit food intake by binding to GLP-1 receptors on afferent nerves in the liver and subsequent activation of a hepatic vagal pathway is also possible. Central administration of GLP-1 is also known to inhibit feeding in rodents, however, the ligand for central GLP-1 receptors is likely to be GLP-1 released in the CNS rather than GLP-1 of peripheral origin.

2.3.2.3. Gastrin Releasing Polypeptide (GRP). GRP is the mammalian homolog of bombesin, a peptide first identified in amphibians. GRP is produced by endocrine cells in the gastric mucosa and is involved in the physiological regulation of gastrin secretion. Peripheral (intravenous) infusion of GRP or bombesin reduces food intake in animals including nonhuman primates, and central administration of bombesin decreases meal size. GRP induces sensations of satiety and decreases food intake in human subjects. To what extent the effect of peripheral GRP administration is mediated via inhibition of gastric emptying is unclear.

2.3.2.4. Other Gastrointestinal Peptides. Many other peptides of peripheral origin affect feeding behavior, nearly all having an inhibitory effect on food intake. For example, the pancreatic islet hormones, glucagon and pancreatic polypeptide, are secreted from the pancreas in response to eating, primarily via a cholinergic mechanism and peripheral administration of these hormones inhibits feeding in rodents. Enterostatin, a cleavage product of pancreatic colipase, selectively inhibits fat intake and induces weight loss. Peripheral infusion of somatostatin has been shown to reduce food intake in rodents and baboons, and lower hunger and induce satiety in humans. The islet peptide, amylin, and the related calcitonin gene related peptide (CGRP), have a peripheral action to inhibit food intake in rats. Gastric inhibitory polypeptide, also known as glucose-dependent insulinotropic polypeptide (GIP), is released from intestinal endocrine cells in response to carbohydrate ingestion. Inhibition of GIP reverses the reduction of food intake and increases satiety in response to intraduodenal carbohydrate in humans, however, it is again unclear how much of this effect is because of alterations of gastric emptying. Despite evidence that administration of these and a number of other peptides of peripheral origin strongly influence ingestive behavior, the role of most of these gastrointestinal peptides, other than CCK, in the physiological regulation of food intake remains unclear at this time.

2.4. Insulin and Leptin Serve as Important Long-Term Regulators of Food Intake and Energy Balance

2.4.1. Insulin

There is a considerable body of evidence that insulin, acting in the brain, functions as a long-term regulator of food intake, energy balance, and adiposity (Fig. 3). Insulin is produced by the beta cells of the endocrine pancreas in response to ingested carbohydrate and protein. In addition, some gastrointestinal peptides released in response to food intake, particularly GIP and GLP-1, contribute to the insulin response to nutrient ingestion. Despite the wide moment-to-moment variability in plasma insulin levels measured over the course of a day, both fasting and meal- or glucose-stimulated insulin concentrations are well correlated with body fat content. Thus, overall circulating insulin concentrations are proportional to both adipose tissue stores and recent energy intake.

Receptors for insulin are located in several brain areas involved in food intake regulation, notably the hypothalamus, olfactory region, and hippocampus. Insulin is not synthesized in the brain, but gains access to the CNS via a specific and saturable transport system. Central administration of insulin reduces food intake, and the combination of a low dose of intraventricular insulin with a dose of intravenous CCK, that alone does not significantly inhibit food intake, results in a dramatic suppression of meal size in nonhuman primates, demonstrating that insulin in the CNS

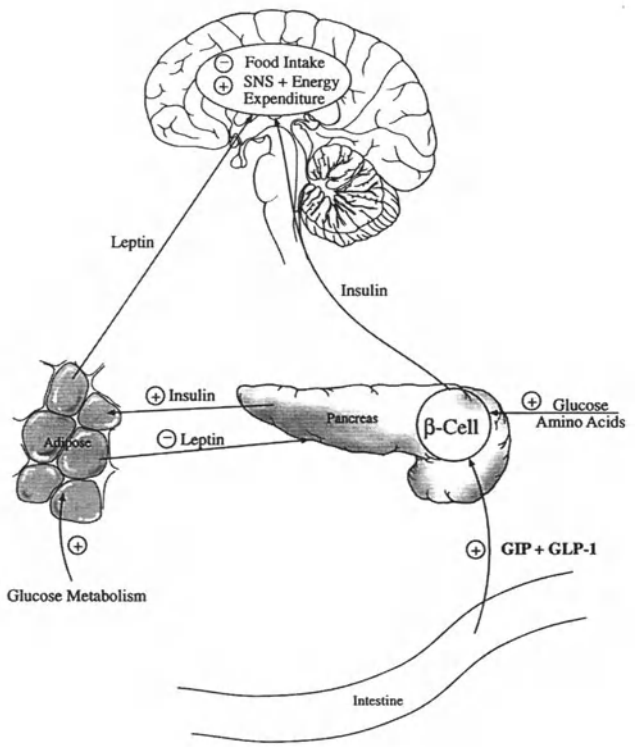

Fig. 3. Schematic diagram indicating the principal signals mediating long-term regulation of body composition, and the primary factors regulating their synthesis and secretion.

increases the sensitivity to the satiety effects of this gut peptide. Central insulin administration leads to a selective reduction of fat intake, relative to carbohydrate and protein intake. The effect of insulin to inhibit food intake is likely to involve interactions with several of the same hypothalamic neuropeptides involved in the regulation of feeding behavior by leptin (*see* Section 3.). Specifically, central insulin administration has been shown to inhibit expression of NPY in the arcuate nucleus. In addition to its effects on food intake, it is important to consider that insulin also increases sympathetic nervous system activity and thermogenesis. Thus, the overall effect of insulin on regulation of energy balance by the CNS is to cause weight loss by both suppression of feeding and increased energy expenditure.

2.4.2. Leptin

Leptin was discovered in 1994, when the gene responsible for obesity in the ob/ob mouse, a genetically obese animal, was positionally cloned. The ob or leptin gene is expressed in adipose tissue and codes for a 16 kD protein. In the ob/ob mouse, a mutation of the leptin gene causes leptin deficiency and results in hyperphagia, decreased energy expenditure, severe obesity, and insulin-resistant diabetes. Other animal models of obesity such as the db/db mouse and the fatty Zucker rat are obese as a result of genetic defects in the leptin receptor. Several forms of the leptin receptor have been identified and the receptors have homology with members of the family of cytokine receptors. The long form of the receptor (OB-Rb) appears to utilize a JAK-STAT signal transduction pathway.

Investigation of the actions of leptin to date has been conducted primarily in rodents. Central or peripheral administration of leptin to rodents leads to acute decreases of food intake, and with chronic administration, marked reductions of body fat. In addition to its effect on food intake, leptin has also been shown to increase energy expenditure, an action that appears to be mediated via activation of the sympathetic nervous system. The efficacy of small doses of centrally administered leptin and the presence of leptin receptors in areas of the CNS involved in regulating energy balance, such as the hypothalamus, demonstrates that the brain is an important site of leptin action.

The importance of normal leptin production and signaling in the regulation of energy balance in humans is convincingly demonstrated by the marked obesity that accompanies congenital leptin deficiency or leptin-receptor defects in humans. Leptin administration reduces food intake and activates the sympathetic nervous system in rhesus monkeys, providing additional evidence that leptin is involved in regulating energy balance in primates. Furthermore, increased sensations of hunger correlate well with decreases in circulating leptin during a prolonged, moderate energy deficit in women, further suggesting that leptin has a role in the regulation of human appetite.

Circulating leptin concentrations are highly correlated with adiposity in humans and animals. Leptin concentrations are higher per unit adiposity in obese vs normal weight subjects, a finding that has been interpreted to indicate resistance to leptin action in obese subjects. Women have higher leptin levels than men, even after correcting for greater adiposity in women. Because adiposity-corrected leptin levels are similar in pre- and postmenopausal women and in postmenopausal women who are taking or not taking hormone replacement, this difference does not appear to be because of an effect of female reproductive hormones. It is possible that the gender difference is a result of inhibitory effects of androgens and/or differences in body fat distribution between men and

women. In rats, the gender difference is reversed with males having higher leptin concentrations than females, a finding that is most likely because of the greater amount of body fat in male rats.

Although circulating leptin concentrations are highly correlated with adipose tissue mass, adiposity is not the sole determinant of circulating leptin. Recent studies indicate that energy intake also is a major factor because fasting, or energy restriction, acutely decreases leptin concentrations whereas refeeding and overfeeding acutely increase leptin, even in time-courses in which there are either no or very small changes in adiposity. Effects of recent energy intake on leptin secretion are likely to be regulated by changes in plasma insulin and glucose concentrations. Insulin increases *ob* gene expression and leptin secretion in vitro and in vivo. Not surprisingly, glucose infusions also raise leptin secretion. The effects of glucose and insulin to increase leptin are not observed until about 4 h after the initiation of the infusions, suggesting that these effects may be mediated at the level of transcription or translation. Infusion of small amounts of glucose, sufficient to prevent the decline of glycemia and insulinemia during fasting in humans, prevents the fasting-induced decrease in plasma leptin concentrations. In addition, decreases of circulating leptin during energy restriction in humans are proportional to the decrease of plasma glucose. Inhibition of glucose transport or glycolysis decreases *ob* gene expression and reduces leptin secretion in proportion to the reduction of glucose utilization in isolated adipocytes. This is observed even in the presence of high concentrations of insulin suggesting that adipose glucose utilization is an important determinant of insulin-mediated leptin expression and secretion. Therefore, shifts in adipose tissue glucose metabolism resulting from changes in insulin and glucose are likely to be involved in the effects of fasting and refeeding on circulating leptin concentrations in vivo.

Circulating leptin concentrations are not constant over the course of a day and exhibit a diurnal pattern in human subjects with a nocturnal peak that typically occurs after midnight. This nocturnal peak is not because of a true circadian rhythm because it does not occur when subjects are fasted, is related to meal-induced insulin secretion and can be entrained to meal timing. In humans, consuming high-carbohydrate low-fat meals, which induce large insulin and glucose excursions, postprandial increases of leptin are observed 4–6 h after meals, and the nocturnal peak is larger than in the same subjects after low-carbohydrate high-fat meals. Thus, leptin is acutely regulated by the macronutrient content of meals. Decreased leptin production may contribute to the effects of high-fat diets to promote increased energy intake, weight gain, and obesity.

2.4.3. Other Endocrine Signals can Influence Energy Balance

2.4.3.1. Cytokines/TNFα. Several cytokines are known to inhibit food intake. Most notably, interleukin-1 and tumor necrosis factor alpha inhibit food intake when administered centrally or peripherally. Cytokines may also have indirect effects to influence food intake via actions on insulin sensitivity or leptin production. Although these cytokines are involved in the anorexia induced by infection and neoplastic processes, the role of cytokines in regulating food intake under physiological conditions is less well understood.

2.4.3.2. Glucocorticoids. In contrast to the central actions of insulin and leptin to promote responses that are primarily catabolic, the actions of glucocorticoids in the brain are primarily anabolic. Administration of glucocorticoids into the CNS increases food intake, and glucocorticoids induce changes in hypothalamic neuropeptide systems that are largely opposite to those produced by insulin and leptin. In contrast, glucocorticoid deficiency, occurring in Addison's Disease, is associated with decreases in adiposity. Interactions between glucocorticoids, insulin, and leptin are crucial in the long-term regulation of food intake. For example, hyperphagia in genetically leptin-deficient or leptin-resistant animals is attenuated by adrenalectomy. In addition, adrenalectomy increases an individual's sensitivity to the effect of central insulin to reduce food intake, and glucocorticoids appear to impair the transport of insulin into the CNS.

2.4.3.3. Thyroid Hormones. Although it has been long known that thyroid hormones play an important role in regulating energy balance, surprisingly, the mechanisms by which thyroid hormones influence food intake are poorly understood. It is likely that the effects of thyroid hormone administration or endogenous hyperthyroidism to increase food intake are largely secondary to the thyroid hormone modulation of energy balance. Hyperthyroidism increases basal metabolic rate (BMR) often markedly so, which creates a negative energy balance, weight loss, reduced leptin and insulin, and hence, increased food intake. Hypothyroidism has an opposite effect (decreased BMR, weight gain, and reduced food intake), however,

hypothyroidism does not usually lead to frank obesity. Effects of hypothyroidism may be mediated in part by the fact that decreased levels of thyroid hormone would lead to increased hypothalamic thyrotropin-releasing hormone (TRH) expression by removing negative-feedback inhibition, and TRH is known to suppress food intake.Throid hormone may also influence food intake via modulation of leptin secretion in that thyroidectomized animals have increased circulating leptin concentrations, whereas administration of exogenous thyroid hormone suppresses leptin secretion.

2.4.3.4. Growth Hormone/Insulin-Like Growth Factors (IGF). The hypothalamic-growth hormone-IGF axis has a profound influence on energy balance. Growth hormone administration generally increases food intake. Similar to insulin, central administration of IGF-1 inhibits food intake, whereas central injection of IGF-II does not affect feeding. Interactions between growth hormone and IGFs with insulin and leptin appear likely to play a role in the ability of these hormones to regulate appetite. For example, IGFs can have both insulin-like actions and antagonize insulin action in peripheral tissues via effects on insulin receptors. Growth hormone, on the other hand, generally inhibits insulin action, and administration of exogenous GHRH, the hypothalamic stimulatory peptide regulating the growth hormone axis, can stimulate leptin secretion.

3. NEURAL PATHWAYS REGULATE FOOD INTAKE AND BODY WEIGHT

3.1. The Hypothalamus Plays an Important Role in the Central Regulation of Food Intake and Body Weight

Since the turn of the century it has been clear that some kinds of obesity are caused by dysfunctional pathways in the CNS. A number of early case reports described individuals who developed severe obesity in association with tumors affecting mediobasal parts of the brain. These clinical observations sparked researchers to identify and characterize the neural substrates responsible for regulating food intake and body weight. Early in this endeavor it became apparent that the hypothalamus is the key integrative CNS site determining body weight and food intake. By the mid-1950s a popular hypothesis of a dual regulatory system had emerged based on the results of lesion studies in experimental animals. This hypothesis proposed that the lateral hypothalamus was the site of neural systems inhibitory to food intake, whereas the ventromedial hypothalamus was the site of neural systems that could profoundly stimulate food intake. However, further studies revealed that appetite and body weight regulation is not simply a matter of two distinct sites, each with opposite actions on food intake. Other hypothalamic areas, such as the paraventricular nucleus and arcuate nucleus, have now been shown to play critical roles not only in the central neural control of food intake, but also in the control of energy expenditure. The emerging picture of CNS sites involved in regulating body weight has become increasingly complex, with the hypothalamus playing a central role in integrating energy-related information obtained from various internal (*see* Figs. 1–3) and external (i.e., taste, smell, social stimuli) sources.

Although initial clinical observations of hypothalamic obesity came from observing man, this syndrome is rare in primates and very few of the studies that have followed have been carried out in primate species. Rather, most of our current knowledge about hypothalamic integration of body weight homeostasis has come from experiments carried out in rodents. The hypothalamus is a phylogenetically well-conserved area of the brain and there is reason to believe that most of the conclusions drawn from rodent studies are also applicable to primate physiology. The topographical organization of the rodent hypothalamus is almost identical to that of the primate, although it is not clear if this is also true for the pattern of neural connections within the hypothalamus. There is also evidence that the hypothalamic distribution of several neuropeptides shows clear species differences. In addition, the complexity of feeding behavior in primates is considerably higher than in rodents, which calls for a critical comparison of species specific behaviors determined by neural circuits interconnecting the hypothalamus and higher limbic structures. Despite these limitations, most of the experimental evidence used for description of neural systems orchestrating feeding behavior has come from studies in rodents.

3.2. Multiple Hypothalamic Nuclei are Involved in the Regulation of Food Intake and Body Weight

In the lateral-medial direction, the hypothalamus is grossly divided into lateral, medial, and periventricular regions. In the rostro-caudal direction, the hypothalamus is divided into anterior, middle, and posterior regions. Neurons involved in regulating feeding

behavior are preferentially organized in nuclei within the medial region, including the paraventricular (PVN), ventromedial (VMH), and arcuate (ARC) nuclei. However, neurons of the lateral hypothalamic area (LH) are also intimately involved in the regulation of feeding and of other motivated behaviors. The connectivity of these nuclei, as will be described below, is highly diversified with some nuclei responding primarily to endocrine factors, others receiving information from the gastrointestinal tract, and yet others receiving their primary stimuli from sensory systems processing external cues.

The PVN is situated dorsally in the medial area of the hypothalamus and consists of anatomically and functionally distinct cell groups, easily recognized by different cytoarchitecture, neurotransmitter content, and connectivity. The overall function of the PVN appears to be that of an integrator processing incoming information about the internal environment of the body from other hypothalamic nuclei, the limbic cortex, the amygdala, and from numerous brainstem nuclei. The PVN indirectly receives information about circulating levels of energy from parameters such as thyroxin, leptin, insulin, glucocorticoids, and blood glucose. Information about on-going ingestion, digestion, and absorption of macronutrients is conveyed from the gastrointestinal tract via brainstem nuclei and hormonal input to other hypothalamic nuclei to the PVN. The PVN is also intimately interconnected with limbic structures involved in regulation of motivated behavior, including the lateral hypothalamus and central amygdala. The outflow of the PVN is organized in principally three types of fibers: hypothalamo-neurohypophysial, hypophysiotropic, and conventional neuronal. Hypothalamo-neurohypophysial fibers are neuroendocrine and emanate from magnocellular cell bodies and project to the posterior pituitary, from which the systemic hormones, oxytocin and vasopressin, are released. Hypophysiotropic fibers are also neuroendocrine and emanate from parvicellular cell bodies in the periventricular region. These neurons project to the external zone of the median eminence from where they release their contents of hypophysiotropic factors into the portal vessels connection the hypothalamus with the anterior pituitary. The conventional neural output of the PVN arises from intermediate sized cell bodies and projects among target sites to preganglionic autonomic cell bodies in the lower brain stem and spinal cord. At present, the neurochemical identity of PVN neurons mediating effects on feeding behavior are uncertain, but descending pathways to preganglionic autonomic nuclei are involved in determining body energy consumption.

Like the PVN, the ARC is a highly heterogenous nucleus with distinct outflow patterns from individual subnuclei. The ARC is situated in close apposition to the median eminence, which is the blood brain barrier-free circumventricular area penetrated by hypothalamic-pituitary portal vessels. It seems likely that this area serves a dual purpose. In addition to being a site of release of hypothalamic factors regulating anterior pituitary function, it probably serves as a window to the systemic circulation and thereby monitors plasma levels of insulin, leptin, and other hormones and metabolic substrates influencing food intake and body energy consumption. At present, it is uncertain whether insulin and leptin are actively transported from the median eminence into the adjacent ARC, but it is known that the ARC constitutes the central site that is most sensitive to the anorectic actions of both of these hormones. The ventral portion of the ARC contains neurons synthesizing a variety of neuropeptides affecting food intake, including stimulatory NPY, and inhibitory melanocortins and CART (cocaine amphetamine regulated transcript).

The ventromedial hypothalamic nucleus is a highly heterogenous structure housing neurons that play a role in regulating diverse functions, including sexual dimorphic behavior, reproduction, aggression, and feeding. Its importance for feeding came from postmortem observations from patients with neoplasms or other lesions involving the VMH. However, experimental studies have shown that lesions within the nucleus are not sufficient to induce hypothalamic obesity, suggesting that adjacent areas like the ARC and medial forebrain bundle need to be damaged before overt obesity develops. The neurons of the VMH that regulate feeding are situated in the dorsomedial aspect of the nucleus and this region is reciprocally connected to the dorsomedial hypothalamic nucleus (DMH). Neurons of the VMH are sensitive to glucose and some respond with increased firing to glucose, whereas others decrease their activity.

The DMH is not as easily delineated in primates as in rodents. Besides a role in ingestive behavior, not much is known about the functional significance of the DMH. Tract tracing studies carried out in rats, have shown that the DMH is intimately connected to numerous brain stem nuclei, including the nucleus tractus solitarii or nucleus of the solitary tract (NTS), the locus coerules, the parabrachial nucleus, and the ventrolateral medulla. In the hypothalamus, the DMH is connected to the PVN and the lateral hypothalamic

area. Also, the DMH rather profusely innervates limbic cortical structures including the hippocampus and cingulum. It has been speculated that these projections, together with projections from the lateral hypothalamic area to the cortical mantle, constitute the anatomical basis for food-seeking behavior.

The neurons of the LH area are diffusely interspersed between projection fibers of the medial forebrain bundle. The lack of clear-cut anatomical organization in this region makes lesion experiments particularly difficult to interpret. Thus, it is uncertain whether decreased food intake resulting from electrolytic lesions within the LH is caused by damage to local nerve cells or destruction of ascending fibers in the medial forebrain bundle. The LH contains a high number of glucose-sensitive neurons and direct application of local anesthetics into the LH decreases the sensitivity to glucoprivic stimuli, suggesting that LH neurons are involved in the regulation of blood glucose levels. The LH is also the site of synthesis of a number of newly discovered neuropeptides characterized by their stimulatory effect on feeding behavior. Both MCH and orexins A and B (OrA, OrB) are present in elevated levels in LH neurons during states characterized by increased food intake (i.e., in leptin deficiency).

3.3. Hypothalamic Neurons Respond to Altered Energy Availability and On-going Digestive Processes

3.3.1. Glucose Availability can Provide a Strong Signal to CNS Systems Regulating Food Intake and Energy Expenditure

Throughout the study of energy homeostasis, it has been suggested that hunger is closely related to a central site, a "hunger center," which responds with increased activity to a decrease in blood glucose levels. Such perturbations in blood glucose would call for immediate action and consequently would be responsible for acute regulation of food intake, whereas long-term regulation would rely on assessment of energy stores in liver, muscle, and fat. Despite the popularity of the "glucostat" hypothesis, that is, a hunger center made up of neurons that are either directly or indirectly responsive to blood glucose levels, physiological fluctuations in blood glucose have little effect on hunger sensations in humans. More dramatic changes in glucose availability can stimulate feeding behavior, and although there is evidence for hypothalamic involvement in glucoprivic feeding, and although glucose- and insulin-sensitive neurons are found within hypothalamic nuclei, there is strong evidence that information about glucose availability is conveyed to the hypothalamus from a glucose-sensitive circuitry involving neurons of the NTS and ventrolateral medulla of the lower brain stem. Interestingly, the NTS and the adjacent area postrema also contain insulin receptors, suggesting that this site acts as a window to circulating levels of both glucose and insulin. In the periphery, receptors monitor glucose and fatty acids in the hepatoportal circulation, and this information is conveyed via vagal afferents to the NTS. Thus, on-going absorption of nutrients from the upper gastrointestinal tract is constantly monitored by the lower brain stem, which, in turn, regulates the overall input to hypothalamic feeding circuits.

3.3.2. Sensory Stimuli can Strongly Influence Food Intake

Obviously, much more than absorbed nutrients have implications for hunger and satiety. Olfactory information impacts numerous limbic structures, including the hypothalamic neurons regulating feeding. Also, orosensory information about food texture, taste, and other physical and chemical properties is conveyed to the CNS via trigeminal, facial, glossopharyngeal, and vagal afferents. The seemingly simple fact that pungent or bitter tasting food leads to repulsion of such food items, relies on a very complex neuroanatomical set of pathways. Gustation has both visceral and somatic components, as evidenced by the fact that gustatory afferents overlap with abdominal afferents in a common target area within the NTS. Most of the outflow from taste-activated NTS neurons project to the lateral parabrachial nucleus, which is considered an important relay station for visceroception. Subnuclei of the lateral parabrachial nucleus send third-order gustatory neurons to the thalamus, which, in turn, project to agranular insular cortex, adjacent to the primary sensory representation of the tongue. The lateral parabrachial subnuclei are also directly connected to several hypothalamic nuclei and the central amygdala, which all constitute areas involved in regulation of feeding and other motivated behaviors.

3.3.3. Information about Body Energy Stores also Regulates Feeding Behavior

Information about energy stores in muscle, liver, and fat are also important determinants of hunger and satiety. As discussed in Section 2.3., circulating levels of the fat-derived hormone, leptin, can be an important regulator of long-term food intake patterns

(*see* Fig. 3). Leptin mediates its action on food intake via hypothalamic neurons in the ARC and VMH. The overall action of leptin on hypothalamic neurons is inhibitory because of hyperpolarization via interaction with sulfonylurea-sensitive potassium channels. Also, sex steroids and adrenocortical steroids have a profound effect on metabolism and overall energy consumption. These hormones exert their action by regulating transcriptional activity of lipases and other enzymes involved in deposition of triglycerides in adipose tissue.

3.4. Neurochemically Well-Defined Pathways are Involved in the Regulation of Food Intake and Energy Expenditure

A functionally comprehensive way of describing central pathways involved in the regulation of energy homeostasis is by defining connections based on their content of neurotransmitters. The transmitter systems of the CNS involved in the regulation of feeding behavior can be divided into two chemical classes, one consisting of classical biogenic amines, and the other consisting of neuropeptides. Early studies of the regulation of food intake focused on the classical neurotransmitters, such as noradrenaline and serotonin, but more recent studies have revealed that a large number of neuropeptides exert profound effects on feeding behavior via interaction with specific hypothalamic receptors. Again, most of our understanding of these pathways comes from animal experiments, carried out predominantly in the rat. However, it is not even certain that what has been established in one rodent species holds true for another rodent species. Thus, a number of compounds that appear to regulate food intake in rats are ineffective in mice, including noradrenaline, galanin, and OrA and OrB. Thus, caution against automatically assuming that specific neurochemical pathways have similar levels of influence on the control of food intake in diverse species is warranted.

3.4.1. Catecholaminergic Neurons

All endogenous catecholamines present in the CNS have been demonstrated to have effects on feeding. Both noradrenaline and adrenaline stimulate food intake via receptors in the medial hypothalamic region, whereas the actions of dopamine appears far more complex. The PVN and VMH are the central components of a CNS noradrenergic circuitry regulating food intake. Injections of either noradrenaline or noradrenaline reuptake inhibitors into the PVN increases food intake, and chronic infusion renders animals obese. The stimulatory effect of noradrenaline on feeding is mediated via α_2-adrenoceptors, situated within the PVN and VMH, whereas α_1-adrenoceptor activation in the PVN causes inhibition of food intake. Furthermore, β-adrenergic receptors in the LH probably mediate a suppressive effect of catecholamine on food intake. Glucocorticoids have permissive effects on noradrenaline-induced feeding and it is of interest that increased carbohydrate craving coincides with the circadian peak of plasma glucocorticoids. Thus, it appears that glucocorticoids are major determinants of the intake of readily available energy normally associated with the start of the activity period in animals and humans. The catecholaminergic input to the PVN arises in the NTS and VLM of the lower brain stem and pontine locus coeruleus. Neurons of the NTS and VLM are activated by vagal afferents and probably convey information about on-going digestion, as well as circulating levels of glucose and insulin to the PVN, VMH, and DMH. It is of interest that a number of anorectic agents like amphetamine, mazindol, and phentermine bind particularly well to these medial hypothalamic nuclei, and the decline in binding sites associated with food deprivation is believed to reflect receptor downregulation because of an increased noradrenergic tone.

Direct administration of dopamine into the LH decreases food intake whereas prior administration D_2 receptor antagonists abolishes this action. This effect is probably caused by antagonizing D_2-mediated instrumental responses to eating, because D_2-antagonism in the LH also reinforces reward effects analogous to self-stimulation. Dopamine is released intrahypothalamically both in the medial and lateral areas in conjunction with the onset of a meal. Experiments in nonhuman primates have shown that hypothalamic taste-responsive neurons are also responsive to dopamine. In diabetic rats, intrahypothalamic dopamine concentrations exceed normal levels consistent with delayed termination of time spent on a meal for these animals. Thus, it appears that hypothalamic dopaminergic systems may both initiate and inhibit feeding behavior dependent on the setting. Dopaminergic fibers and terminals in the hypothalamus derive from several sources, including intrahypothalamic cell groups and neurons of the ventral tegmental area. By projecting heavily to the nucleus accumbens, the latter cell group constitute the mesolimbic dopaminergic system, which is intimately involved in regulating reinforced motivated behavior, as in classical conditioning. This system is highly active during self-stimulation and eating. Starvation decreases dopamine

content in the nucleus accumbens. Starvation also potentiates self-stimulation and, of course, eating, which probably serves to restore dopamine content in the accumbens. It has been speculated that by increasing food intake or by taking drugs that restore dopamine, people with low endogenous dopamine levels in the accumbens can normalize their content of this catecholamine.

3.4.2. Serotonergic Neurons

Analogous to catecholamines, increased release of serotonin (5-HT) and subsequent activation of hypothalamic nuclei coincides with the onset of the activity period. Generally, increased serotonin release is detected at elevated levels of arousal and the overall effect on feeding of such excitement-induced serotonin release is inhibitory. The density of serotonin terminals in hypothalamic areas is largest in lateral areas, whereas medial nuclei, in particular the PVN, are sparsely innervated by serotonin fibers. It has not been possible to detect altered levels of serotonin synthesis in response to perturbations in energy homeostasis, suggesting that most effects of serotonin on feeding are short-lasting. However, site-directed injections of serotonin or analogs into medial hypothalamic sites have revealed clear and lasting anorectic effects. Serotonergic innervation of hypothalamic nuclei arise from mesencephalic and pontine median and dorsal raphe nuclei, which send rather widespread projections to the entire forebrain.

At first, it seems paradoxical that serotonin activity increases during a state characterized by increased food intake, but it has been proposed that serotonin acts as an endogenous postingestive satiety factor. This action should take place in synergy with a number of neuropeptides, including CCK. By combining lesion studies with agonist pharmacology, a circuit-mediating serotonin-induced anorexia emerges. In the rat, receptors of the 5-HT_{1B} and 5-HT_{2C} subtypes in the ARC mediate 5-HT-induced anorexia. Studies from mice having the expression of 5-HT_{1B} receptors knocked out clearly demonstrate that anorexia normally associated with increased CNS levels of 5-HT is absent. The human equivalent of the 5-TH_{1B} receptor is the $5\text{-TH}_{1D\beta}$ and it remains to be demonstrated that this receptor is responsible for serotonin-mediated anorexia in primates. A number of the anorectic drugs currently available increase extracellular serotonin concentrations by inhibiting its reuptake form the synaptic cleft (*d*-fenfluramine and sibutramine).

3.4.3. Histamine

In the CNS, histamine is synthesized in a distinct group of magnocellular neurons situated in the tuberomammillary nuclei. These neurons send projections to other hypothalamic nuclei and a variety of other forebrain areas including cortex, basal ganglia, and thalamus. Cortical histaminergic projections participate in modulation of arousal, whereas hypothalamic projections are crucial in maintaining energy homeostasis and thermoregulation. Thus, upon agonist activation of H_1-receptors or antagonism of H_3-autoreceptors in the VMH and PVN, food intake is suppressed. Depletion of endogenous histamine synthesis by α-fluoromethylhistidine renders animals hyperphagic. The fact that histamine synthesis is markedly decreased in genetically obese Zucker rats, supports a role for histamine in the pathophysiology of some forms of obesity. Histamine may also be responsible for mediating the satiation that often follows a period of energy deficiency. Histamine synthesis and hypothalamic release is increased during states of hypoglycemia, and histamine increases glycogenolysis in the hypothalamic astrocytes, ensuring energy availability during conditions of food deprivation. Histamine also plays an important role in determining the duration and volume consumed during a meal. The duration of a meal is strongly regulated by descending histaminergic fibers interacting with trigeminal motoneurons, whereas the volume of a meal is negatively correlated with histamine turnover in the VMH.

3.4.4. Gamma-Aminobutyric Acid (GABA)

GABA is one of the most abundant neurotransmitters within the CNS. Several types of GABA receptors are present, but those most abundantly present in the hypothalamus are inhibitory $GABA_A$-receptors. Injection of GABA into the VMH increases food intake, whereas injections aimed at the LH inhibit food intake. Both of these actions appear to be mediated via $GABA_A$ receptors, because the selective antagonist, bicucculine, abolishes the response whereas benzodiazepines stimulate feeding. Because GABA is present in almost half of the hypothalamic neurons including ARC NPY-containing cells, it is very difficult to pinpoint which are the best candidates involved in physiological relevant GABAergic modulation of feeding. Many antiepileptic drugs act by increasing the GABAergic tone either via direct interaction with $GABA_A$ receptors or via modulation of GABA reuptake. The clinical experience that most of these drugs also cause modest elevation of body

weight suggests that GABA exerts physiological relevant actions on feeding.

3.4.5. Neuropeptide Y (NPY)

NPY causes a robust stimulation of food intake, and if it is administered chronically in experimental animals, it leads to an obesity syndrome accompanied by hyperglycemia and hyperinsulinemia. In the rodent, the principal pathway responsible for triggering NPY-induced feeding is the ARC-PVN projection. The ARC neurons respond to decreased food intake by increasing their synthesis of NPY, and increased release of NPY in the PVN accompanies states of energy shortage. Specific damage of ARC NPY neurons makes animals hypophagic and less responsive to starvation. The PVN also receives NPY innervation from ascending brain stem projections arising in the locus coeruleus and the NTS, but these circuits probably serve other roles because altered NPY synthesis in response to starvation has never been detected at these distant sites. It is interesting that in nonhuman primates, in addition to the population of NPY neurons in the ARC, there are NPY-containing neurons in the parvocellular region of the PVN, the SON, and the DMH. Moreover, all of these populations of hypothalamic NPY-containing neurons are responsive to changes in food intake. Thus, in primates the modulation of food intake by NPY appears to be more extensive than in the rat.

The ARC NPY neurons contain leptin receptors, and their activity is suppressed by leptin, explaining some of the anorectic actions of leptin. ARC NPY neurons are also affected by changes in circulating levels of insulin, adrenocortical, and sex steroids, explaining at least in part how these hormones influence feeding behavior. ARC NPY neurons also coexpress agouti-related peptide (AGRP; *see* Section 3.4.10, below). Thus, activation of ARC NPY neurons leads to a stimulation of food intake by two mechanisms, the release of NPY, and the release of AGRP, which blocks the inhibitory action of hypothalamic POMC peptides on food intake.

A number of pharmacological studies have focused on the receptor subtypes involved in mediating the feeding response elicited by NPY, and both Y1 and Y5 receptors appear to be involved. Both the ARC and PVN appear to express the mRNA encoding for these two NPY receptors. Targeted knock out of either Y1 or Y5 receptors render the feeding response of mice less responsive to starvation. Application of Y1 antagonists to nonhuman primates abolishes fasting-induced feeding, suggesting that such sites are also of importance in primates.

3.4.6. Galanin

The functional importance of this neuropeptide relates to the ingestion of fat. Central administration of galanin produces a strong feeding response characterized by preference of high-fat diets. Moreover, blocking synthesis of endogenous galanin with antisense oligonucleotides specifically decreases fat consumption. The anatomical substrate for this action appears to be a projection from the PVN to the median eminence. The curious thing about this projection is that ingestion of fat further stimulates its activity resulting in a positive feedback further increasing food intake. It is, however, possible to inhibit the galanin production in the PVN with massive increases in circulating fat, indicating that when certain critical fat intake is exceeded the central drive to continue is diminished. A number of endocrine factors influence PVN galanin synthesis, with female sex steroids being stimulatory, whereas insulin inhibits galanin synthesis. At present, three different galanin receptor subtypes are present in the hypothalamic nuclei related to feeding, but it is uncertain whether all are involved in mediating the orexigenic effects of this peptide. Further analysis of this issue awaits specific antagonists recognizing either of the receptors.

3.4.7. Corticotropin-Releasing Hormone (CRH)

The isolation of CRH was the result of a search for hypothalamic factors regulating the corticotropes of the anterior pituitary lobe. As with most other hypothalamic releasing factors, this peptide also functions as a central neurotransmitter. Central administration of CRH influences a large number of motivated behaviors, including feeding. CRH occurs downstream to many other anorectic peptides. Central release of CRH favors a state of negative energy balance characterized by decreased food intake and increased sympathetic output and chronic administration causes long-lasting reduction in food intake. Another CRH-like peptide, urocortin, has recently been identified in brain structures related to feeding and it is likely that both peptides constitute the endogenous activators of anorexia activating CRH receptors. Urocortin has a higher affinity for CRH_2 receptors in the VMH, which are believed to be at least partially responsible for CRH-induced anorexia. Antagonists acting at these receptors enhance basal feeding. The CNS also produces a CRH binding pro-

tein that may serve as an endogenous buffer of released CRH and urocortin, but the physiological role of this binding protein remains to be established.

CRH is synthesized in neurons of the PVN, and a functional continuum consisting of the central amygdala and lateral portion of the bed nucleus of the strea terminalis. Much focus has been on the PVN as a source of endogenous CRH mediating anorexia, but given the fact that the VMH is massively innervated by the amygdala and not by the PVN, suggest that the emphasis should be diverted to CRH projections from the central amygdala influencing CRH_2 receptors in the VMH. Other CNS areas targeted by these nuclei are diverse, but the latter nuclei are intimately connected to brain stem parabrachial nucleus and the NTS. This pathway is involved in regulation of learned aversions including decreased feeding.

3.4.8. CCK

This gut-brain peptide acts both in the periphery and the CNS to inhibit feeding. Its effects on vagal afferents and the NTS to inhibit feeding are described above in Section 2.2.1. The central neurons that provide CCK-mediated inhibition to food intake project from the NTS to the PVN, with the prevailing receptor in this pathway being the CCK_B receptor, although CCK from the periphery acting at the NTS influences food intake through the CCK_A receptor.

3.4.9. Opioid Peptides

The central effects of opioid peptides on feeding behavior are complex and best understood in a framework focusing on reward of motivated behavior. Like food deprivation, opiates potentiate self-stimulation triggered by lateral hypothalamic sites. This observation has led to the hypothesis that palatable tastes stimulate a natural incentive to eat via kappa opioid receptors in the LH. However, kappa receptors in the nucleus accumbens are involved in terminating various reinforced behaviors, including food intake, suggesting that multiple dynorphin systems are involved in the regulation of food intake. Dynorphin is synthesized in neurons of the LH, and a well-defined projection from LH to the parabrachial nucleus has been described and may play a role in the regulation of food intake.

Another opioid, ß-endorphin, is derived from the common proopiomelanocortin molecule (POMC). This peptide interacts with mu-receptors and stimulation of these receptors increase food intake both in animals and humans. Expression of POMC in the CNS is restricted to the ARC and NTS, both of which send dense projections to the PVN. Thus, it is likely that the primary target of β-endorphin's effects on food intake is within the PVN.

3.4.10. Melanocortins and Agouti-Related Peptide (AGRP)

In a series of relatively recent experiments, it has become clear that a number of peptides known as melanocortins influence food intake. The most prominent melanocortin in the CNS is α-melanocyte stimulating hormone (α-MSH), which in its acetylated form inhibits feeding. Melanocortins are derived from post-translational processing of POMC, which is also the source of the orexigenic opioid peptide, β-endorphin (described above). Thus, processing of POMC gives rise to both anorectic and orexigenic peptides, but the functional implications of this antagonism are not understood. The major pathway mediating the anorectic effects of melanocortins is the ARC-PVN projection. All of the functional studies with these peptides have been conducted in rodents, but anatomical studies have clearly demonstrated the presence of a similar system in human and nonhuman primates, suggesting a similar functional role in most mammals. The central sites responsible for anorectic actions of melanocortin are hypothalamic MC4 receptors. Inactivation of endogenous MC4 receptor synthesis by gene knock out gives rise to an obese phenotype, suggesting that a stimulatory tone is normally present at the MC4 receptors. Leptin receptor is present in the ARC POMC expressing neurons, and stimulation by leptin lowers POMC synthesis. Pharmacological experiments support the idea that leptin mediates part of its anorectic effect via the melanocortinergic pathway.

Hypothalamic ARC neurons also produce an endogenous antagonist to the MC4 receptor, which is known as agouti-related peptide (AGRP). AGRP blocks stimulatory actions of melanocortins at MC3 and MC4 receptors, and overexpression of GARP leads to obesity, providing further evidence that a stimulatory tone by melanocortins is normally present. As discussed above (Section 3.4.5), ARC NPY neurons are one of the neuronal populations that produce AGRP. Release of AGRP and NPY thus provide a dual stimulation to feeding by releasing an orexigenic peptide and an endogenous inhibitor of an anorexic peptide.

3.4.11. Glucagon-Like Peptide 1 (GLP-1)

The post-translational processing of preproglucagon in the brain is different from the pancreas, giving

rise mainly to GLP-1 and GLP-2. Administration of GLP-1 centrally, particularly in the PVN leads to profound anorexia. Endogenous GLP-1 probably acts as a satiety factor because central administration of the GLP-1 receptor antagonist, exendin 9-39, results in hyperphagia and weight increase. The perikarya of central GLP-1 synthesizing neurons are restricted to the caudal portion of the NTS, which receives vagal afferents conveying information mainly from the lower gastrointestinal tract. The PVN is densely innervated by ascending GLP-1 fibers and possesses a high density of GLP-1 receptors, and the PVN is thought to be the primary site of central GLP-1 regulation of food intake.

3.4.12. Orexins

Two novel hypothalamic peptides, orexin A and B (OrA, OrB) have recently been discovered. These peptides are derived from a common precursor, which is expressed in LH neurons. Two closely related G-protein-coupled receptors mediate the actions of OrA and OrB, with the latter having a shorter duration of action. The central distribution of binding sites is at present unknown. In rodents, the synthesis of both orexin peptides is markedly elevated during starvation, whereas refeeding decreases their synthesis.

3.4.13. Melanin Concentrating Hormone (MCH)

Like the orexins, this peptide is expressed in neurons of the LH. MCH is believed to be of importance in initiating the onset of feeding because its synthesis in the LH is elevated at the onset of natural feeding cycles. This hypothesis is congruent with the finding that acute administration of MCH stimulates food intake in rodents, whereas chronic administration has little effect on body weight. However, there is some evidence that MCH may play a role in the chronic regulation of food intake, in that *ob/ob* mice overexpress MCH in the LH, and a similar increase in expression is seen in states of starvation.

3.4.14. Cocaine Amphetamine Regulated Transcript (CART)

The recent discovery of this endogenous satiety factor has revealed yet another central neuropeptide whose synthesis is stimulated by leptin. CART is rather diffusely synthesized in numerous brain sites, but only neurons of the ARC and DMH have leptin regulation of CART synthesis. Central receptors for CART have not yet been localized, but CART activates neurons in the PVN, DMH, and NTS with exogenous administration. CART is likely to play a role in limiting the duration of meal intake, in that administration of a neutralizing antiserum to CART during the course of a meal prolongs meal intake.

4. THE CONTROL OF FOOD INTAKE CAN BE DRAMATICALLY ALTERED IN PATHOLOGICAL CONDITIONS

4.1. Diabetes

4.1.1. Multiple Processes Involved in the Long-Term Control of Food Intake are Disrupted in Diabetes

Uncontrolled insulin-deficient diabetes is associated with marked hyperphagia, and insulin treatment lowers food intake. As discussed in Section 2.3., insulin is one of the primary peripheral signals playing a role in the long-term regulation of food intake. In that insulin suppresses the central neural systems to stimulate food intake, it is not surprising that with diabetic hypoinsulinemia and the resulting decrease in insulin delivery to the central nervous systems, the drive to eat would increase. A role for decreased central insulin in diabetic hyperphagia is supported by the finding that in diabetic animals insulin infusion directly into the brain, in concentrations too low to alter circulating insulin or glucose concentrations, food intake is suppressed.

In diabetes, leptin, the other peripheral hormone that plays a primary role in the long-term regulation of food intake, also shows a marked change in secretory pattern. Diabetic hyperphagia is associated with very low circulating leptin levels. Insulin treatment increases plasma leptin concentrations in proportion to the degree of glucose lowering. Interestingly, central insulin administration to experimental animals reduces, but does not normalize food intake, suggesting that a factor other than hypoinsulinemia contributes to the hyperphagia. This factor is likely to be low-circulating leptin levels.

4.1.2. Multiple Central Neural Systems Show Altered Function in Diabetes

Changes in central neural systems that may be mediating the diabetic hyperphagia include an increase in hypothalamic NPY neuronal activity, and a decrease in CRH expression in the PVN. Insulin is known to be an important inhibitor of NPY gene expression, however central insulin administration reduces, but does not normalize either NPY gene expression or food intake, suggesting that an additional peripheral signal, such as leptin, plays a role

in altering NPY gene expression in diabetic states. The reduction in CRH gene expression in diabetes is at least in part, a result of elevated levels of circulating glucocorticoids, that are inherent to this condition when individuals are not receiving insulin treatment.

4.2. Obesity

4.2.1. Obesity is a Contributing Factor in a Number of Disease States, but is Likely to be Advantageous from an Evolutionary Point of View

Obesity is a major contributing factor in a number of costly and prevalent diseases, including, but not limited to, cardiovascular disease, hypertension, some types of cancer, and insulin resistant/type 2 diabetes mellitus. The prevalence, and thus, the costs, of obesity are increasing progressively not only in the United States and industrialized countries, but worldwide, including developing nations. The reasons underlying this increased prevalence are incompletely understood. Obesity is an extremely complex disease resulting from a combination of genetic and environmental factors. Major environmental factors include decreased physical work and inactivity and the availability of relatively inexpensive, palatable foods with a high energy content. Clearly, from an evolutionary point of view, it would be advantageous to be able to readily store energy as body fat at times when increased quantities of food are available. Therefore, genes that allow greater energy intake at times when surplus food is available and genes that confer metabolic efficiency are likely to be selected for, particularly in populations that experience recurring food scarcity. It has been estimated that somewhere between 40–70% of the variability in phenotypes of adiposity is heritable.

4.2.2. Most Forms of Obesity are Polygenic

Genetic approaches to understanding obesity and for finding genes regulating food intake, energy expenditure, and body weight have led to a number of major discoveries, most prominently the cloning of the genes for leptin and its receptor (*see* Section 2.3.). Based primarily on work in animal models, a number of candidate genes regulating body weight have been identified. Obesity in humans resulting from single gene mutations are extremely rare. To date, an aggregate of nine individuals have been identified with single gene defects in the leptin gene, the leptin receptor gene, and in the carboxypeptidase gene involved in post-translational hormone processing. Obesity, in the vast majority of humans, is a polygenic disorder which is likely to involve more subtle and coexisting mutations in several genes. Linkage studies have suggested a relationship between body mass index and extreme obesity and the region of chromosome 7 containing the leptin gene. A region on chromosome 2 containing the POMC gene and a glucokinase regulatory protein has been strongly linked to body fat mass and serum leptin levels. Other studies have found linkage between a number of candidate obesity genes including TNFα, melanocortin receptors, and the beta-3 adrenergic receptor with obese phenotypes. However, other genetic studies have failed to find such genetic relationships between obesity and putative obesity genes. Given that energy expenditure is at least as important as energy intake in overall energy balance, the novel uncoupling proteins, UCP-2 and UCP-3, are prime candidates as genetic contributors to obesity. In one example, UCP-2 expression is increased in mice that are resistant to developing obesity on a high-fat diet, whereas UCP-2 expression does not change in another mouse strain that is sensitive to diet-induced obesity.

4.2.3. High Leptin Levels are Common in Obesity

A small subset of obese subjects have leptin levels that are much lower than expected for their body fat mass, and these individuals may have a relative defect in the ability to produce enough leptin for normal body weight regulation. That is, it appears likely that their inability to produce leptin leads to a decrease in inhibitory signaling to the central food intake systems and thus results in hyperphagia and obesity. In contrast, the majority of obese individuals have a many-fold higher concentrations of leptin than lean individuals. That is, leptin production remains proportional to body fat content even in the obese state (as discussed in Section 2.3.). Because this higher level of leptin might be expected to act to promote decreased food intake and weight loss, this finding has been interpreted to suggest that the majority obese subjects exhibit resistance to the actions of leptin. Resistance to leptin could occur at several points in the leptin signaling pathway. Leptin enters the CNS across the blood-brain barrier via a saturable transport system. Mice with diet-induced obesity and polygenic obesity are resistant to effects of peripheral, but not central, leptin administration to reduce food intake, suggesting a potential defect in transport of leptin into the CNS. Reduced CNS transport of leptin may

also occur in obese humans because the cerebrospinal fluid to plasma leptin ratio is reduced in obese compared with lean individuals.

Another potential mechanism for leptin resistance is the failure of leptin to activate the leptin receptor, as evidenced by the complete resistance to leptin action in *db/db* mice or in humans with leptin receptor defects. However, subtle leptin receptor polymorphisms have not been generally found in obese humans suggesting this is not a common mechanism leading to leptin resistance. Alternatively, postreceptor defects in the leptin signal transduction pathway or a failure of leptin to act on its hypothalamic targets such as NPY or MCH neurons could result in an apparent resistance to leptin action. Accordingly, the agouti (Ay) mouse, which develops obesity due to ectopic production of an antagonist of melanocortin receptors, exhibits resistance to both peripheral and central leptin administration, suggesting a defect downstream of the leptin receptor.

An additional mechanism that could contribute to an apparent resistance to the action of leptin to inhibit food intake is that the leptin signal to the hypothalamus may be overcome by the hedonic qualities of highly palatable foods. In animal studies central leptin administration has been shown to be more effective in suppressing food intake when standard diets are being consumed than when a more palatable high energy diet is consumed. This is an important consideration in that consumption of highly palatable, high-fat diets are known to contribute to obesity in humans. Plasma leptin levels over a 24-h period are higher in humans consuming low-fat high-carbohydrate meals, which induce larger postprandial insulin and glucose excursions, than when the same subjects consume high-fat low-carbohydrate meals. This lowered leptin response to high-fat meals could contribute to the long-term effects of high-fat diets to promote weight gain and obesity, whereas higher leptin production could contribute to weight loss on low-fat high-carbohydrate diets.

4.2.4. Most Current Treatments for Obesity are Ineffective

Although there are numerous approaches for treating obesity, ranging from behavioral modification to pharmacological intervention, current treatments available for obesity are rendered largely ineffective by a high rate of relapse. Both weight loss and energy restriction lead to lowered circulating leptin levels (*see* Section 2.3.). Because lowered leptin is likely to contribute to the fall in metabolic rate and increased sensations of hunger that occur during restricted energy intake, decreased leptin production could contribute to the strong tendency for weight regain after successful dieting. Thus, replacement of leptin by exogenous leptin administration or by augmenting endogenous leptin production could theoretically help maintain weight loss, and in fact, early clinical trials suggest such treatment may aid in the maintenance of weight loss in obese individuals. However, because food intake and body adiposity are regulated by multiple and redundant systems, it is likely that more than one pathway may need to be targeted to effect consistent weight loss. Other potential new targets for obesity treatment include the central neuroeffectors of leptin and insulin action such as the NPY and melanocortin systems, GLP-1, and the orexins, or systems regulating energy expenditure such as uncoupling proteins and beta-3 adrenergic receptors. It is also important to consider that pharmacological treatment is likely to be most effective and health-enhancing when combined with diet and exercise and not as an alternative to lifestyle changes.

4.3. Anorexia and Bulimia Nervosa

4.3.1. Elevations in Central Serotonergic Systems May Underlie the Severely Inhibited Food Intake Characteristic of Anorexia Nervosa

Anorexia nervosa is a disorder of unknown etiology that is characterized by the maintenance of a dramatically low body weight, accompanied by an intense fear of gaining weight or becoming fat. Most anorexics have markedly reduced food intake, although some have binge episodes that are followed by purging. Many individuals with this disorder have enough symptoms to warrant an additional diagnosis of obsessive-compulsive disorder, and depression and anxiety are also often comorbid with this eating disorder.

Studies of anorexic patients when they are normal weight have contributed to our understanding of neural systems that may underlie this disorder, without the confounding effects of changes that occur secondary to the profound weight loss associated with the disorder. Long-term weight-restored anorexics have elevated CSF 5-HIAA, the primary metabolite of serotonin, suggesting that they may chronically have increased serotonergic neuronal activity. This is in contrast to patients in the weight loss stage of this

disorder, who have decreased levels of CSF 5-HIAA. Treatment of anorexics with serotonin reuptake inhibitors (SSRIs) improves weight gain in many individuals and relieves some of the obsessional and depressive symptoms that they exhibit. In that serotonergic systems have been shown to suppress food intake (*see* Section 3.4.2), it is possible that elevated serotonergic neurotransmission directly underlies the tendency to decrease food intake. However, it must be kept in mind that serotonergic systems serve a number of roles in the brain, and thus it is not known if the elevated CSF 5-HIAA emanates from the serotonin projections that regulate food intake. An alternative possibility is that increased serotonergic neurotransmission underlies the obsessive compulsive or depressive aspects of this disorder, and that patients find that they can "self-medicate" and reduce elevated serotonin by restricting food intake.

4.3.2. Serotonergic Mechanisms are also Likely to Underlie the Binge/Purge Pattern of Food Intake Characteristic of Bulimia Nervosa

Individuals with bulimia nervosa, like those with anorexia nervosa, have an obsession with body weight and a fear of becoming fat, but they do not continuously restrict food intake and lose a great amount of body weight. Instead, they show a pattern of restrictive food intake that is broken by periodic large eating binges. CSF 5-HIAA levels have been reported to be generally within the normal range in bulimia nervosa, but challenge studies with serotonin agonists show a decreased responsiveness to serotonin. Treatment with SSRIs or serotonin receptor agonists are effective in normalizing eating patterns in some patients. However, as with anorexia, the mechanism by which serotonergic drugs modulate food intake in this eating disorder is unknown.

SELECTED READINGS

Bray GA. Static theories in a dynamic world: a glucodynamic theory of food intake. Obesity Research 1996; 4:489–492.

Campfield LA. Metabolic and hormonal controls of food intake: highlights of the last 25 years—1972–1997. Appetite, 1997; 29:135–152.

Campfield LA, Smith FJ, Burn P. Strategies and potential molecular targets for obesity treatment. Science, 1998; 280:1383–1387.

Caro JF, Sinha MK, Kolaczynski JW, Li Zhang P, Considine RV. Leptin: The tale of an obesity gene. Diabetes 1996; 45:1455–1462.

Figlewicz DP, Schwartz MW, Seeley RJ, Chavez M, Baskin DG, Woods SC, Porte D, Jr. Endocrine regulation of food intake and body weight. Journal of Laboratory and Clinical Medicine 1996; 127:328–332.

Havel PJ. Leptin production and action: relevance to energy balance in humans. American Journal of Clinical Nutrition 1998; 67:355–356.

Hoebel BG. Neurosciene and appetitive behavior research: 25 years. Appetite, 1997; 29:119–133.

Kaye WH. Anorexia nervosa, obsessional behavior, and serotonin. Psychopharmacology Bulletin 1997; 33:335–344.

Woods SC, Seeley RJ, Porte D, Jr, Schwartz MW. Signals that regulate food intake and energy homeostasis. Science 1998; 280:1378–1383.

Schwartz MW, Figlewicz DP, Baskin DG, Woods SC, Porte D, Jr. Insulin in the brain: a hormonal regulator of energy balance. Endocrine Reviews 1992; 13:387–414.

Turton MD, O'Shea D, Bloom SR. Central effects of neuropeptide Y with emphasis on its role in obesity and diabetes. In: Grundemar L, Bloom SR, eds. Neuropeptide Y and Drug Development San Diego, Academic, 1997; 15–39.

2 Neuroendocrine Regulation of Learning and Memory

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Contents

1. INTRODUCTORY REMARKS

Behavior of an organism is regulated by complex influences exerted by earlier experiences, i.e., by learning. Usually, memory is considered to be the end product of learning. However, the concepts of learning and memory are not strictly separable. Learning is synonymous with the acquisition of new information, skills, or behavioral patterns. It involves information storage (consolidation), as well as retrieval (remembering).

Learning and memory are essential requirements for every living organism to cope with environmental demands, which enable the organism to adapt to changes in the conditions of life. Hormones may influence learning and memory indirectly by affecting arousal, vigilance, attention, and motivation or by a direct effect on learning and memory processes through trophic effects on synaptic contacts by changes in plasticity to establish a memory trace or network.

Historically, much of the literature dealing with neuronal correlates of learning and memory has focused on a single or a small set of brain structures. However, memory "traces" for different types of learning are not restricted to one brain structure. Many modalities take part in the formation of a memory trace or network. Separate regions of the brain seem to simultaneously carry out computations on stimuli from the external and internal environment and even "localized" memory "traces" may include multiple brain sites. Neocortical structures are connected with all brain areas—limbic structures in particular—that are involved in learning and memory processes. These regions are richly innervated by fibers containing neurotransmitters and numerous neuropeptides. Every neuropeptide possesses multiple CNS effects, whereas every particular brain function is affected by a multitude of neuropeptides and other neurotransmitters and neuromodulators. Learning and memory are a good example of such a brain function.

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2. NEUROPEPTIDES

2.1. General Aspects

The classical neurotransmitters are formed from amino acids in small vesicles on the nerve ending, the synapse, near the site of their release. The neuropeptides are synthesized as large precursor molecules following gene expression, and are generally colocalized with other neurotransmitters and neuropeptides. They are stored in larger vesicles further from the synaptic cleft, slightly more to the side of the synapse. If the content of the vesicle is to land in the synaptic cleft, the vesicle's wall must fuse with that of the synapse. This happens when an electrical stimulus reaches the synapse. One characteristic of the migration of the large vesicles with neurotransmitters and neuropeptides is that it only occurs following repeated stimulation. The assumption is, therefore, that neuropeptides are only active when activity in the brain increases, as in the case of stress, learning and memory, pain, brain injuries, etc. Although some neuropeptides function as neurotransmitters, most have a neuromodulating function such as influencing the activity of the neurotransmitters that are stored with them. They have a persisting influence, so that they are well suited to maintain for a sufficient length of time a particular behavior that must take precedence, such as searching for food and water, sexual behavior, rearing of offspring, etc.

The precursor proteins of neuropeptides as vasopressin and oxytocin, and those of proopiomelanocortins as well, are distributed throughout the brain along neural pathways from the sites where they are produced. The various receptors for the neuropeptides also occur at a number of sites in the brain. This explains their involvement in so many different kinds of behavior. Many neuropeptides affect one particular behavior, whereas on the other hand, learning and memory are influenced by numerous neuropeptides. This may be related to the fact that learning plays a role in many adaptive behaviors.

The term neuropeptide(s) denotes peptides that influence the nervous system. The precursor protein, which in itself is biologically inert, can be cleaved by proteolytic enzymes yielding active neuropeptides of the "first generation." There is an enormous variety of neuropeptides. Over 50 genes that encode for neuropeptides have already been identified; of these, a neuron usually only expresses one or two. This choice is already determined during the development of the brain. Sometimes different cells, for example, nerve cells or thyroid cells utilize the same gene in different ways, producing different precursor molecules. The conversion of precursor proteins to form neuropeptides of the "first generation" also depends on the cell in which it occurs. An example is proopiomelanocortin which is the precursor protein of adrenocorticotropin hormone (ACTH), various melanocyte-stimulating hormones (MSH's), β-lipotropin and a number of endorphins.

Neuropeptides can undergo chemical changes (acetylation, amidation etc.) that may have a powerful effect on their biological activity. They can also be further converted extracellularly to neuropeptides of the "second generation." For example, vasopressin and oxytocin are precursors of neuropeptides of the "second generation" in the brain. These "second generation" neuropeptides are generally small fragments of their "first generation" precursors, such as the (4–9) and (4–8) fragments of Arg^8-vasopressin (AVP). Compared to that of their precursor, the influence of the second-generation neuropeptides can be the same, more selective, different, or the reverse. They often have little if any "classical" endocrine effect, such as the stimulating influence of ACTH on the production of corticosteroids from the adrenal, or the antidiuretic effect of vasopressin.

2.2. Proopiomelanocortin Fragments

2.2.1. Introduction

Early this century, a pituitary hormone was discovered that induced darkening of frog skin and changes in skin color in several fish species. This hormone, originally called "intermedin" because of its presence in the intermediate lobe of the pituitary, is presently named melanocyte-stimulating hormone, (MSH). Although evidence for the presence of MSH in the brain had already been found in the 1930's, little attention was paid to its possible central nervous system (CNS) effects. In the mid-1950s effects of MSH, ACTH, and their fragments on behavior suggested their implication in brain function. In the mid-1970s, it was found that a family of peptides, including MSH and ACTH, are derived from a large precursor molecule proopiomelanocortin (POMC). This precursor molecule is produced not only by the pituitary, but also by the nervous system. The processing of the POMC, yielding MSH and ACTH, is a cell-specific phenomenon; whereas ACTH is formed in the corticotrophs of the adenohypophysis, successive cleavage to α-MSH takes place in the melanotrophs and probably

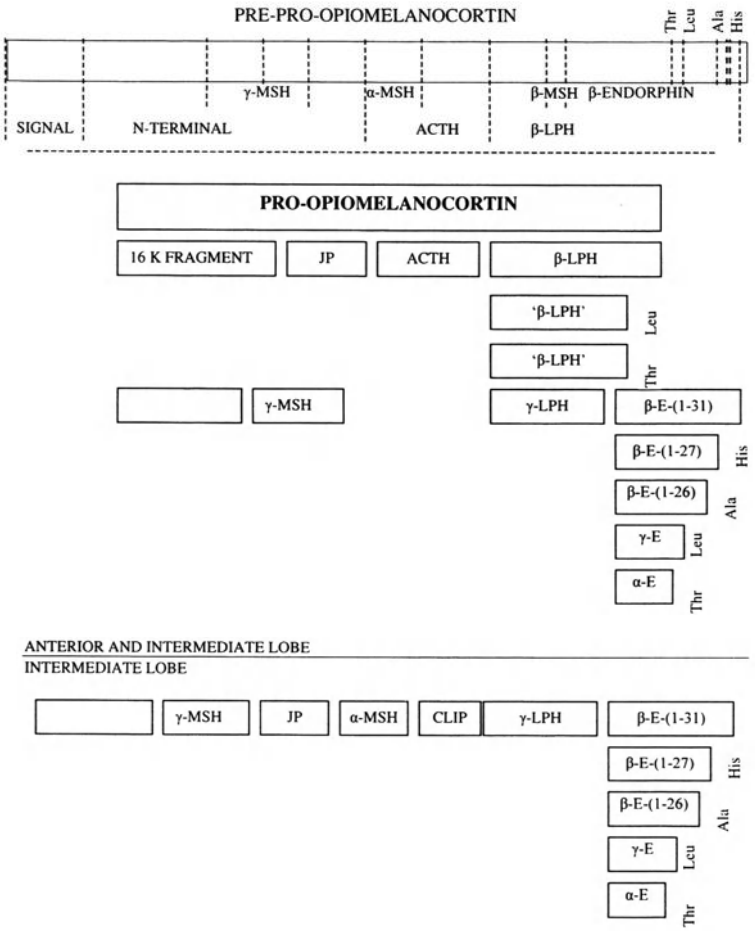

Fig. 1. Schematic representation of the rat proopiomelanocortin (POMC) precursor molecule and the products of its enzymatic processing in the anterior and intermediate lobes of the pituitary gland. ACTH, corticotropin; β-LPH, β-lipotropin; MSH: melanotropin; CLIP: corticotropin-like intermediate lobe peptide: E: endorphin; JP: joining peptide.

also in the brain. Cleavage of POMC results not only in the production of α-, β-, various γ-MSHs and ACTH, but also in that of α-, β-, and γ-endorphin, corticotropin like intermediate lobe peptide (CLIP) and related fragments (*see* Fig. 1). α- and β-MSH structurally resemble ACTH. γ-MSH appears to have a slightly different amino-acid composition (*see* Fig. 2).

2.2.2. Effects on Learning and Memory

2.2.2.1. ACTH/MSH. In the early 1960s, it was found that the pituitary gland possessed a profound effect on learning and memory processes in rats. Removal of the adenohypophysis together with the intermediate lobe or the whole pituitary attenuated acquisition of avoidance behavior in a shuttle-box test, used to test learning and memory processes. It is a two-way active avoidance task in which a rat is trained to jump over a fence to the other identical side of the cage in response to a sound stimulus on penalty of an electric footshock. This abnormality could be restored by treatment with ACTH, or α-MSH.

In rats ACTH/MSH peptides or melanocortins facilitate acquisition and delay extinction of shuttle box avoidance behavior and one way active avoidance behavior, as pole-jumping avoidance behavior, in which the rat is trained to jump onto a pole in response to a light signal to avoid footshock. They also facilitate passive avoidance behavior of intact rats (for explanation of the test see Section 2.2.2.2.), but only when administered prior to retention and not when given after the learning trial (*see* Fig. 3). In addition, they reverse retrograde amnesia induced by various amnesia treatments, thus, retrieval and not storage of information is enhanced.

In positively reinforced paradigms α-MSH improves acquisition or delays extinction of a complex maze response for food or sexual reward. In these paradigms, hungry rats learn to run to or to find a route to a source of food, or sexually deprived (male) animals learn to find access to a receptive (female) partner.

Classical endocrine activity of peptides can be clearly dissociated from behavioral effects. Structure-

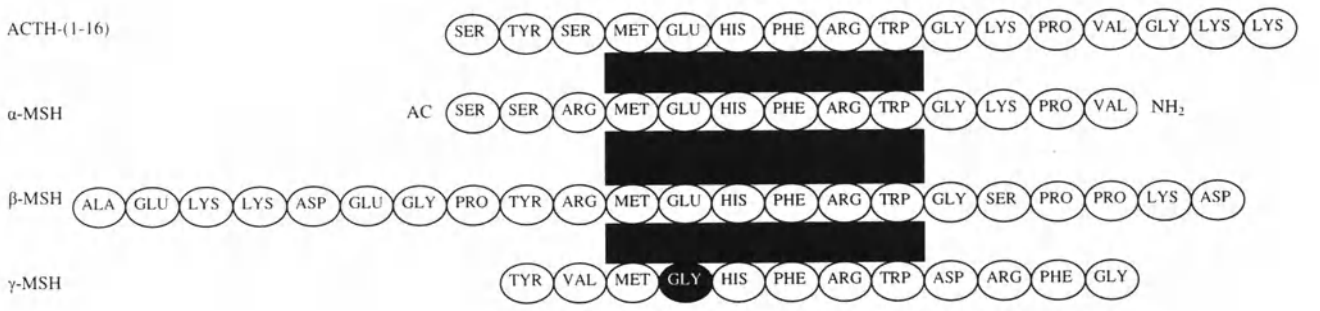

Fig. 2. The amino acid sequence of the proopiomelanocortin (POMC) products ACTH-(1–16), α-MSH, β-MSH, and γ-MSH. ACTH-(4–9) is present in three of the four peptides.

activity studies showed that ACTH-(4–7) was the smallest peptide to be fully active on learned behavior. It possesses no significant adrenocorticotropic effect, but it is as potent as the whole ACTH molecule on avoidance behavior.

2.2.2.2. One-Trial-Learning Passive Avoidance Paradigm. This test uses the innate preference of rats for the dark rather than the light. The experimental apparatus consists of a dark compartment equipped with a grid floor, to which an elevated and illuminated platform is attached. During passive avoidance conditioning the illuminated platform is the only light source in the room. When a rat is placed on the illuminated platform it enters immediately the dark box. During the learning trial the rat receives a single scrambled electric footshock for 2 s through the grid floor of the cage immediately upon entering the dark compartment. Retention is tested 1 d after the learning trial by measuring the entrance latency up to a maximum of 300 s. Memory is reflected in a prolonged entrance latency to enter the box. In this test substances can be distinguished on their effects on consolidation or retrieval of information. When a substance, administered within a critical time after the learning trial, facilitates or attenuates passive avoidance behavior during the retention test, it is considered to affect storage processes. If given prior to the retention test, it is considered to influence retrieval processes.

Different hypotheses have been offered to explain the behavioral influence of ACTH/MSH neuropeptides. From numerous studies, it appeared that such neuropeptides increase motivation, attention, concentration and/or vigilance. Indeed, ACTH/MSH peptides induce an increase in mean and peak frequencies in theta activity (a slow, synchronous rhythm in response to stimuli) in the hippocampus of rats during stimulation of the mesencephalic reticular formation. Mean and peak frequency of theta activity are correlated with the neural activity within the mesodiencephalic limbic structures. This suggests that these peptides augment the excitability in the limbic-midbrain system. Also, in man, these peptides were found to increase attention and vigilance and to attenuate habituation. Furthermore, endogenous ACTH and MSH in the brain undoubtedly are involved in brain processes underlying active and passive avoidance behavior. More, in particular, endogenous ACTH (and MSH) in the rat brain plays a role in the retention of a learned response by improving the retrieval of stored information.

2.2.2.3. γ-MSH. γ-MSH resembles α-MSH. However, one alteration in the amino acid composition in the behavioral active sequence (4–9) alters its biological activity completely (*see* Fig. 2). It has only minor melanotropic activity and it acts opposite to ACTH and related peptides in several paradigms. γ-MSH thus inhibits the acquisition of shuttle-box avoidance behavior, facilitates extinction of pole-jumping avoidance behavior and attenuates passive avoidance behavior. Such opposite effects in the same neuropeptide system (see also Section 2.3.1.3.) may have local homeostatic value.

2.2.2.4. Endorphins. The endorphins are another important class of peptides generated from the POMC molecule (*see* Fig. 1). β-Endorphin has an interesting dose-dependent biological activity on behavior; in low doses it facilitates passive avoidance behavior, whereas in high doses it inhibits this type of behavior. In this respect, it is of interest that β-endorphin can be converted in the brain to γ-endorphin, α-endorphin and smaller fragments. The γ-type endorphins inhibit acquisition and facilitate extinction of active avoidance behavior and attenuate passive avoidance behavior. In contrast, α-endorphin has opposite effects; it delays extinction of avoidance behavior. Also in some other behavioral tasks the α- and γ-endorphins show opposite effects. These are independent of their opi-

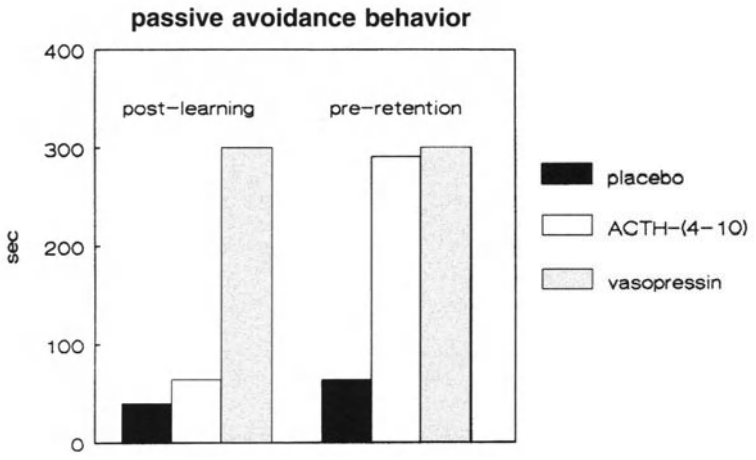

Fig. 3. Preretention but not postlearning administration of ACTH-(4–10) enhances passive avoidance retention, reflecting an effect on retrieval, but not on storage of information. Vasopressin enhances avoidance behavior at preretention as well as postlearning treatment, indicating effects on retrieval and storage processes. Vertical axis represents avoidance latency time in sec.

ate-like effects because removal of the N-terminal amino acid residue tyrosine, which is essential for activating opiate receptors, eliminates the opiate-like, but not the behavioral effects. Moreover, results of a variety of behavioral tests suggest that α-endorphin possesses amphetamine-like, i.e., psychostimulant activity, whereas γ-endorphin (and various active fragments thereof) possesses neuroleptic-like activities. Effects of these peptides on the performance of animals in a learning situation might be secondary to the psychostimulant- and neuroleptic-like effects.

2.2.3. Distribution and Sites of Action

The major population of cell bodies, which produce POMC, resides in the nucleus arcuatus of the hypothalamus. It projects to many limbic and brain stem regions as amygdala, bed nucleus of the stria terminalis (BNST), the locus coeruleus, the septal area, periventricular nucleus of the thalamus, periaqueductal gray, reticular formation, etc. (*see* Fig. 4).

Hormones, neuropeptides or steroids, modulate learning, and/or memory processes directly or indirectly by acting at brain structures, which mediate information processing and memory formation and/or retrieval. The difference in profile of effects of various neuropeptides (and steroids) on behaviors that are related to learning and memory may be caused by different modes of action, different sites of action, and/or by different receptors. Attempts have been made to determine the sites of action of the hormones in the brain in relation to their behavioral effect. For this purpose, various approaches can be used such as experiments with animals bearing lesions in the brain or local administration of behavioral active substances or their antagonists or antibodies in restricted brain areas. Each of these approaches, however, has its own limitations and needs a specific interpretation. Nevertheless, combination of the various techniques available may provide a reliable answer to the question where the central sites of behavioral action of hormones affecting learning and memory processes are located.

2.2.4. Lesion Studies and Microinjections

Bilateral lesions in the midposterior region of the thalamus, which destroy the parafascicular nuclei completely, block the inhibitory effect of α-MSH and ACTH-(4–10) on the extinction of an active avoidance response. Also, extensive bilateral lesions in the (rostral) septal area destroying the medial septal area completely and damaging (part of) the hippocampocortical tract, the lateral septal nuclei and the nucleus accumbens, completely block the inhibitory effect of ACTH-(4–10) on extinction of an active avoidance response. Moreover, lesions in the antero-dorsal hippocampus and lesions destroying the central and basolateral parts of the amygdaloid complex strongly interfere with the inhibitory effect of ACTH-(4–10) on extinction of a pole-jumping avoidance response.

However, ACTH and some of its congeners may not act on a single anatomically well-defined site of the brain, but they may need an intact limbic system in order to display their behavioral effects related to learning and memory function. This hypothesis obtained support from studies with rats in which the fornix was transected, interrupting the main afferent and efferent connections of the hippocampus and amygdala. In fact, fibers projecting to and/or from the septum, the preoptic area, the corpora mammillaria, and the amygdala are transected by this intervention. It abolished the effect of ACTH-(4–10) on extinction of an active avoidance response. Microinjections of ACTH fragments in the same areas have led to similar conclusions. Thus, various limbic-

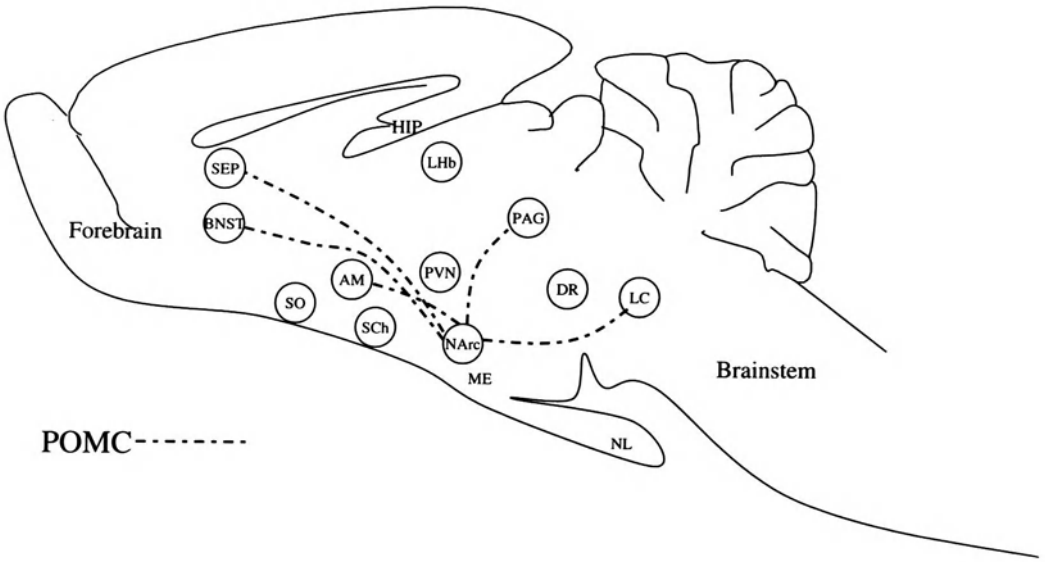

Fig. 4. Main proopiomelanocortin (POMC) pathways in the brain.

Abbreviations:

AM	Amygdala	NArc	Nucleus Arcuatus
BNST	Bed Nucleus of the Stria Terminalis	PAG	Periaqueductal Grey
DR	Dorsel Raphe Nucleus	PVN	Nucleus Paraventricularis
HIP	Hippocampus	SCh	Nucleus Suprachiasmaticus
LC	Locus Coeruleus	SEP	Septal Area
LHb	Lateral Habenular Nucleus	SO	Nucleus Supraopticus
ME	Median Eminence		

midbrain regions (dorsal hippocampus, amygdala, septum), are anatomical substrates for the effect of ACTH fragments on behavior related to learning and memory processes. Furthermore, an effective site of ACTH fragments in delaying extinction of active avoidance behavior is located in the posterior thalamic region and includes the parafascicular nuclei; in this area, mesencephalon and diencephalon merge. This region is involved in relaying information via the ascending reticular formation to the cortex.

2.2.5. Receptors

Five melanocortin receptor subtypes for the ACTH/MSH-like neuropeptides are now known that mediate their influence on the adrenal cortex, on pigment metabolism, and on the brain. The so-called MC_1 receptor is the receptor expressed in melanocytes—cells playing a role in skin pigmentation in amphibians or color changes in fish—whereas the MC_2 receptor is the classical ACTH receptor expressed in the adrenal cortex and exclusively activated by ACTH. The MC_3 receptor which is expressed in the limbic system and in the hypothalamus (in particular the nucleus arcuatus) binds γ-MSH best of the 5 MC-receptors. The MC_4 receptor is much more widely distributed throughout the brain, in particular the PVN, brain stem, and spinal cord. It binds α-MSH, β-MSH, and ACTH with equal potency and is exclusively expressed in the central nervous system. Finally, the MC_5 receptor is a typical MSH-receptor showing affinity for α-, β-, and γ-MSH. It is also expressed in the brain, but in addition in many peripheral tissues including the zona glomurulosa of the adrenal cortex. The behavioral effects of ACTH/MSH-like neuropeptides and their fragments on learning and memory, make it likely that the receptor mediating these effects is either the MC_3, or the MC_4. However, the relatively low-intrinsic activity of ACTH-(1–10) and ACTH-(4–10) for these receptors suggests that other receptors for the effect(s) of melanocortins on learning and memory processes (attention, motivation, vigilance, etc.) may be found.

2.3. Neurohypophyseal Hormones

2.3.1. Effects on Learning and Memory

Whereas the effects of most of the POMC-derived neuropeptides on learning and memory are of short-term nature, the neurohypophyseal hormones vasopressin and oxytocin exert a long-term effect on learn-

Vasopressin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-$GlyNH_2$
Oxytocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-$GlyNH_2$
ACTH	Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-$ValNH_2$
β-endorphin	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu
CRH	Ser-Glu-Glu-Pro-Pro-Ile-Ser-Leu-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Glu-Val-Val-Glu-Met-Ala-Arg-Ala-Glu-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser-Asn-Arg-Lys-Leu-Met-Glu-Ile-$IleNH_2$
CCK-8	Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-$PheNH_2$
VIP	His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-$AsnNH_2$
Galanin	Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-His-Ala-Ile-Asp-Asn-His-Arg-Ser-Phe-Ser-Asp-Lys-His-Gly-Leu-Thr-NH_2
NPY	Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Leu-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-$TyrNH_2$

Fig. 5. Amino acid sequence of various neuropeptides regulating learning and memory.

ing and memory processes (for amino acid sequences *see* Fig. 5). In the passive avoidance paradigm, rats treated with vasopressin show an increased latency to enter the box, suggesting improved memory function. Time gradient studies indicate that vasopressin not only improves retrieval of information but in contrast to ACTH, also storage of information (*see* Section 2.2.2.2. and Fig. 3). Vasopressin has also been shown to prevent and reverse retrograde amnesia induced by various amnestic treatments, which is another measure for effects on retrieval processes. Moreover, vasopressin inhibits extinction of an active avoidance response in the shuttle-box as well as in the pole-jumping paradigm. This inhibition of extinction is also of long-term nature and lasts far beyond the actual presence of the injected material in the body showing that vasopressin strongly improves the maintenance of a learned response. The effect of vasopressin on learning and memory processes in some non-aversive (food rewarded, sexually motivated) tasks is controversial. Social recognition is another model used to study memory processes in rodents (see Section 2.3.1.1.) is also facilitated by vasopressin, i.e., a reduction in investigation time during the second encounter with the conspecific juvenile.

2.3.1.1. Social Recognition Test. Social recognition is a procedure to investigate social memory in rats. The test is based on the observation that under laboratory conditions an adult, i.e., sexually mature male rat, will investigate a conspecific juvenile less on repeated exposure, provided that the time interval between encounters is no longer than 30 min. This phenomenon is considered as recognition, since it is absent, if another juvenile rat is presented on the second encounter. If the interexposure interval between first and second encounter with the same juvenile is prolonged and amounts to 2 h, the investigation times displayed by the adult rat are similar.

Oxytocin and related peptides have the opposite effect and impair acquisition and facilitate extinction of an active avoidance response and attenuate passive avoidance behavior. It has been proposed that oxytocin and related neurohormones are amnesic neuropeptides. This hypothesis was substantiated by the fact that oxytocin and various treatments known to induce retrograde amnesia (e.g., electroconvulsive shock therapy, or inhibition of cerebral protein synthesis) cause comparable degrees of retrograde amnesia. Oxytocin might also have anxiolytic properties. Vasopressin and oxytocin are derived from an evolutionary older precursor vasotocin, which has depending on the dose a positive or negative effect on avoidance. The presence of opposite effects in the same neuropeptide systems as mentioned before may have homeostatic value.

Although the CNS is the primary site of action for the memory effect of the neurohypophyseal neuropeptides, peripheral variables might be of importance for this effect as well. In addition to other evidence, the finding that vasopressin and oxytocin may be centrally metabolized into highly selective peptide fragments, which are extremely potent in affecting active and passive avoidance behavior, but are devoid of peripheral endocrine effects on blood pressure, water retention, and glucose metabolism, suggests that the neurohypophyseal hormones influence learning and memory processes by a direct action on the brain. However, peripheral influences cannot be excluded. For example, CNS effects of various other neuropeptides (cholecystokinin (CCK), neuropeptide Y (NPY), etc.) are mediated partly through the vagus.

2.3.2. Distribution and Sites of Action

The neurohypophyseal hormones are widely distributed in the brain (*see* Fig. 6). Synthesis of vasopressin and oxytocin precursors takes place in hypothalamic nuclei in magnocellular and parvocellular neurons. Magnocellular neurons produce vasopressin and oxytocin precursors that are transported to the posterior pituitary and released into the circulation upon osmotic, pressure, and stress stimuli. PVNs project from the hypothalamus to the median eminence

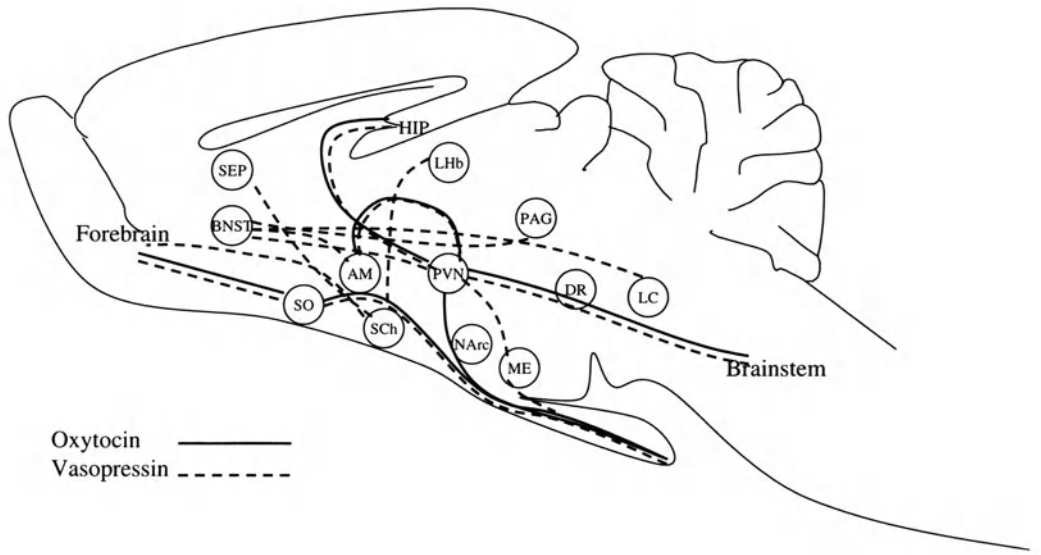

Fig. 6. Main vasopressin and oxytocin pathways in the brain. See for abbreviations Fig. 4.

to regulate corticotroph activity in the anterior pituitary and to the brainstem and spinal cord to modulate autonomic functions. Other vasopressin producing neurons are found in the suprachiasmatic nucleus (SCH), the BNST, and the medial amygdala that project to several limbic structures as the lateral septum and hippocampus, areas involved in learning and memory processes. The central amygdala is also innervated by fibers from the BNST and is rich in vasopressin receptors. The level of expression of vasopressin is gender-dependent and greater in males than females.

2.3.3. Lesion Studies

The results of experiments using rats with lesions in various brain regions in active avoidance behavior paradigms suggest that nearly the same brain areas sensitive to the melanocortins are sites of action for vasopressin. However, the parafascicular area seems less essential for vasopressin than for ACTH, and also lesions in the anterodorsal hippocampus as well as transections through the fornix are less effective in blocking the inhibitory effects of vasopressin than of ACTH on extinction of an active avoidance response.

2.3.4. Microinjections

Marked facilitating effects of the neurohypophyseal hormones are found in passive avoidance behavior. The dorsal septal area and the dorsal raphe nuclei are important for the effect of vasopressin on the storage (posttrial administration), whereas the central nuclei of the amygdala take part in effects on retrieval (preretention administration). The dentate gyrus of the hippocampus and the subiculum seemed to be the anatomical substrate for the effect of vasopressin on both storage and retrieval processes.

Similarly to vasopressin, oxytocin has also been found to exert site-specific effects on learning and memory processes. The hippocampal dentate gyrus, the subiculum, and the midbrain raphe nuclei are involved in the suppressive effect of oxytocin on storage processes, whereas the amygdala might be involved in its inhibitory influence on retrieval.

The effects of vasopressin on learning and memory might be explained by an influence of the neuropeptide on excitation of limbic structures (hippocampus, septum). Neurons in the lateral septum and the ventral hippocampus respond to vasopressin with an increase in single-unit activity in the same way as the excitatory neurotransmitter glutamate increases the activity of these neurons. Thus, a neurotransmitter-like effect of vasopressin in limbic brain structures is likely. However, in the majority of neurons vasopressin potentiates glutamate-induced increase in single unit activity. This points to a modulatory role of vasopressin. This effect is of long term nature. Vasopressin is also involved in long-term potentiation (LTP) (*see* Section 2.3.4.1.). LTP is believed to be an electrophysiological basis of memory processes.

2.3.4.1. LTP. LTP can be defined as a stable and long lasting increase in synaptic transmission after conditioning of a pathway in the brain, for instance by a burst of high-frequency pulses applied to the afferent fibers. In fact, this long-lasting change in

synaptic efficacy is a form of synaptic plasticity that occurs in the excitatory (glutamate) pathways of the hippocampus and in other pathways within the CNS.

Vasopressin is involved in the maintenance of LTP in lateral septum slices; LTP could not be maintained in the presence of a vasopressin (V1) receptor antagonist. The same occurred in Brattleboro rats, which lack vasopressin due to a genetic failure, and could be restored by vasopressin. The amount of LTP after high-frequency stimulation in lateral septum neurons was highest in rats with good performance in a shuttle box avoidance task. Furthermore, a long-lasting enhancement of synaptic excitability of lateral septum neurons was found in good performers, whereas rats with low performance showed a long-lasting depression of these neurons. The hippocampus is a highly sensitive locus of action for the effects of vasopressin on learning and memory effects. A pronounced effect of vasopressin and one of its behavioral active fragments (4–8) was found on eliciting a long-lasting enhancement of the excitability of particular neurons of the ventral hippocampus. Taken all these electrophysiological findings together, it looks as if vasopressin as well as the behavioral more selective fragment AVP-(4-8) increase transmission plasticity in those brain structures involved in learning and memory processes.

Vasopressin and oxytocin also exert regional effects on the metabolism and turnover of neurotransmitters. An intact coeruleo-telencephalic noradrenergic bundle is critical for the memory effect of vasopressin. Destruction of this system interferes with vasopressin effects on passive avoidance behavior only when vasopressin is injected immediately after the learning trial, but not when injected prior to the retention test. This suggest that the coeruleo-telencephalic noradrenergic system is needed for memory storage. Vasopressin also affects dopamine activity. The dopamine system projects to the amygdala and the nucleus accumbens. These structures are involved in retrieval processes.

Various approaches have been used to establish the physiological role of endogenous neuropeptides in learning and memory, such as the vasopressin deficient Brattleboro rat, local administration of specific antagonists into a restricted brain site followed by studying behavior. Another technique consists of the administration of specific antisera, in this way binding and neutralizing the endogenous neuropeptide, and the observation of the behavioral effect of this treatment. Also, antisense targeting is an appropriate technique which uses antisense oligonucleotides to inhibit the synthesis of a given neuropeptide or receptor protein. Finally, local release of neuropeptides can be measured by the push–pull technique, which uses a special cannula by which material can be injected and/or collected into/from a restricted brain area. The results of these studies show that endogenous vasopressin, present in the dorsal and ventral hippocampus and in the dorsal raphe nucleus, plays a role in the improvement of processes involved in the storage of information as well as in the retrieval of stored information. Moreover, endogenous vasopressin in the dorsolateral septum and in the habenular nucleus is involved in retrieval only. In contrast, endogenous oxytocin present in a limbic structure as the ventral hippocampus has been shown to play a role in both storage and retrieval processes. Endogenous vasopressin in the dorsal and ventral hippocampus as well as in the septum also plays a crucial role in social recognition. As far as endogenous oxytocin is concerned, only its presence in the ventral hippocampus is important for social memory. That vasopressin, in more brain areas than oxytocin, is involved in learning and memory processes and may be related to the finding that both cell bodies, as well as terminal fields of the vasopressin system in the brain, are more widely extended than those of the oxytocin system.

2.3.5. Receptors

As in the periphery, various receptors have been found in the brain. They belong to the g-protein-coupled receptor family, to which the receptors for the classical neurotransmitters belong as well. V1a, V1b, and V2 vasopressin receptors as well as receptors for oxytocin have been described. The V1a and V1b vasopressin receptor are recently also classified as V1 and V3 receptor. The cardiovascular and renal effects of vasopressin are reasonably well characterized and are mediated by the V1a (V1) receptor and the V2 receptor, respectively. Also, the stimulatory role of vasopressin on ACTH-release from the anterior pituitary by its binding to a unique pituitary vasopressin (V1b or V3) receptor, which is distinct from the V1 and V2 receptor, is reasonably well documented. However, further documentation is needed regarding a receptor for the CNS effects of vasopressin, where it acts as neurotransmitter or modulator in the regulation of functions as blood pressure, body temperature, and learning and memory. Subtypes of the oxytocin receptor have not been found yet. This receptor is not very specific; in the uterus, it has the same affinity for vasopressin and oxytocin. This may

have to do with the common evolutionary origin of the two neurohormones. The genes for both neurohypophysical hormones are present in the same chromosome. High-affinity binding sites for oxytocin are present in the olfactory system, basal ganglia, some parts of the limbic system, cortical areas, thalamus, hypothalamus, brain stem, and spinal cord. These sites are also recognized by vasopressin. V1b (V3) receptors that were originally found in the pituitary have been found in multiple brain regions. In the hippocampus, both oxytocin and V1a (V1) receptors have been found. The receptors for the memory effects of neurohypophyseal hormones, however, are not known at present. It appeared that the influence of the neurohypophyseal hormones and related peptides on passive avoidance behavior can be blocked by specific V1, V2, and oxytocin-receptor antagonists. The behavioral (amnesic) effect of oxytocin is also blocked by the oxytocin antagonist and the V1 antagonist, whereas the V2 antagonist was less effective. In view of this a neurohypophyseal hormone receptor complex was postulated, which might be present in the ventral hippocampus, on which vasopressin and related peptides act as agonists and oxytocin and related peptides as 'inverse' agonists.

Vasopressin and oxytocin are converted in the brain to metabolites of which the C-terminal 4–9 sequence is the main product. These fragments are more selective (without peripheral endocrine effects) and much more potent on avoidance behavior than the parent molecules. These vasopressin metabolites do not possess the peripheral endocrine effects of the precursor molecule vasopressin and oxytocin and have no CNS effects other than those involved in learning and memory. Moreover, the effects of these fragments on passive avoidance behavior are also blocked by the antagonists that block the effects of vasopressin and oxytocin themselves. However, the fragments do not display affinity for vasopressin- and oxytocin-receptor sites in the brain, and other receptors in the brain have to be found. Presently, attempts are being made to clone this receptor, but so far no biochemical evidence for its existence in the brain has been found.

2.4. Releasing, Release-Inhibiting Hormones and Gastro-Intestinal Neuropeptides

Multiple neuropeptides, in addition to POMC products and neurohypophyseal hormones, modulate learning and memory processes and induce various behavioral effects. Among those (corticotropin-releasing hormone (CRH), somatostatin, cholecystokinin (CCK), neurotensin, neuropeptide Y (NPY), bombesin, vasoactive intestinal peptide (VIP), and galanin) only some have been studied more extensively and will be discussed in this chapter (for their amino acid sequences *see* Fig. 5).

2.4.1. CRH

CRH as the link between the adrenal cortex and the adrenal medulla, plays a pivotal role in stress and in learning and memory processes. CRH is a 41-amino acid peptide that is found in the parvocellular neurons of the PVN. Its axons terminate in the median eminence to release CRH into the portal vessel system. Half of these neurons also contain vasopressin and many express additional neuropeptides as angiotensin (AngII), CCK, enkephalin, and VIP. Some of these are also colocalized with oxytocin. These project to the posterior pituitary. A terminal field of vasopressin and CRH containing fibers from the PVN also reaches nuclei in the brain stem involved in the regulation of the autonomic nervous system. Extrahypothalamic sites of CRH within the CNS project to the limbic system, in particular the amygdala and to the olfactory bulb, certain thalamic nuclei, the nucleus tractus solitarius (NTS), the periaqueductal gray, cortex, cerebellum, the spinal cord, and to sympathetic and parasympathetic nuclei (*see* Fig. 7). The PVN has been implicated as a center of integration for the stress response either through visceral stimulation from the NTS, or through blood brain factors, influences from limbic regions and regulatory effects from the hypothalamus and the preoptic area. In this way the PVN integrates neuroendocrine, autonomic, and behavioral activities and relays them to the hypothalamo-pituitary-adrenal (HPA) axis.

Stress results in a rapid response of early genes in the PVN followed by activation of CRH, vasopressin, enkephalins, and other mRNA transcripts. A single exposure to stress can increase the vulnerability to subsequent stressors. Stress-induced sensitization is reflected by behavioral inhibition and exaggerated ACTH and corticosteroid responses. Interestingly, this is associated with an increase in expression and production of vasopressin in a vasopressin-containing subset of CRH neurons in the external zone of the median eminence.

Because CRH is the most important physiological stimulus for the release of POMC-derived peptides from the corticotrophs in the adenohypophysis, many other neuropeptides and neurotransmitters may act synergistically with CRH. These include vasopressin, angiotensin-II (AngII), VIP, and the catecholamines,

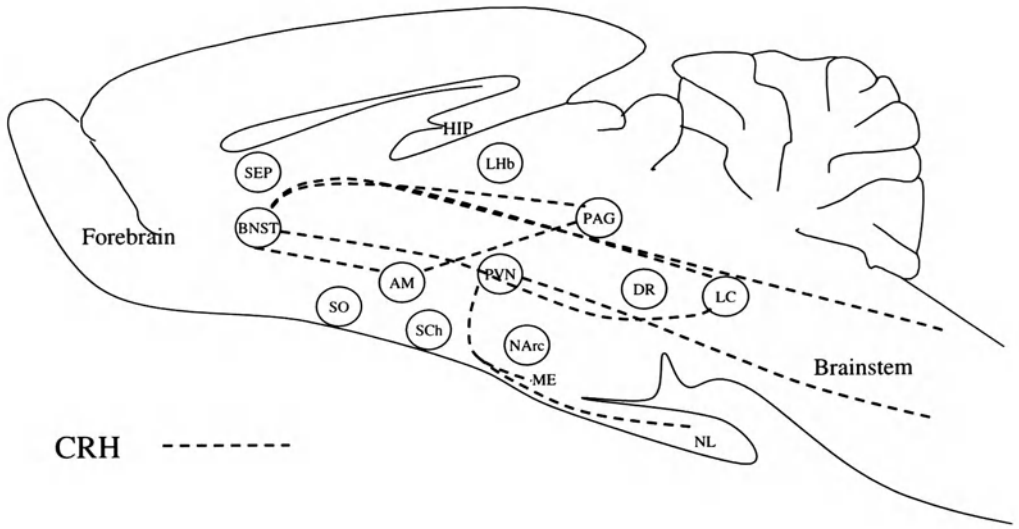

Fig. 7. Main CRH pathways in the brain. See Fig. 4 for abbreviations.

norepinephrine (NE), and epinephrine (E). Indirectly, the release of CRH is modulated by multiple factors through the PVN. These peptides and the various releasing- and release-inhibiting hormones have effects on the CNS, which may be independent of their influence on the pituitary. Central administration of CRH, for example, results in a variety of stress-related phenomena, such as increases in blood pressure, heart rate, blood glucose, an increase in locomotion and grooming and plasma levels of catecholamines, which is mediated by CRH-containing fibers that innervate the nuclei of the autonomic nervous system in the medulla oblongata and the locus coeruleus in the brainstem to activate the cortex. Moreover, CRH also affects learned behavior; it facilitates the extinction of pole-jumping avoidance behavior and attenuates or facilitates passive avoidance behavior, depending on the dose and route of administration (central or peripheral). This "dual action" is difficult to interpret, but could have to do with the activation of the autonomic nervous system, which may result from the intracranial administration of CRH and/or the release of ACTH upon peripheral injection of CRH.

The hypothesis has been put forward that CRH primarily exerts anxiogenic effects and has arousing properties. Anxiety has profound effects on learning and memory processes and induces bell-shaped (or inverse U-shaped) dose-dependent effects on behavioral performance; moderate to high level of arousal facilitates performance, whereas extremely intensive arousal inhibits performance. The bell-shaped relationship between anxiety and performance and the bell-shaped dose-response curve of CRH on avoidance behavior may be explained by the anxiety-inducing effect of this neuropeptide; three receptors have been found: CRH, $CRH_{2\alpha}$, and $CRH_{2\beta}$. The stimulation of the HPA axis is mainly mediated by type 1 CRH receptors. This receptor also mediates anxiety and depression. CRH_1 receptor is present in discrete regions of the CNS, including pituitary and cortical structures. CRH_2 receptor has two isoforms. The expression of $CRH_{2\alpha}$ is more confined to subcortical structures, whereas the $CRH_{2\beta}$ receptor is localized in nonneural elements. The type 2 CRH receptors are widespread distributed in subcortical areas which project to autonomic brain stem areas and therefore they may be involved in autonomic regulation. They also seem to mediate CRH-induced feeding behavior.

The locus coeruleus is the anatomical substrate for the effects of CRH on learning and memory. This brainstem nucleus gives rise to ascending noradrenergic neurons of the coeruleo-telencephalic tract to the cortex and has been implicated in attention and behavioral arousal. It has been suggested that the dorsal noradrenergic pathway is involved in the effects of CRH on behaviors related to learning and memory. Because the locus coeruleus is regarded as an anatomical substrate for anxiety, CRH may indeed enhance memory processes through its anxiogenic actions.

Only scattered and isolated data are available from studies on the physiological involvement of various other neuropeptides in the brain in learning and memory function. However, the presence in the brain of various hormones, which were found originally in the gastro-intestinal tract, initiated extensive research on the behavioral effects of these hormones; in particular,

CCK in the cingulate cortex and the amygdala and VIP, and galanin in the hippocampus are probably of physiological importance in learning and memory.

2.4.2. Cholecystokinin (CCK)

CCK was originally isolated from porcine intestine as CCK-33. The C-terminal pentapeptide of CCK-33 and gastrine are identical. In the CNS, CCK is mainly found as the octapeptide CCK-8. Pathways of CCK-8 have been demonstrated in the cerebral cortex as well as in subcortical structures in particular limbic areas, thalamus, amygdala, and raphe nuclei. Receptors are classified as CCK-A receptors, mainly present in the alimentary tract, and CCK-B receptors, which are predominantly found in the brain. Effects of agonists and antagonists in various behavioral paradigms, such as a one-trial passive avoidance test, acquisition of shuttle-box avoidance behavior, extinction of bench-jumping avoidance behavior, and food-motivated conditioned approach behavior and spatial memory tests, suggest that these peptides facilitate learning and memory processes.

2.4.3. Vasoactive Intestinal Peptide (VIP)

VIP is a 28 amino acid neuropeptide that is widely distributed throughout the central and peripheral nervous system. The cerebral cortex and hypothalamus contain significant amounts of VIP. It is colocalized with several other neuropeptides and neurotransmitters. It causes amnesia in mice in a shock avoidance task in a T-maze, facilitates extinction of a pole-jumping avoidance response in rats and attenuates passive avoidance behavior, when administered before the retrieval test. Although scattered data on VIP effects on behavior related to learning and memory processes are not always in agreement, it has been suggested that VIP might play a role in memory modulation or in some behavioral function that is important for optimal performance in tests associated with retrieval. Interestingly, the inhibitory effects of VIP on conditioned avoidance behavior are similar to or comparable with the ones observed after administration of corticosteroids (*vide infra*) and it has been suggested that VIP controls neural mechanisms underlying behavior that are also affected by these steroids.

2.4.4. Galanin

Galanin a 29 amino acid neuropeptide, affects diverse processes throughout the nervous systems and induces various behavioral effects. It is densely concentrated in the hypothalamus, where it is colocalized with GnRh. In the cortex, where it is colocalized with acetylcholine, it inhibits cholinergic transmission. Furthermore, it also inhibits the release of acetylcholine in the hippocampus and it decreases hippocampal theta activity and septal neural activity. This probably explains its negative effect on learning and memory processes. It impairs choice accuracy in learning and memory paradigms in rats as well as retention of a one-trial discriminative reward learning task when administered after the learning trial and not when administered before the retention trial. Thus, galanin may interfere with storage processes rather than retrieval.

Galanin is an inhibitory neuropeptide on vasopressin and ACTH secretion. Galanin pathways exist that run through the medial preoptic area and the PVN and project to the median eminence.

2.4.5. Neuropeptide Y (NPY)

NPY is an amidated 36 amino acid peptide with a wide distribution in the central and peripheral nervous system. The main effect of NPY is on food intake. It is synthetized in neurons of the arcuate nucleus and secreted from terminals originating from this nucleus into the PVN and other brain regions.

With respect to learning and memory the potential influence of NPY on memory and cognition is of particular interest. Time-gradient studies in mice reveal that NPY in relatively high doses improves retention of a T-maze active avoidance response without affecting acquisition of the response. This enhanced retention therefore probably reflects enhanced retrieval of previously stored information. In addition, NPY was found to reverse retrograde amnesia induced by various drugs. NPY produces its effcts on memory at both the amygdala and hippocampus.

The effect of NPY on food intake is initiated by a postsynaptic (Y1) receptor, whereas the influence on learning and memory is mediated by a presynaptic (Y2) receptor. This latter receptor is sensitive to the whole 36 amino acid neuropeptide as well as to the fragment NPY (20–36) which is localized in the hippocampus.

In summary, the available evidence on brain sites involved in the neuroendocrine regulation of learning and memory shows that some neuropeptides exert highly specific local effects in the brain. However, this selective involvement of brain structures in the effects of neuropeptides on learning and memory processes cannot be viewed independently of other aspects of selectivity such as behavioral and neurochemical ones. Cortical and limbic structures are primarily involved in mechanisms of information pro-

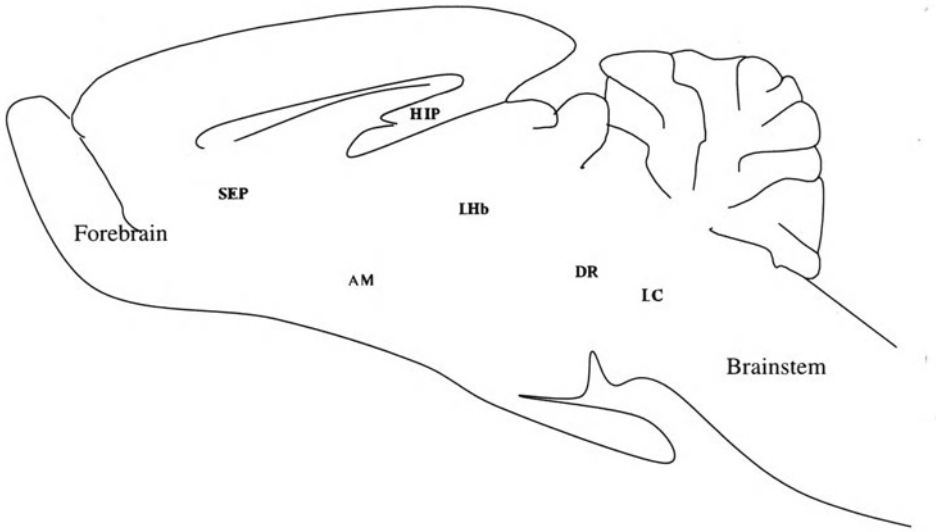

Fig. 8. General overview of brain areas that are important for the effects of various neuropeptides on learning and memory. See Fig. 4 for abbreviations.

cessing, whereas the ascending reticular activating system mainly triggers arousal and activity. The sensitivity of the latter is modulated by neuropeptides and steroids (discussed later). It is interesting to notice that the ventral and dorsal hippocampus, the raphe nucleus, septum, and amygdala are sensitive areas for the effects of vasopressin on learning and/or memory processes. Local sensitivity exists for other neuropeptides as well: ACTH (septum, hippocampus, amygdala, posterior thalamus), oxytocin (septum, hippocampus, raphe nuclei), CRH (locus coeruleus), CCK (cingulate cortex, amygdala), VIP (hippocampus), galanin (hippocampus, septum), and NPY (amygdala, hippocampus, *see* Fig. 8). In most of these cases, receptors are present for the respective neuropeptides, and it is tempting to assume that the neuropeptide-receptor interaction takes place in these brain regions and that these are involved in learning and memory processes. In this way a neuropeptide in a certain brain area may more or less selectively affect a particular aspect of these processes.

3. STEROID HORMONES

3.1. Adrenocorticosteroids

3.1.1. Effects on Learning and Memory

Various corticosteroids attenuate acquisition and retention of passive avoidance behavior and conditioned taste aversion. Corticosterone (the physiologically most important corticosteroid hormone in rats) and related steroids facilitate the extinction of a conditioned avoidance response. This effect may be related to the negative feedback action of corticosterone mediated by glucocorticoid receptors on ACTH release from the pituitary, because ACTH has been shown to exert an opposite effect, i.e., it delays extinction of conditioned avoidance behavior. However, the observation that corticosterone also facilitates extinction in hypophysectomized rats indicates that this effect of the steroid is partly independent of its effects on the release of ACTH. Nevertheless, extinction of shuttlebox avoidance behavior is attenuated in adrenalectomized rats. This may be caused by an increased release of ACTH. Implantation of cortisol in the median eminence region of the hypothalamus, which inhibits ACTH release, indeed facilitates extinction of shuttle-box avoidance behavior. The more the release of ACTH is suppressed, the stronger the behavioral effect. However, cortisol implantation in the mesencephalic reticular formation, which hardly reduces ACTH release, also facilitates extinction of the avoidance response as well. Structure-activity relationship studies indicate that glucocorticoid rather than mineralocorticoid activity is responsible for the facilitatory effect on extinction of a conditioned avoidance response. This behavioral effect of the steroids is not directly related with their glucocorticoid activity, being that progesterone and pregnenolone are as potent in facilitating extinction of the avoidance response as corticosterone. This also argues against an effect mediated by the glucocorticoid receptor because progestagens do not effectively block the release of ACTH. It is, therefore, not clear which mechanism is involved in the negative influence of

steroids on aversively motivated behavior. Most probably these effects are not mediated by an intracellular steroid receptor, but rather by a site in the GABA-A/benzodiazepine/barbiturate-receptor complex in the brain, which is sensitive to these steroids. Corticosteroids have been reported to enhance GABA turnover in the hypothalamus. Corticosteroids have an effect opposite to that of ACTH/MSH peptides on mean and peak frequencies of hippocampal theta activity following stimulation of the reticular mesencephalic formation. It suggests that corticosteroids decrease selective arousal and thereby facilitate the elimination of behavior, which is no longer relevant. It is not known whether progesterone and pregnenolone have a similar effect. Two corticosteroid receptors (glucocorticoid (GR) and mineralocorticoid (MR), as discussed later) participate in the expression of behavior, although their exact role is as yet not clear. Under certain conditions the MRs are important. This can be shown when forced extinction is used as a measure of behavior. In this test a rat, which is put into a place, where it previously encountered an aversive experience, responds with immobility. This response is absent in adrenalectomized rats, and can be restored by corticosterone but not by dexamethasone, which has high affinity for the GR (*see* Section 3.1.2.). The GR is involved in spatial memory. GR-antagonists cause a disturbance in this type of behaviour.

3.1.2. Receptors

In the brain, corticosterone actions are mediated by glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs). The GRs have a widespread distribution and are present in both the pituitary and the brain, in so-called "stress-centers," which are brain regions involved in the organization of the stress response. These receptors show affinity for corticosterone, but have higher affinity for potent synthetic glucocorticosteroids as dexamethasone. They are saturated only at stress levels of circulating corticosterone and are involved in the feedback action of corticosteroids by terminating the release of elevated levels of ACTH. They are found in CRH/AVP containing neurons in the hypothalamus (PVN), as well as in the hippocampus, the septum, and the brain stem ascending catecholaminergic neurons. MRs have high affinity for corticosterone and are abundantly expressed in behaviorally important limbic brain areas as hippocampus, septum, and amygdala. They are relevant for the sensitivity of the neuroendocrine stress response. They control the basal activity of the pituitary-adrenal system through a tonic inhibitory hypothalamic control of the pituitary through the hippocampus. Second, they are involved in the organization of circadian driven daily fluctuations in HPA activity.

3.2. Gonadal Steroids

Pregnenolone, ethylestrenol, a complex synthetic anabolic steroid sharing certain structural characteristics with testosterone, estradiol and pregnenalone, improve passive avoidance behavior. Also pregnenolone and dehydroepi-androsterone and their sulfates—neurosteroids synthesized in the nervous system—as well as testosterone, enhance retention of an active avoidance task in a T-maze. Moreover, the concentration of pregnenalone sulfate decreases with age in the rat hippocampus and this coincides with memory deficit in aged rats. Apparently, sex steroids and their precursors/metabolites exert memory enhancing effects.

Also it is worthwhile to mention that some gonadal steroid hormones have transcriptional effects on vasopressin gene expression and on its receptors in the brain. This may be related to behavioral differences, including learning and memory, between the sexes as has been observed in rats. The development of the vasopressin system is delayed in female rats as compared to males. Circulating gonadal steroids are a major determinant of the level of expression of the vasopressin gene in the BNST and the medial amygdala neurons and cause sexual dimorphism of this system in the adult rat brain. Although it is not clear yet whether testosterone, its metabolites, estrogen, and/or 5α-dihydrotestosterone, are responsible for this action, testosterone is regarded as the main candidate.

Some evidence exists that oestrogens may improve memory processes and epidemiologic surveys report that the incidence of Alzheimer is less in postmenopausal women on hormone replacement therapy (HRT).

4. CATECHOLAMINES

Catecholamines are released from the adrenal medulla, sympathetic nerve endings and in the CNS during stress. This prepares the body for flight or fight responses. The limbic system, in particular the amygdala and hippocampus, are activated by catecholamines. Mild stress increases NE release in the amygdala and facilitates the consolidation of aversive events, which enables the organism to avoid such stimuli in the future. Also, exogenous administration of catecholamines affects learning and/or memory. In

studies using a passive avoidance task, the E effect on memory exhibits an inverted U-dose response curve; low and moderate doses enhance, whereas high doses impair retention of the response. This U-shaped phenomenon may be caused by the rate of activity of the system in the structures (amygdala, prefrontal cortex) involved in learning and memory processes. Excessive amounts of catecholamines released into the prefrontal cortex blocks information processing. E enhances the development of LTP (see paragraph on neurohypophyseal hormones). This effect also follows the inverted U-shape dose response curve, with maximal facilitation at doses comparable to those effective in behavioral studies. E enhances memory in various aversively and appetitively motivated single and multiple learning paradigms.

Central, as well as peripheral, adrenergic influences are involved in the effects on memory. Through these influences, the adrenomedullary hormones maintain optimal activity of the limbic system structures, i.e., keep these structures in a state to respond to neuropeptides and other neuromessengers. Moreover, vasopressin requires the presence of E to improve learning and memory processes. On the other hand, studies using vasopressin deficient Brattleboro rats showed that the E effects on memory require the presence of vasopressin. Also, the effects of ACTH (fragments) and opioid hormones on memory involve interactions with central catecholaminergic (and cholinergic) systems, E seems essential for ACTH-related memory retrieval mechanisms and opioid hormones may impair memory by inhibiting the central release of neurotransmitters. Acquisition of new behavioral patterns requires the adrenomedullary hormones, such as E and NE. Removal of the adrenal gland or the medulla only shortly after training, blocks passive avoidance behavior. This can be restored by the administration of adequate amounts of E before the retention test. This is a state-dependent effect in which the absence of a particular factor during acquisition interferes with the retrieval of information. Thus, catecholamines are essential for the storage and retrieval of information, whereas the presence of corticosteroids is not essential because administration of this steroid is without effect. In rats, which have been deprived of corticosteroids by adrenalectomy and therefore have a low occupation of the type 1 (MR) receptor, the sensitivity for E is much greater than when the receptor is fully occupied as a result of substitution with corticosteriods.

In summary, catecholamines released during stress are also essential for learning and memory processes. They activate limbic structures, which is a condition for the effects of neuropeptides such as vasopressin and ACTH on learning and memory.

5. LEARNING AND MEMORY AND STRESS

Learning and memory processes are modulated by multiple factors such as neurotransmitters, neuropeptides, and steroids. In particular, the catecholamines, the melanocortins, the neurohypophysial hormones, CRH, a few other brain hormones, and the adrenal steroids seem to be the major players in these processes. Limbic midbrain structures that are involved in learning and memory processes are densely innervated by fibers containing catecholamines, acetylcholine, melanocortins, and neurohypophyseal hormones and contain receptors for these principles and for corticosteroids. Also, this suggests that higher forms of adaptation as learning and memory are under the control of the same factors as those which defend the organism against noxious stimuli. These principles are needed to restore homeostasis and to facilitate adaptation to changes in the living conditions. Learning and memory processes are higher forms of adaptation and enable the organism to cope adequately with environmental demands. Acquisition of new skills or knowledge are continuously added to the repertoire of the organism that enables it to survive in case of living in the wild or to be more or less successful in every day life. This is achieved by learning to avoid danger, predators, aversive situations and pain, to look for food and water, for mates, to feel well, to establish and maintain good social contacts, and to seek other rewards.

The melanocortins increase the activity of the ascending reticular system, thereby augmenting arousal, attention, and concentration that facilitate the acquisition of new information. CRH and under conditions of stress also vasopressin, activate the pituitary-adrenal system. In addition, they stimulate the sympathetic nervous system through the autonomic centers in the lower brainstem, which by virtue of the influence of catecholamines, also increase the vigilance of the organism. The neurohypophyseal hormones and related neuropeptides modulate the storage and retrieval of information by acting on limbic midbrain structures. The corticosteroids may have a permissive action in physiological amounts and in supraphysiological amounts (during stress). They decrease the activity of the ascending reticular system which attenuates performance of a learned response.

From the animal studies reviewed above it is clear

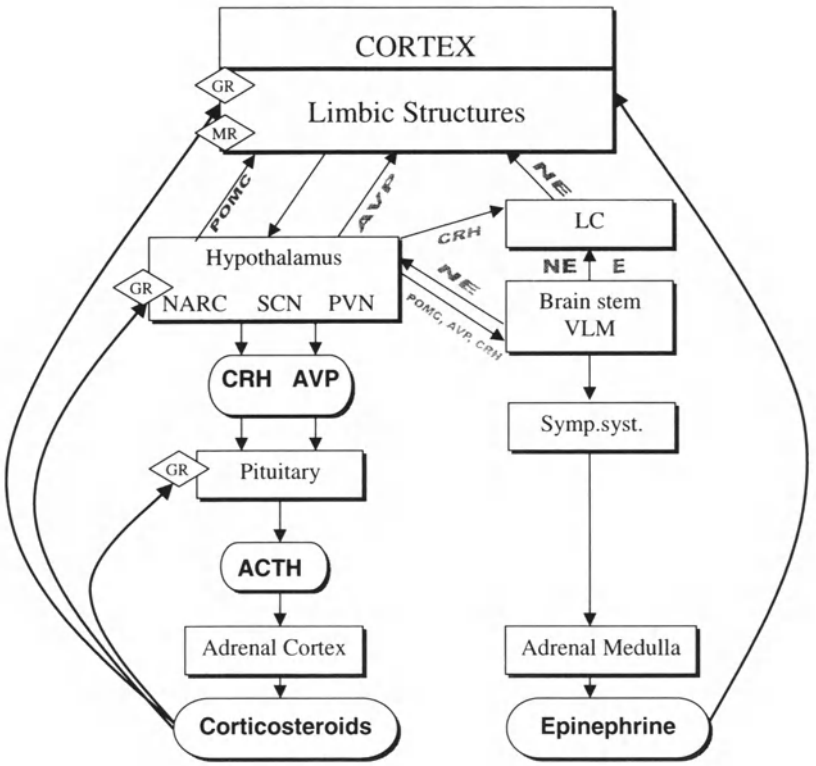

Fig. 9. Schematic overview of structure/organs and their respective products involved in the stress response and in behavioral adaptation.

AVP	vasopressin	MR	mineralocorticoid receptor	PVN	paraventricular nucleus
CRH	corticotropin releasing hormone	NE	norepinephrine	SCN	nucleus suprachiasmaticus
GR	glucocorticoid receptor	NARC	nucleus arcuatus	Symp syst	sympathetic nervous system
E	epinephrine	POMC	proopiomelanocortin	VLM	ventral lateral medulla
LC	locus coeruleus				

The primary hormones involved in response to stress are CRH, AVP, ACTH, and the hormones of the adrenal cortex and medulla. The parvocellular neurons of the PVN produce CRH, and some of these also AVP, which potentiates the action of CRH on the corticotrophs of the adenohypophysis to release ACTH, which in turn, activates the adrenal cortex to produce corticosteroids. Additional neuropeptides are involved. CRH neurons also project to the sympathetic nuclei in the brainstem to activate the adrenal medulla to release epinephrine and to the LC to activate NE pathways. Corticosteroids exert their effect in the brain through two types of receptors: the mineralocorticoid receptor (MR), which has high affinity for corticosterone and the glucocorticoid receptor (GR) with a lower affinity for corticosterone but a higher affinity for synthetic glucocorticosteroids. MRs are expressed abundantly in limbic structures whereas GRs, also expressed in these structures, are widely distributed in the brain in neurons and glia, in particular, in the PVN. These are involved in the feedback of stress-induced activation of the HPA axis by blocking the release of CRH and AVP from the PVN and ACTH from the adenohypophysis. MRs control basal activity and circadian-driven activation of the HPA-axis and determine its sensitivity to stress. Epinephrine (E) from the adrenal medulla and central norepinephrine (NE) pathways activate bodily functions and vigilance needed to prepare the organism in its defense to stress to adapt to environmental changes. Learning and memory processes are modulated by the same hormones as the body uses to mobilize the organism's defense against stress. Vasopressin and oxytocin modulate memory consolidation and retrieval. These effects are exerted mainly on limbic structures. The melanocortins (MCs), and probably also other neuropeptides, affect learning by modulating attention, concentration, motivation, and vigilance through activation of the ascending reticular formation (selective arousal). CRH activates the sympathetic nervous system, peripherally and centrally, to release E and NE, which affect vigilance and that play a permissive role in the memory effect of vasopressin. Conversely, vasopressin is needed for the memory effect of E. The corticosteroids, through MRs, participate in limbic structures in the expression of behavior. The GRs that are also found in limbic structures, but in particular, in the PVN and which are involved in the feedback action of corticosteroids, attenuate behavior that is no longer relevant (facilitation of extinction of aversively motivated behavior). This effect may be mediated by the GABA A/benzodiazepine/barbiturate receptor complex through a decrease in selective arousal. Learning and memory processes are part of the stress response to guide the organism's coping in its response to environmental changes. Thus, they can be regarded as the highest form of adaptation. It is, therefore, not surprising that the factors involved in stress and cognitive behavior are more or less the same.

that learning and memory is associated with stress and emotion. In Fig. 8, a schematic overview is presented with brain structures and neuropeptides, steroid hormones and catecholamines involved in learning and memory as well as in the stress response. From the animal studies reviewed earlier, it is clear that learning and memory is associated with stress and emotion. Acquisition of new behavior, whether aversively motivated or positively reinforced, elicits a marked increase in pituitary-adrenal activity, secreting the same hormones that are involved in the response of the organism to stress. There is ample evidence for this from studies on pituitary-adrenal activity during acquisition of active and passive avoidance behavior. Other paradigms as separation distress, which occurs when a pup is separated from the mother, the withholding of a reward (food, water), the exposure to an intruder, and even novelty also elicit an increase in pituitary-adrenal activity.

6. CONCLUDING REMARKS

Despite intensive experimentation and theorizing, the discussion on the role of neuropeptides in learning and memory processes makes no claim to completeness. One of the conclusions is that the mammalian brain does not possess a single neuropeptidergic mechanism that could account for the modulation of learning or memory processes. On the contrary, a symphony of neuropeptides of different chemical nature, localization, and origin seem to act in concert with each other and with classical transmitters, and in some instances, one of them may become more effective or even more specific in a particular behavioral situation. What makes the role of some of these neuropeptides very attractive is that they may contribute to changes in plasticity and thus in the connectivity of neurons whose relationships are reconstructed during learning and memory formation. Learning and memory in a broad sense, are involved in drug and alcohol addiction and development of tolerance (recognition/remembering the drug/alcohol), muscle hypertrophy as a result of exercise (learning/training), or antigen-antibody reactions (memory for antigens). Although it is far too early to determine their exact roles, there exists abundant evidence that neuropeptides modulate learning and memory processes at the behavioral, the cellular and the synaptic level. The belief that the neurosciences, in general, and neuropeptide mechanisms in particular, are important for an understanding of some of the most fascinating and important of all human psychological functions provides a strong motivation for all of those who devote their research to the neuroendocrinology of learning and memory.

SELECTED READINGS

Bohus B. Humoral modulation of learning and memory processes: physiological significance of brain- and peripheral mechanisms. In: Delacour J, ed. Advanced Series in Neuroscience, Vol. 4, The Memory System of the Brain. Singapore: World Scientific, 1994; 337–364.

Dantzer R, Bluthé R. Vasopressin and behavior: from memory to olfaction. Regulatory Peptides 1994; 45:121–125.

De Kloet ER, Vreugdenhil E, Oitzl ME, Joels M. Brain corticosteroid receptor balance in health and disease. Endocrinol Rev 1998; 19(3):269–301.

De Wied D. The effects of neurohypophyseal hormones and related peptides on learning and memory processes. In: Frederickson RCA, McGaugh JL, and Felten DF, eds. Peripheral Signaling of the Brain. Role in Neural-Immune Interactions and Learning and Memory. Neuronal Control of Bodily Function: Basic and Clinical Aspects, Vol. 6, Toronto, Ont., Canada, Hogrefe en Huber, 1991; 335–350.

De Wied D. Neuropeptides in learning and memory processes. Behav Brain Res 1997; 83:83–90.

De Wied D, Jolles J. Neuropeptides derived from pro-opiocortin: behavioral, physiological and neurochemical effects. Physiol Rev 1982; 62:976–1059.

De Wied D, Croiset G. Stress modulation of learning and memory processes. In: Jasmin G and Proschek, L, eds. Stress Revisited. 2. Systemic Effects of Stress, Methods Achieve. Exp. Pathol. Vol. 15, Basel: Karger, 1991; 167–199.

Kovács GL, De Wied D. Peptidergic modulation of learning and memory processes. Pharmacol Rev 1994; 46:269–291.

Landgraf R. Commentary: Antisense targeting in behavioural neuroendocrinology. J Endocrinol 1996; 151:333–340.

Landgraf R, Wotjak CT, Neumann ID, Engelmann M. Release of vasopressin within the brain contributes to neuroendocrine and behavioral regulation. Progr Brain Res 1998; in press.

Levine S, Coe C, Wiener SG. Psychoneuroendocrinology of stress: a psychobiological perspective. In: Brush FR, Levine S, eds. Psychoendocrinology, San Diego, CA: Academic, 1989; 341–377.

McGaugh, JL and Gold PE. Hormonal modulation of memory. In: Brush FR, Levine S, eds. Psychoendocrinology, San Diego, CA: Academic, 1989; 305–340.

Morley JE, Flood JF. Neuropeptide Y and memory processing. In: Allen JM, Koenig JI, eds. Central and Peripheral Significance of Neuropeptide Y and Its Related Peptides. Ann NY Acad Sci 611, 1990.

Sapolsky RM. Neuroendocrinology of the stress response. In: Becker JB, Breedlove SM, Crews D, eds. Behavioral Endocrinology, Cambridge, MA, MIT, 1992; 287–324.

Van Wimersma Greidanus, TB and Rigter H. Hormonal regulation of learning performance, In: Brush FR, Levine S, eds. Psychoendocrinology. San Diego, CA: Academic, 1989; 271–304.

Van Wimersma Greidanus TB, Van Ree JM. Behavioral effects of vasopressin. In: Ganten D, Pfaff D, eds. Current Topics In neuroendocrinology. Berlin, Germany: Springer Verlag 1990; 10:61–79.

21 Neuroendocrine Correlates of Aging

Phyllis M. Wise, PHD

CONTENTS

1. INTRODUCTION

1.1. Definitions of Aging

Neuroendocrine systems display dramatic changes with age. A deeper understanding of the factors that regulate the rate and process of aging and the repercussions of aging on these neuroendocrine axes will be important because, if the central nervous system (CNS) is a key driver of neuroendocrine senescence, then studying the neuroendocrinology of aging may allow us to gain a clearer view of the fundamental process of brain aging. We will limit our discussion to the growth hormone, reproductive, and adrenocortical axes because more information is known about the regulation of these axes than others. Comparisons and contrasts among each of these neuroendocrine axes provide insights into the unique aspects of aging of each and commonalities that regulate the aging of all of them.

Suspicion that neuroendocrine systems are involved in the process of aging dates back more than 100 years. Brown-Sequard's legendary reports that he was able to rejuvenate himself using testicular extracts were not supported by later studies and objective interpretation of data. Nevertheless, they stimulated the scientific community to consider the possibility that endocrine interventions may delay the effects of aging. The fundamental importance of the brain and particularly the hypothalamus in integrating environmental and internal neural information to peripheral organ systems crystallized in the 1950s. Since then, much work has focused on the question of whether changes at the level of the hypothalamus and higher neural centers can explain the alterations that occur in the anterior pituitary and target organ function or whether changes in target organs occur first and alter their feedback actions to influence age-related changes in the CNS.

Recent advances in the neuroendocrinology of aging have been possible because of several methodological advances, such as optimization of in vitro methods. These methods include: (1) adult and aging cell culture; (2) patch clamping of single cells, which permits quantitative measurement of single channel activity; (3) molecular biological techniques including *in situ* hybridization, which allows analysis of gene expression in individual cells; (4) tract-tracing methods that enable investigators to follow neuronal pathways for considerable distances; (5) micro-

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dialysis of specific brain regions, which permits monitoring of neurochemical events over time within an individual animal; (6) generation of cell lines from neuroendocrine cells and transgenic mice that overexpress or knock out relevant genes. Using these techniques, we are slowly beginning to decipher the neural events that regulate endocrine changes in the periphery and feedback mechanisms that mediate homeostasis.

1.2. Choice of Animal Models in Aging Research

Numerous animal models have been used to study aging processes. It is important because this allows us to identify potential primary aging events and permits us to assess the generality of a putative candidate that influences the aging process. It seems likely that primary aging processes are similar for all members of a class of animals, such as mammals. Several criteria have been considered when choosing a given animal model for aging research:

1. Life table data are important because they provide information to investigators so that they can know what percent of the population can be expected to survive at any given age and may predict when functional decline may be expected.
2. When possible, species with short life-spans are advantageous because they permits investigators to perform longitudinal studies and follow the entire life-span of individual animals.
3. Fewer variables are likely to confound interpretation of experimental results, when environmental conditions can be defined and known for the entire life-span.
4. Information on age-associated pathologic lesions allows investigators to differentiate between age-related pathologies and normal healthy aging.
5. The genetic characteristics of the species and/or strain should be known and preferably fairly uniform among individuals in the population.

No animal model meets all of these criteria, so neuroendocrinologists have had to compromise based on the experimental questions being asked. Laboratory rodents tend to meet most of these criteria. For this reason, they are the major mammalian order used for aging research. Some studies have used hamsters or guinea pigs, but the laboratory mouse and rat are used most frequently for aging studies. The rat has been preferred for neuroendocrinological studies because of their larger blood volume allows investigators to draw multiple blood samples at frequent intervals. However, with the increasing interests in the genetic basis for age-related changes, the availability of transgenic mice will become an important factor in choosing an animal model.

1.3. Unique Challenges of Aging Research Relative to Neuroendocrinology

There are several issues that gerontologists must face when designing their studies. Each of these will impact upon the interpretation of findings and will influence the design of future studies. We will discuss only three of the particular challenges of aging research: (1) definitions of development compared to aging; (2) advantages and disadvantages of longitudinal vs cross-sectional studies; and (3) confounds presented by less healthy older individuals.

There is no consensus on the definition of aging. Some biologists argue that aging begins at or even before birth. However, most investigators accept the definition that development includes prenatal and postnatal changes involving differentiation of cells and tissues to allow setting down of new tissue and arrangement of organs and organ systems, specialization of functions, growth, and initiation of adult-like function. Aging, on the other hand, is defined as post-maturational changes that make organisms less able to cope with environmental and internal perturbations that lead to decreased ability to survive. Even when biologists accept these definitions of development and aging, it is difficult to determine when development ends and aging begins in an organism or a species because different physiological systems appear to achieve mature "adult"-like characteristics at different times during the life-span. For example, sexual maturity is frequently reached before growth of the skeleton ends; and reproductive senescence in females begins before some other physiological systems reach their peak maturity. Thus, it should be clear that the chronological age of experimental groups that are considered "young," "middle-aged," or "old" must depend upon the system or cells that are under investigation. Even when the community of scientists can agree on what characteristics define maturity, the time when these occur can depend on gender, and can be influenced by environment, nutrition, and other external factors. These factors can affect certain systems more than others. Indeed, they can affect one element of a neuroendocrine axis more than another. Thus, it is impossible to identify a specific time or physiological event that marks the end of development and the beginning of aging in organisms.

When interested in understanding important factors

that regulate a process that often takes months, years, or decades, investigators must make decisions as to whether to design their study in longitudinal manner, in which one measures an experimental endpoint from the same population of individuals at a given interval, or whether to use a cross-sectional approach, in which different individuals are used at each stage of aging.

Clearly, each experimental design has advantages and disadvantages. The major advantage of longitudinal studies is that investigators can detect changes within a single individual, and can assess whether baseline values of an end point affect that individual's ability to respond to a stimulus. Statistical methods of analysis using repeated measures can control for variation from individual to individual. If the investigator measures multiple end points in a longitudinal study, one can be more certain that a change chronologically precedes or follows another in time and can then design studies to assess whether the relationship between these two end points is causative or merely correlational. If an experimental perturbation is used in a longitudinal design, conclusions regarding causality can be more definitive. However, there are some disadvantages of the longitudinal approach. First, one has to decide whether tissue or blood samples will be assayed immediately upon collection or preserved until the study is complete. If samples are assayed immediately and the study encompasses a long period of time, assay methods may change, and interassay coefficients of variation may prevent detection of small changes. If samples are preserved until the end of the study and then assayed together, they may deteriorate during the interval, leading to under or over estimation of end points of the earlier samples relative to the most recently obtained samples. Second, studies that must span many years may be impossible to perform using longitudinal approaches, because it may be impossible for investigators to follow the same procedures for the entire experimental period. Third, unpredictable and random environmental changes may prevent investigators from maintaining or monitoring the interactions of the environment with the end points to be measured. Fourth, subjects may drop out of or may be eliminated from the study for any number of reasons. Researchers must decide whether subjects will be included only if they complete the entire experiment or statistical analysis must take into account incomplete sample sizes.

Cross-sectional studies have the advantages that investigators can gather data from subjects in different age groups within a short period of time. In addition, the experimental conditions at the time of sample collection are clearly less variable than under conditions of longitudinal studies. However, there are disadvantages of a cross-sectional approach. First, usually considerably larger sample sizes are necessary to detect small age-related changes because of interindividual variation. Second, if one is comparing an end point in young, middle-aged, and old subjects, the population that survives at each experimental group may be different. It is important to consider that only the most healthy and most fit survive to the oldest age. Thus, one may not detect age-related differences because the samples are from an inherently different population in each age group. Third, one does not know and cannot control for different past experiences of the experimental subjects. If these past experiences affect the end point to be measured, it can prevent investigators from detecting subtle, but real and significant age-related differences or can lead to erroneous conclusions.

Gerontologists must pay particular attention to the way laboratory animals are maintained or the health status of human populations that are used in aging studies. For example, it is questionable to compare a group of young healthy students with older individuals who are chosen at random from patients in a geriatrics clinic or a nursing home. Likewise, it is important to compare young virgin animals with old virgins (not retired breeders) because exposure to steroids of pregnancy and lactation may influence all neuroendocrine axes. It is critical to be able to differentiate between changes that occur with normal aging from ones that are attributable to age-related pathologies. Many earlier studies were performed using healthy young individuals and older individuals of questionable health status or animals that were maintained in laboratory conditions that were not clean or "pathogen-free." Some of the alterations that were attributed to aging could not be repeated when only healthy individuals were included in studies.

2. COMMON FEATURES OF AGING OF NEUROENDOCRINE SYSTEMS

2.1. Pulsatile Endocrine Rhythms

It is now well established that all anterior pituitary hormones are secreted in a pulsatile manner. The frequency and amplitude of the periodic discharge of these hormones determine the response of target organs. For example, high-frequency GnRH pulses favor release of LH; whereas lower frequency pulses

favor FSH secretion. Chronic secretion of hypothalamic hormones can down-regulate and desensitize secretion of targeted pituitary hormone. In general, the pulsatile pattern of pituitary hormone secretion is thought to be a reflection of the pattern of secretion of the hypothalamic factors that regulate the pituitary. Thus, age-related changes in the patterns of the pituitary hormones are thought to reflect changes in the CNS and not to be caused by inherent changes in the pituitary gland. In a few instances, changes in pituitary responsiveness have been documented. Frequently, however, differences in pituitary responsiveness disappear upon repeated administration of the hypothalamic releasing hormone. This suggests that changes in responsiveness of the pituitary gland may result from long-term lack of stimulation from the hypothalamus, but are not permanent or inherent in the pituitary gland.

It would be ideal to measure directly the effects of age on the pattern of hypothalamic hormone release. However, this is not possible because hypothalamic hormones are in such low concentrations in the peripheral blood that assays are not sensitive enough to be able to detect them. In some cases, hypothalamic hormones have been measured in the hypophyseal portal blood using very invasive methods. However, even in these instances, it is impossible to perform aging studies over several months, which would be required. Therefore, the only alternative is to measure pituitary hormone secretion as an indirect index of hypothalamic hormone secretion with the hope that changes in the frequency of pulses of pituitary hormone levels faithfully reflect the frequency of pulses of the hypothalamic hormone that is being secreted into the hypophyseal portal circulation.

Aging affects the frequency and the amplitude of many of the pituitary hormones examined thus far (Fig. 1). In addition, some investigators have noted that the duration of pulses increases with age, suggesting that the ability of hypothalamic neurons to release hormones in discrete bursts may deteriorate with age. Whether this is because of a gradual lack of synchrony of messages from higher brain centers or whether this is because of an inability of releasing hormone-containing neurons to respond rapidly to afferent signals is unclear. Frequently, the initial age-related changes are so subtle that the average level of hormone is not affected. Thus, our ability to assess multiple blood samples over an interval of time has revealed age-related changes that were not appreciated earlier.

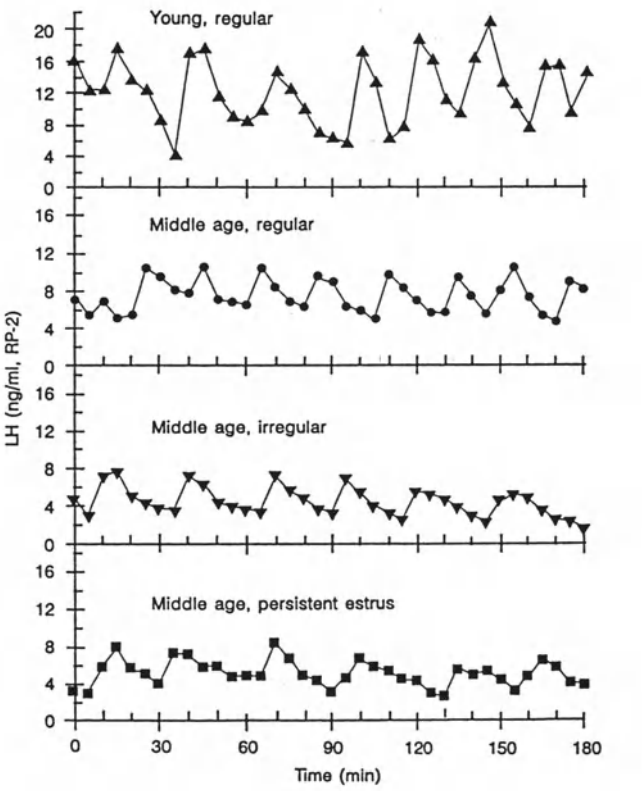

Fig. 1. LH concentrations in representative ovariectomized young and middle-aged rats. Young rats were regularly cycling at the time of ovariectomy. Middle-aged rats were at progressive stages of reproductive aging. The second panel shows the pattern of LH secretion of a rat that was regularly cycling; the third panel shows LH concentrations of a rat that was already irregularly cycling at the time of ovariectomy; the bottom panel displays the pattern of LH secretion of a rat that had completed the transition to persistent estrus. The data show that amplitude and frequency of LH pulses decreases with age and reproductive senescence. In addition, the duration of pulses increases (From Scarbrough K, Wise PM. *Endocrinology* 1990; 126:884–890).

2.2. Circadian and Diurnal Endocrine Rhythms

In addition to being pulsatile, the patterns of secretion of most anterior pituitary hormones exhibit diurnal rhythms, that is, rhythms whose periods are approximately 24 h. In virtually all organisms 24-h rhythmicity has evolved in response to the temporal organization of the environment. When a rhythm is endogenous, that is it persists under constant environmental conditions, it is considered circadian. When a 24-h rhythm requires external cues to be expressed, such as a regular light:dark cycle, it is considered diurnal. Even endogenous circadian rhythms are entrained by environmental cues, such as the

light:dark cycle, food, sleep/wake cycles, etc. The suprachiasmatic nuclei of the hypothalamus are the major circadian neural pacemakers in mammals. This region of the brain, which is sometimes called the "biological clock," contains cells that exhibit inherent circadian rhythmicity in electrical activity, gene expression, glucose metabolism, and secretion of peptides. The suprachiasmatic nuclei receive both photic input from the retina and nonphotic input from other regions of the brain. These inputs allow the suprachiasmatic nuclei to be influenced by the environment. In addition, the suprachiasmatic nuclei drive virtually all circadian outputs through complex efferents that reach many areas of the brain. The hypothalamus is one of the most heavily innervated regions.

Aging influences both the amplitude and period of many circadian rhythms. Some rhythms are more affected than others, leading to a loss of temporal organization in the relationships among the rhythms of some of the pituitary hormones with each other. In addition, both the timing of the peak and nadir of these rhythms are influenced by sleep/wake patterns and by the timing of meals. Even in carefully controlled environmental conditions, the rhythms of several hormones change with age. The maintenance of diurnal rhythms in pituitary hormone secretion is an index of: (1) the ability of the brain, specifically the suprachiasmatic nuclei to maintain endogenous 24-h rhythmicity in its activity; (2) the ability of the suprachiasmatic nuclei to drive outputs that, in turn, regulate hypothalamic releasing hormones; (3) the ability of hypothalamic releasing hormone containing neurons to respond to outputs from the suprachiasmatic; and (4) the ability of stimuli, such as sleep, light, or activity to entrain neuroendocrine rhythms.

Studies have shown that the 24-h profiles of several pituitary hormones changes with age (Fig. 2). The failure to monitor hormonal levels for extended periods of time in some studies is probably the cause of contradictory findings regarding basal neuroendocrine function during aging. With frequent sampling throughout the 24-h cycle, it has been shown unequivocally that the rhythm of virtually all pituitary hormones changes with age. Whereas circadian rhythms persist, normal aging is associated with earlier timing and blunted amplitude of circadian neuroendocrine hormone release. Because so many hormones in the neuroendocrine axis are altered, it is possible that alterations in the central circadian pacemaker underlie these changes. This concept is also supported by the fact that, in aging humans, the distribution of REM

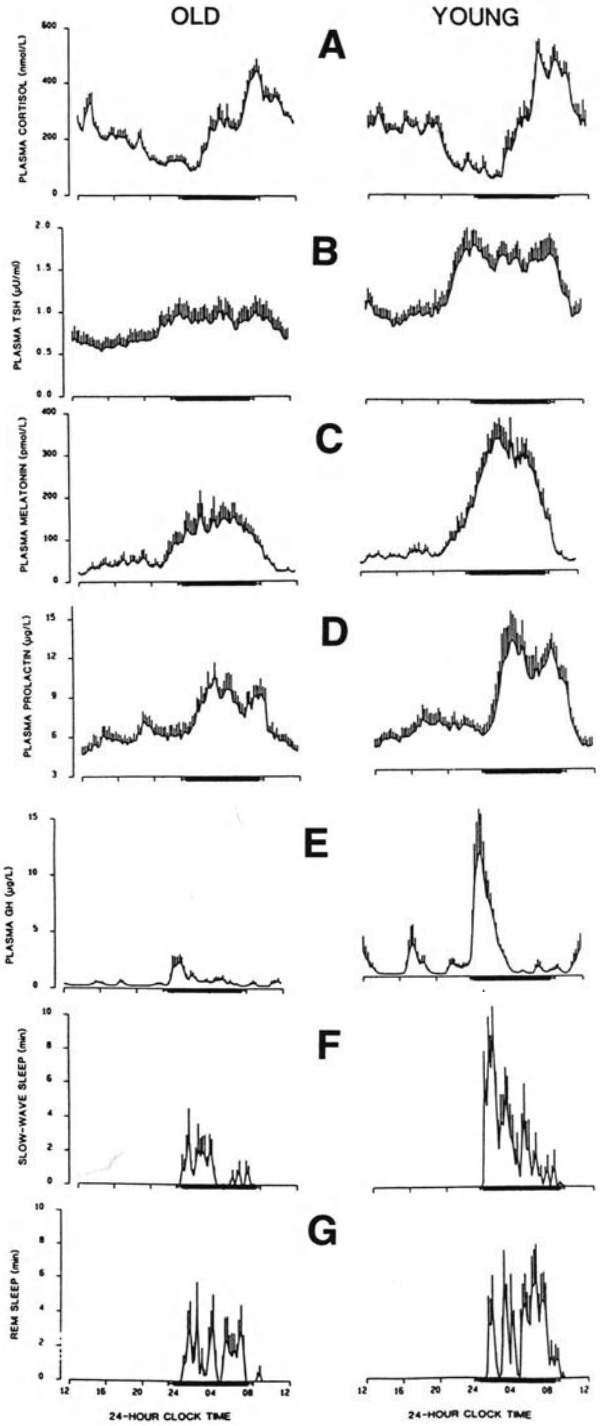

Fig. 2. Mean 24-hour profiles of plasma cortisol (A), thyroid stimulating hormone (TSH, B), melatonin (C), prolactin (D), and growth hormone (E) levels and distribution of slow wave sleep (F) and rapid eye movement (REM, G) stages in sleep in old and young subjects. Vertical lines represent S.E. Black bars correspond to mean sleep period. Data show that both the amplitude and timing of the diurnal patterns of hormone section are altered with age. (From van Coevorden A et al. *Amer J Physiol* 1991; 260E651–661).

sleep, a process that is also regulated by the circadian clock, is also altered compared with that observed in younger individuals.

3. AGING OF THE GROWTH HORMONE RELEASING HORMONE (GHRH)/SOMATOSTATIN (SRIF)/ GROWTH HORMONE (GH)/INSULIN-LIKE GROWTH FACTOR-1 (IGF-1) AXIS

3.1. Introduction

Growth hormone acts on multiple diverse tissues including the liver, bone growth plates, adipose tissue, muscle, and lymphocytes with diverse actions on each of these targets. In some cases GH acts directly, in others it stimulates IGF-1 production by the liver or local target tissues and acts indirectly through this latter hormone. Growth hormone is regulated by the interaction of two hypothalamic peptides: GHRH and SRIF. It is secreted in distinct pulses. The majority of time concentrations are very low and undetectable using less sensitive assays. Basal GH release appears to be primarily under tonic inhibition by SRIF; whereas secretory pulses of GH require both the suppression of SRIF and stimulatory influence of GHRH. Both GH and IGF-1 inhibit GH release in a typical negative-feedback loop. Both GH and IGF-1 also feed back to the level of the hypothalamus to increase SRIF and suppress GHRH release (Fig. 3).

The majority of GH and IGF circulate bound to their respective binding proteins (GH-BPs and IGF-BPs, respectively). However, much less is known about how aging affects these proteins or their role in modulating GH and IGF during aging. Finally, GH-releasing peptide was discovered in 1981 and a high-affinity receptor has been found. Little is known about this peptide or its receptor relative to aging and the GHRH/SRIF/GH/IGF axis.

Age-related changes in GH could be because of alterations in one or more components that regulate its secretion: changes in GHRH, SRIF, or IGF-1, changes in responsiveness to each of these factors or decreased somatotroph mass. Each of these has been studied to assess what role they play in the age-related changes in the pattern of GH secretion. It appears that sensitivity to negative-feedback actions of IGF-1 does not change with age. Also, the number of somatotrophs in the anterior pituitary gland does not appear to change with age. Therefore, these two hypotheses will not be discussed further.

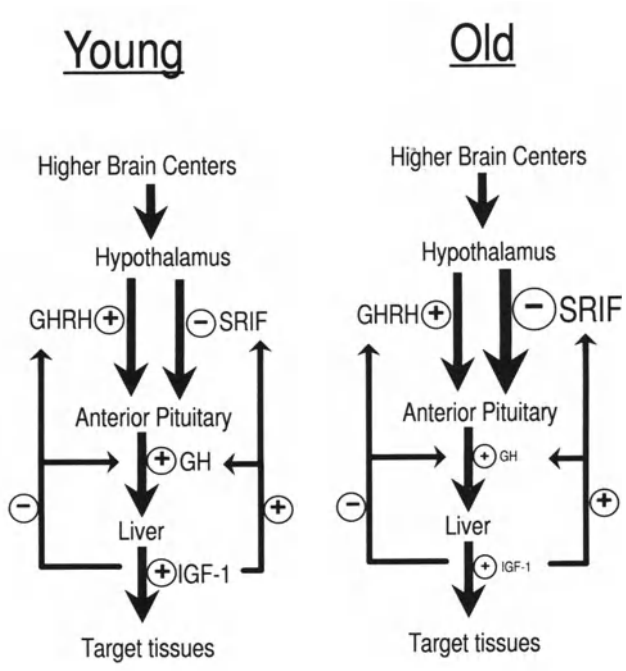

Fig. 3. Neuroendocrine relationships that regulate growth hormone secretion in young and old individuals. Growth hormone decreases significantly with age, both in terms of the number of pulses and the amplitude of each pulse. It appears that SRIF tone increases with age. Although average concentrations of SRIF mRNA does not appear to change with age, the proportion that are within the polysomal fraction increases. This suggests that the amount of mRNA that is actively synthesizing protein increases with age.

3.2. Changes in GH and IGF-1 Secretion with Age

Initial studies, in which a single blood sample was measured, suggested that GH did not change with age. However, numerous GH-regulated physiological functions change with age suggesting that a more thorough analysis of GH secretion should be undertaken: (1) muscle mass and lean body mass as well as and muscle strength decreases with age; (2) bone density declines, particularly in women; and (3) relative fat mass increases in the elderly, even when total body weight does not change. This increase is thought to lead to changes in insulin secretion and increased risk of diabetes, cardiovascular disease and stroke. More recently, when assays became more sensitive and GH was measured across several hours in laboratory animals or over 24-h in humans, marked age-related changes were revealed. Basal concentrations do not appear to change, but the number and the amplitude of growth hormone pulses both decrease in the older individuals (Fig. 4). Also, in younger individuals, there is a marked diurnal rhythm in GH

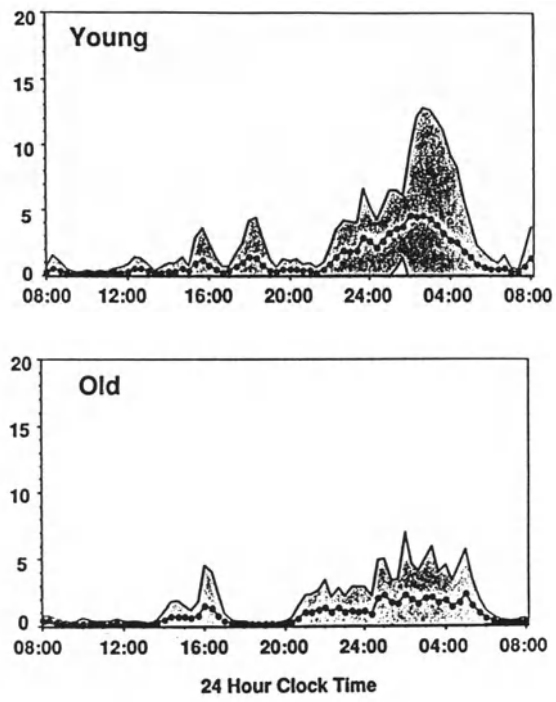

Fig. 4. Mean (± 1 sd) 24 h GH release in nine young (mean age 26 ± 4 yr) and 10 old (68 ± 6 yr) men sampled at 20 min intervals, demonstrating the normal diurnal pattern of GH secretion and the reduced nocturnal peak amplitude in the older men. (From Corpas E et al. *J Clin Endocrinol Metab* 1992; 75:530–535).

secretion with the majority of the pulses occurring at night. In older individuals, although overall secretion is lower, and the diurnal rhythm is blunted, about twice as much GH is secreted at night compared to during the day. Both the number and the amplitude of GH secretory episodes reach a maximum around the time of puberty and decrease gradually with age throughout adulthood. In humans, 24-h integrated means of GH concentrations decrease approximately 15% and the half-life of the hormone decreases approximately 6% each decade after puberty (Fig. 5).

Diminished GH levels may be partly because of decreased responsiveness of GH to stimuli such as GHRH, arginine, or exercise. Responses to all of these GH secretogogues are attenuated in older subjects than in young, although considerable variation exists. This may be because of the heterogeneity of the human populations used in these studies. Interestingly, repeated administration of GHRH results in a normal GH response, suggesting that age-related changes in pituitary responsiveness are not permanent. If they are because of changes in the density of GHRH receptors on somatotrophs, these experimental results suggest that repetitive injections may upregulate GHRH receptors or increase their ability to evoke second messenger systems that mediate the GHRH stimulus. Ultimately, GH does respond to these stimuli in older subjects, therefore, these remain potential treatments for GH deficiency in the elderly. However, it will be important to monitor the effects of chronic treatment with any of these GH stimuli.

When GH levels decline, there is also a decline in IGF-1 levels. Interestingly, decreases in IGF-1 may be greater than those of GH. This may be because of the fact that IGF-1 is regulated by factors other than GH. For example increased body fat, which is characteristic of older organisms suppresses IGF-1 secretion independent of age. A decrease in physical activity may also contribute to the decrease in IGF-1 concentrations with age. Finally, IGF-BPs, which are regulated by GH and IGF, also exhibit an age-related decrease. Thus, the half-life of IGF-1 may decrease with age, as it is accessible to degradative enzymes when it circulates free in blood, not bound to its binding protein(s). All of these changes in factors that regulate IGF-1 lead to the result that the strong positive relationship between IGF-1 and GH is not as cohesive in older compared to younger individuals.

2.3. Changes in Hypothalamic GHRH and SRIF Synthesis and Secretion

It is not possible to measure any of the hypothalamic releasing or inhibiting hormones in the peripheral plasma of humans. All of the studies to determine whether the hypothalamus plays a role in age-related changes in neuroendocrine hormones are performed in animals or by measurement of patterns of pituitary hormone secretion and deductions about the hypothalamus from these indirect indices of hypothalamic hormone secretion.

Evidence suggests that GHRH decreases and SRIF increases with age. This changing balance of decreased stimulatory and increased inhibitory tone could lead to decreased GH synthesis and secretion. GHRH mRNA levels in the hypothalamus decrease in older animals and the ability of GH to regulate GHRH synthesis is impaired. In terms of SRIF activity, when hypothalami are perfused in vitro, greater amounts of SRIF are released. In addition, the amount of SRIF isolated from the pituitary glands of older rats is greater than from younger animals. SRIF mRNA levels in the hypothalamus appear to decrease with age; however, the amount of SRIF mRNA that is recruited onto polysomes to be translated into SRIF protein is elevated in older rats (Fig. 6). It appears that despite lower levels of mRNA, the population

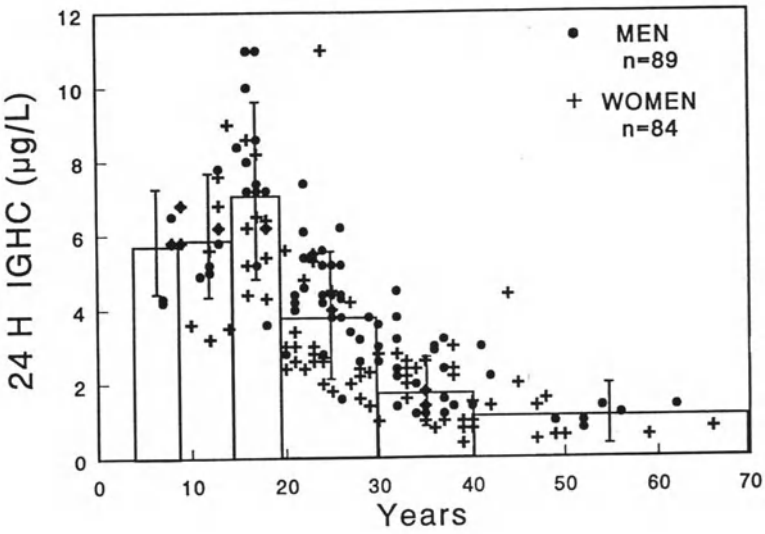

Fig. 5. The relationship between the individual and mean 24-hour integrated GH concentrations and age of 89 normal men (filled circles) and 84 normal women (+). (From Zadik Z et al. *J Clin Endocrinol Metab* 1985; 60:513–516).

of SRIF mRNA that remains is more active and may increase the amount of peptide that is synthesized increases with age.

In summary, major changes in the diurnal and pulsatile pattern of GH are evident with age. It appears that many of these changes are apparent in early adulthood. Evidence from animal studies suggest that the ability of the hypothalamus to secrete SRIF and possibly GHRH may explain the changes in the pattern of growth hormone secretion.

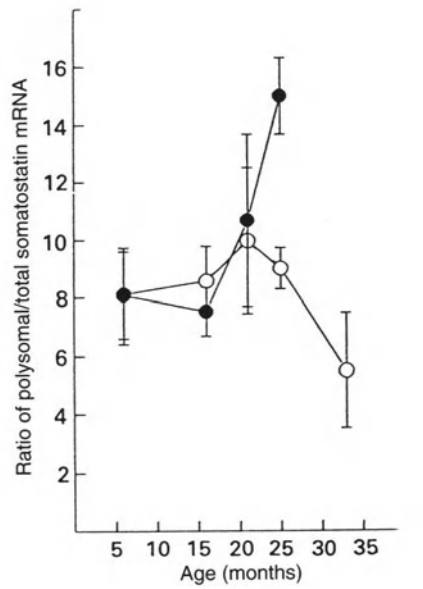

Fig. 6. Ratio of polysomal to total somatostatin mRNA in the hypothalami of aging freely fed (filled circles) and dietary-restricted (open circles) Brown-Norway rats. Data represent mean ± S.E. This figure demonstrates an increased association of somatostatin mRNA with polysomes in aging rats, suggesting that more mRNA is translated in older rats. (From D'Costa et al. *J Reprod Fert* Suppl 1993; 46:87–98).

4. AGING OF THE GnRH/GONADOTROPIN/GONADAL STEROID AXIS

4.1. Introduction

The decline of reproductive function differs dramatically in males compared to females. In females of many mammalian species, reproductive function ceases completely during middle-age. Although changes occur in males, frequently they remain reproductively active and capable of reproducing young until old age, sometimes until the end of the life-span. Several differences exist between the reproductive systems of males and females that may explain the differences between the length of the period of reproductive competence. First, the menstrual cycle requires a complex orchestrated series of events involving higher brain centers, the hypothalamus, the pituitary, and the ovary to occur in precise synchrony. Changes in the amplitude or timing of any of these messages causes disruption of regular cyclicity and decreasing fertility. Animals with irregular reproductive cycles are particularly vulnerable to changes in the dynamics of gonadotropin secretion and ovarian follicular development, resulting in reduced fertility.

The male reproductive system is much more like the other neuroendocrine axes we discuss in this chapter. That is, reproduction depends upon more traditional relationships between the hypothalamus, pituitary, and testes, and the negative-feedback relationships amongst these components of the neuroen-

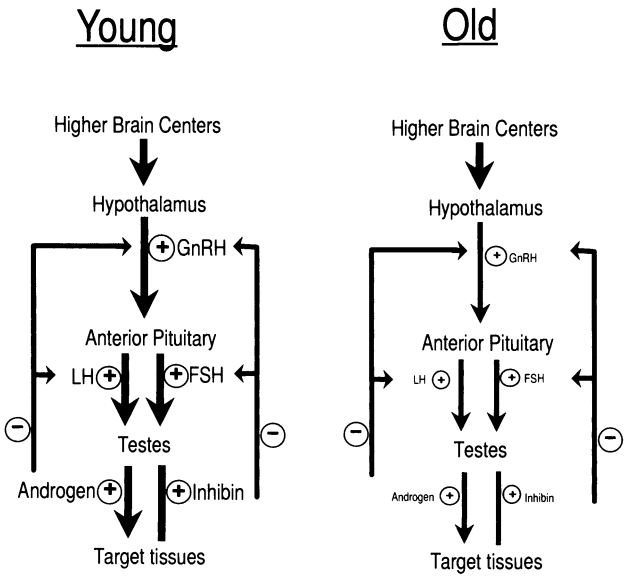

Fig. 7. Neuroendocrine relationships that regulate testosterone secretion in young and old individuals. Testosterone decreases significantly with age. This is thought to be because of decreased stimulation from GnRH and LH.

docrine axis. Thus, the added complexity reproductive cycle is unique to the female. In addition, the female is endowed with a finite pool of germ cells. When the pool of germ cells and surrounding granulosa cells is exhausted, reproductive cyclicity ceases permanently. On the other hand, male germ cells continue to replicate and, therefore, male reproductive function is not limited by the exhaustion of sperm.

Hypothalamic GnRH neurons regulate the synthesis and secretion of two gonadotropins: LH and FSH. These hormones stimulate the synthesis and secretion of androgens and estrogens from the gonads (Figs. 7 and 8). In the male, the predominant and most potent androgen is testosterone, which feeds back negatively at the level of the hypothalamus and pituitary to inhibit GnRH and the gonadotropins. In the female, estradiol, the predominant estrogen, feeds back negatively to inhibit GnRH and the gonadotropins under most circumstances. However, when estradiol is present in high-physiological concentrations for a significant length of time, it feeds back positively to enable a preovulatory GnRH and LH surge. The length of time of elevated estradiol that is required to elicit positive feedback is species specific. This unique biphasic feedback effect of estradiol on the hypothalamic-pituitary axis is not seen in any other neuroendocrine axis. Inhibin, which is synthesized in the Sertoli cells of the testes and the granulosa cells of the follicle, feeds back and selectively inhibits FSH.

The minute-to-minute regulation of each component of the reproductive axis and the feedback loops are tightly regulated. Thus, age-related changes in the hypothalamus, pituitary, or gonad will have immediate repercussions on the other components. The relative roles of each of these components may be somewhat different in the initiation of the transition to infertility from species to species. However, clearly all components of the axis are ultimately involved in the total cessation of cyclicity in the female.

4.2. Aging of the Female Reproductive System

The female reproductive system and the effects of aging upon this system have attracted much attention from neuroendocrinologists because of its complex regulation of cyclicity and because a dramatic decline in function is apparent at middle-age. Menopause occurs at approximately 51 years of age in women and signals the permanent end of fertility. Because the average life-span of humans has increased substantially in the last century, an increasing number and an increasing proportion of women now live a larger fraction of their lives in the postmenopausal state than ever before. Interest in the repercussions of the menopause has increased as we have begun to appreciate that estrogens play a much broader role in the maintenance of normal physiology than we once thought. We now know that estrogen modulates bone and mineral metabolism, cardiovascular function, memory and cognition, and the incidence and progression of age-related disease, such as Alzheimer's disease.

4.2.1. Changes in Ovarian Function with Age

4.2.1.1. Exhaustion of the Pool of Follicles. A hallmark of the postmenopausal state is the total exhaustion of ovarian follicles. In rodent models, which also exhibit reproductive senescence, it is less clear whether the follicular pool is totally exhausted. However, the number of follicles remaining in the ovary is minimal. Females are born with a vast but finite, nonrenewable, postmitotic pool of dormant follicles. Primordial follicles, made up of a germ cell and surrounding granulosa cells, are set down during fetal development: germ cells undergo mitosis for a time and then undergo meiosis and cease growing. Once mitosis ceases during fetal development, no new germ cells will ever be added to the reserve. Throughout life, germ cells reawaken from a dormant state and begin to grow. The vast majority of follicles die as they grow through a process of programmed

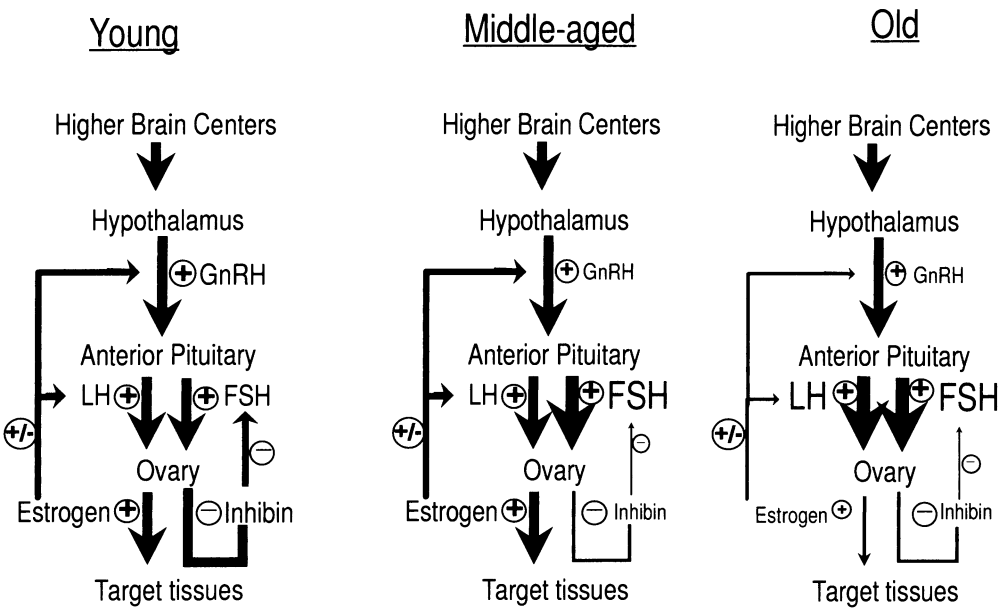

Fig. 8. Neuroendocrine changes in young, middle-aged, and older women. One of the initial changes that occurs during the perimenopausal period is an elevation in FSH concentrations. This may be because of a change in the pattern of GnRH release and decreased inhibin concentrations. Later, when women are at more advanced stages of the menopausal transition and postmenopausal, both LH and FSH are elevated because of the gradual depletion of ovarian follicles and the ensuing decrease in ovarian steroid secretion. This suggests that the finite pool of follicles ultimately limits the reproductive life-span; however, the timing of the initiation of the transition to the perimenopausal state results from complex interactions between the brain and the ovary. In laboratory animal models, the initial rise in FSH and decrease in inhibin occur; however, at later stages, gonadotropin levels are not elevated compared to young controls. This suggests that the brain is the main factor that leads to acyclicity in rodent models.

cell death. A very selected few undergo full growth, final differentiation, and ovulation. The vast majority of follicles die because they do not receive the trophic and hormonal support required for the final stages of growth and differentiation. They reach a critical juncture where specific levels of a repertoire of hormones and factors must act at a time during the menstrual cycle when the proper support is not available.

If one examines an ovary under a microscope, a large number of follicles in nearly every stage of growth is visible. The largest proportion of follicles are small follicles that are dormant or are entering the growing pool. A smaller number of medium- and large-sized follicles can be seen. These follicles are in a rapid growth phase and are undergoing final differentiation in preparation for ovulation. One can visualize a large number of small follicles and many less large follicles because the initial stages of growth are slow and most of the follicles die early during development and will never differentiate into large, Graafian follicles and, thus, will never be ovulated. Many studies have established that the terminal stages of growth and differentiation are tightly regulated by hormones and other factors. However, little is known regarding the factors that determine the size of original follicular endowment, such as (1) how the initial proliferation of germ cells is regulated; (2) what governs the organization of germ cells into discrete follicles; or (3) what influences the extent of cell loss after a follicle reenters the growing pool. From recent analysis of the ovaries of knock-out mice, we know that the initial stages of follicular development do not depend upon gonadotropin stimulated intraovarian steroids.

Many believe that the number of dormant follicles that reawaken and are recruited into the growing pool determines the rate of loss of the follicular pool. This, in turn, is determined by the number of follicles that remain in the ovary. Thus, it has been hypothesized that the vast pool of follicles is a prerequisite to maintain a constant stream of follicles in the developmental pipeline. According to this hypothesis, when the number of follicles falls below a critical number, fine tuning is lost, the number that enter the growing pool becomes less well regulated, the patterns of hormone secretion by the larger follicles become less depend-

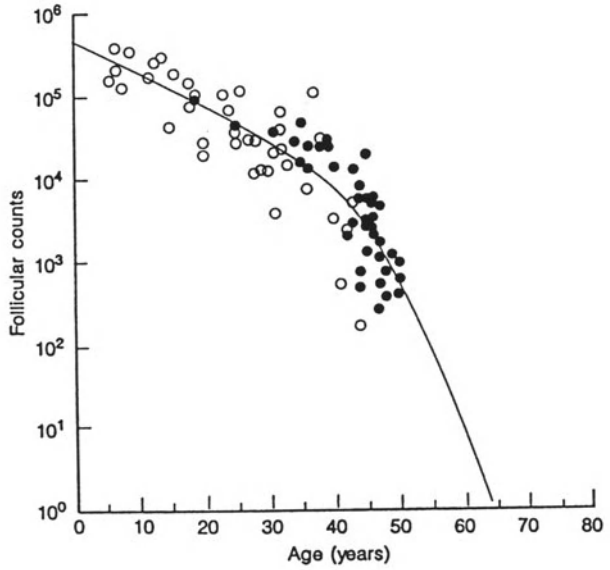

Fig. 9. Numbers of primordial follicles in the human ovary with respect to age. The data show that at approximately 37 years of age, the rate of follicular loss accelerates (From Gougeon A et al. *Biol Repro* 1994; 50:653–663).

able, the length of time between cycles becomes more variable, and consequently fertility declines. The impending menopausal transition is exacerbated because the rate of loss of follicles remaining in the ovary doubles in middle-aged women around 35 years of age (Fig. 9). It is also unknown whether altering the later stages of rapid growth and final differentiation influences the reawakening of the remaining follicles in the stockpile or initial stages of growth of small follicles.

4.2.1.2. Ovarian Hormone Secretion During Aging. Recent findings demonstrate that estradiol concentrations do not decrease prior to the perimenopausal transition in women or the establishment of irregular estrous cyclicity in rodent models. In fact, during the periovulatory phase, estradiol appears to be maintained or is higher in middle-aged animals. However, inhibin, a peptide synthesized by granulosa cells decreases, and may be one of the early signs of ovarian aging. Because this peptide selectively inhibits FSH secretion by feeding back to the pituitary, decreases in this peptide may explain the selective increase in FSH that occurs early during the transition to irregular cycles.

Postmenopausal estradiol concentrations are similar to those observed after ovariectomy. It is thought that most of the estradiol found in peripheral blood is synthesized from androgen precursors in adipose tissue, because the enzymes necessary for the final conversion to estradiol are greatly decreased in the aging ovary.

4.2.2. Changes in GnRH and Pituitary Gonadotropin

The concept that the hypothalamus regulates the timing of the menopause comes from the knowledge that the hypothalamus provides precisely timed neurochemical and neuroendocrine signals that dictate the patterns of secretion of the gonadotropins, which, in turn, govern the development of follicles and the ovulatory surge of LH. Hypothalamic changes, as measured by the incidence of hot flushes, sleep disturbances, and changes in the pulsatile pattern of gonadotropin secretion, become evident in women when they are between 35–40 years old, at the same time as the rate of follicular loss accelerates. Therefore, the acceleration of follicular loss may be a consequence of a change in the pattern of neuroendocrine messages that govern the dynamics of follicular reawakening, recruitment, growth and differentiation.

One of the first signs that the menopausal transition is imminent is the elevated level of FSH during the follicular phase. Similar observations have been reported in middle-aged rats. As discussed above, these alterations may reflect changes in ovarian inhibin feedback. However, it is important to note that a decrease in the frequency of pulses or relatively lower secretion rates of GnRH preferentially increase the release of FSH compared to LH. Therefore, a selective increase in FSH secretion during the perimenopausal transition may result from either changes in ovarian inhibin or changes in the secretion pattern of GnRH.

As with all other neuroendocrine axes, measurement of the patterns of pituitary hormone secretion is our only way to assess indirectly hypothalamic hormone secretion. Alterations in the pulsatile nature of LH secretion during the follicular phase of the cycle have been reported recently in middle-aged regularly menstruating women prior to any change in plasma estradiol. Both the frequency of LH pulses decreases and the duration of the pulse increases in these women. Strikingly similar findings have been reported in middle-aged rats. These observations are particularly provocative from the neuroendocrine perspective. Changes in the frequency of LH pulses and broadening of the width of pulses usually reflect imprecision in the hypothalamic GnRH pulse generator resulting from the gradual uncoupling of the pulse generator from the neurochemical factors that coordinate GnRH secretion.

GnRH secretion is regulated by many neurotrans-

mitters and neuropeptides. Data from several laboratories suggest that during middle age, the precise, synchronized, and interactive patterns of hypothalamic neurotransmitter and neuropeptide activity, which are critical to maintain a specific pattern of GnRH secretion, become less ordered. First, hot flushes, a hallmark of deterioration of the hypothalamic thermoregulatory centers, have been reported in normally cycling women during the fourth decade when more than 25,000 follicles are thought to be present in the normal ovary. Interestingly, in postmenopausal women hot flushes occur in concordance with pulses of LH, suggesting that higher brain centers that regulate both body temperature and GnRH are altered during aging in women (Fig. 10). Second, using animal models of the menopause, changes have been observed in several aspects of neurotransmitter activity in middle-aged animals that still exhibit regular reproductive cyclicity. Some researchers propose that this deterioration in the coupling of neurotransmitter signals that regulate GnRH secretion or an uncoupling of the composite of neurochemical signals from GnRH neurons causes the initial changes in patterns of gonadotropin secretion. The recurring and pervasive feature of these neurochemical changes is that their daily rhythmicity is affected far more frequently than their overall average level of activity or expression.

4.3. Male Reproductive Senescence

Although there is no equivalent of the menopause in men, there is a gradual and subtle decline in reproductive function as measured by a decrease in testosterone levels, the number of Leydig cells that produce testosterone, and an increase in LH and FSH (Fig. 11). These changes are accompanied by changes in reproductive behaviors including frequency of intercourse and impotence. However, one study performed in a longitudinal manner in a selected population of men whose health status was carefully monitored, showed no change in testosterone through the ninth decade of life. These data remind us that it is critical to be aware of age-related pathologies that may confound the interpretation of data in aging studies. Studies performed in rodent models demonstrate decreasing testosterone levels in old male rats and mice. There is significant evidence that alterations in the hypothalamus drive these testicular events, because the pulsatile pattern of LH secretions is altered as well. Both the amplitude and frequency decrease in aging animals and the number of immunocytochemically identified GnRH neurons decreases as well.

In summary, both male and female reproductive function declines with age. The changes that characterize female reproductive aging are among the most dramatic of any neuroendocrine axis. Because they occur at a relatively early age in the life-span of many species, the factors that regulate aging of this axis may serve as a model for the process of aging. In the female, the finite pool of follicles is a major factor that determines the fate of the reproductive system during aging. However, in both males and females, the CNS plays an important pacemaker role in reproductive aging.

5. AGING OF THE CORTICOTROPIN RELEASING HORMONE (CRH)/ ADRENOCORTICOTROPHIC (ACTH)/ GLUCOCORTICOID AXIS

5.1. Introduction

Hypothalamic CRH neurons, anterior pituitary corticotrophs, and the adrenocortical glucocorticoid producing cells of the zona fasciculata function as a hierarchically integrated feedback system (Fig. 12). Stimuli from higher brain centers regulate synthesis of CRH in the medial parvocellular division of the hypothalamic paraventricular nucleus and its secretion into the hypophysial portal circulation. Upon reaching the anterior pituitary gland, CRH stimulates synthesis and secretion of ACTH, which then triggers release of glucocorticoids from the adrenal cortex. Glucocorticoids then exert negative-feedback inhibition of CRH secretion by acting directly upon CRH neurons and indirectly upon higher neural control centers. Among the most important higher centers is the hippocampus. This region of the brain exerts an inhibitory influence on CRH synthesis and secretion. The hippocampus contains the highest concentration of corticosteroid receptors in the brain. These receptors mediate the negative feedback effects of glucorticoids. Neurons from the hippocampus project via the fornix to many hypothalamic regions including the paraventricular nucleus.

5.2. Age-Related Changes in the Hypothalamic-Pituitary-Adrenocortical (HPA) Axis

5.2.1. Changes in Glucocorticoid Levels with Age

In marked contrast to the dramatic age-related changes in levels of LH/ovarian steroids and GH/IGF-1, plasma glucocorticoids change little with age. However, this should not be interpreted to mean that

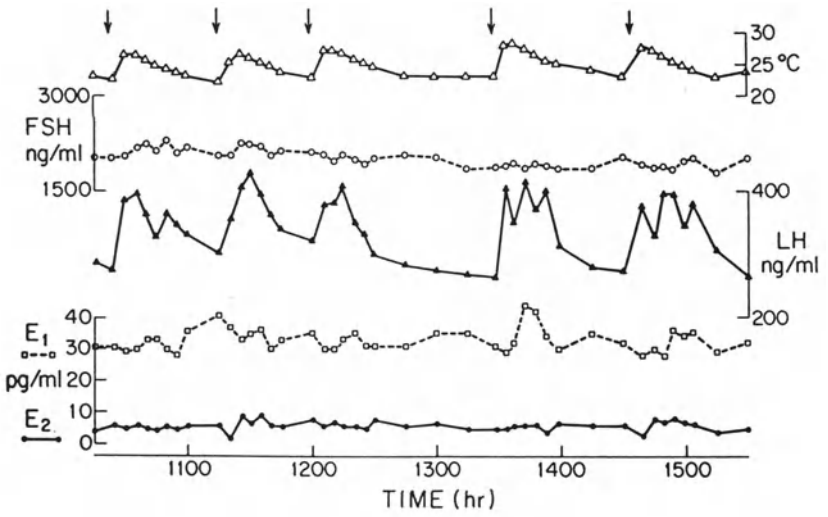

Fig. 10. Serial measurements of finger temperature and serum FSH, LH, oestrone (E1) and oestradiol (E2) in a woman with severe hot flushes. Arrows mark occurrences of hot flushes. (From Meldrum DR et al. *J Clin Endocrinol Metab* 1980; 50:685–689).

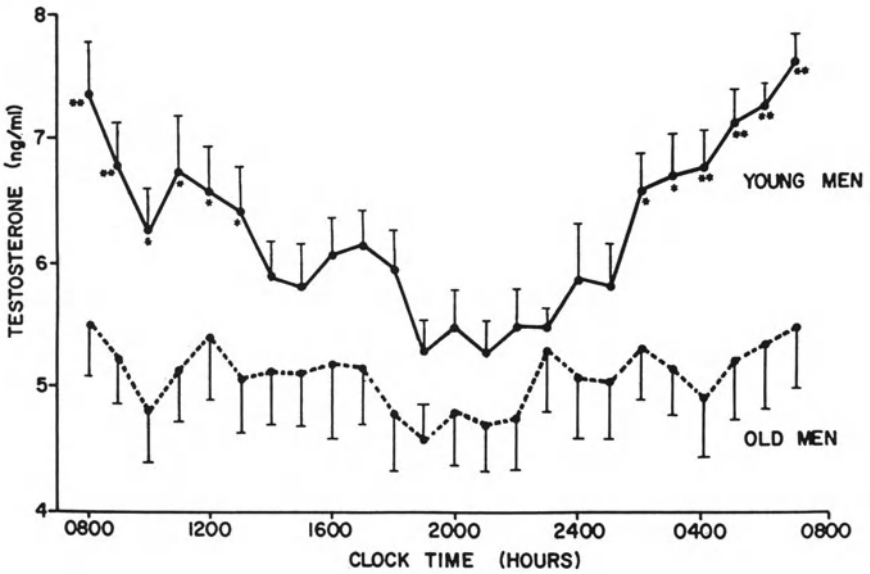

Fig. 11. Hourly serum testosterone levels (means ± SEM) in normal young (n = 17) and old (n = 18) men. Blood samples were obtained by means of an indwelling peripheral venous cannula that allowed free movement and normal sleep. Symbols: asterisk, $p < 0.05$, young versus old at each time point; double asterisk, $p < 0.01$, young versus old at each time point (lack of an asterisk indicates there was no significant difference at that time point). (From Bremner W et al. *J Clin Endocrinol Metab* 1983; 56:1278–1281).

there are no effects of age on the HPA axis. There has been some controversy as to whether basal resting levels of glucocorticoids change, because many studies report no change at all, whereas others demonstrate slight increases in hormone levels in older animals. Because glucocorticoids increase within minutes of handling and exhibit a diurnal rhythm, blood samples must be collected from individuals who have not been disturbed in any way and at carefully monitored times of day relative to lighting conditions and relative to known feeding regimes. When one compares glucocorticoid levels from young and aging animals under these well-controlled conditions, it becomes clear that changes in levels of hormone are very small. Diurnal rhythmicity of glucocorticoid secretion also changes, with the amplitude of the rhythm slightly blunted in older organisms and the timing of the nadir and peak slightly altered (Fig. 13). However, even the daily rhythmicity is remarkably resistant to changes with age compared to much larger changes in the rhythms of other neuroendocrine axes.

5.2.2. Alterations in Negative-Feedback Actions of Glucocorticoids

Although resting levels of glucocorticoids show little change during aging, older organisms exhibit alterations in response to stimulation or stress. There are three aspects of the response that appear to change. First, in young organisms, stress elicits a prompt increase in glucocorticoid secretion, followed by a rapid recovery phase during which hormone levels return to baseline. In older animals, glucocorticoid

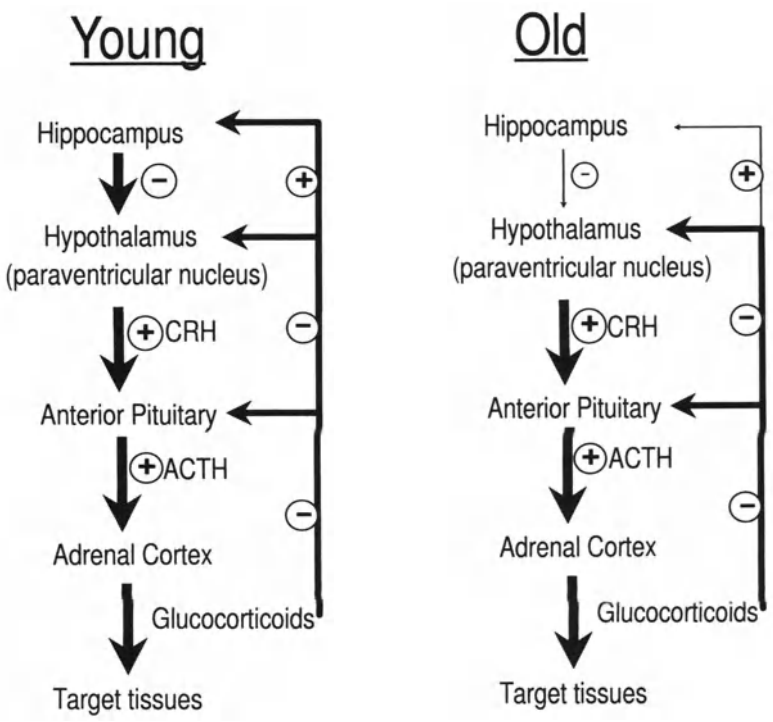

Fig. 12. Neuroendocrine relationships that regulate glucocorticoid secretion in young and old individuals. It appears that the ability of the hippocampus to exert a negative influence on the paraventricular nuclei decreases with age. It is unclear whether this is because of a decreased ability of glucocorticoids to feed back at the level of the hippocampus. The consequence of these changes is that basal levels of glucocorticoids remain largely unchanged, however, the ability to restore basal concentrations of glucocorticoids after a stress is compromised.

Fig. 13. Concentrations of corticosterone in serum at various times of day in ovariectomized, estradiol treated young, middle-aged and old rats. Data represent mean ± S.E. The data show that the diurnal rhythm in corticosterone secretion is blunted with age. (From Weiland NG, et al. *Endocrinology* 1992; 131:2959–2964).

concentrations do not return to baseline promptly, but instead remain elevated for a more prolonged period of time (Fig. 14). Second, in younger animals repeated or chronic stress induces an attenuated response as animals adapt to the stressful environment; whereas older organisms continue to show a robust response to repeated stress. This adds further to exposure of aging animals to elevated glucocorticoids. Third, administration of synthetic glucocorticoid analogs does not feedback negatively and suppress endogenous glucocorticoid levels as effectively in older animals as in young. Over a lifetime, the lack of ability to recover from stress means that older animals are exposed to hypersecretion of glucocorticoids more frequently as they age. Although levels of glucocorticoids at any given time may be only slightly elevated, the cumulative effects of such an increase may be significant.

Glucocorticoids are essential for most physiological functions because they exert major effects on metabolic homeostasis. At the level of the brain, they influence learning, axon sprouting, and dendritic remodeling and neuronal viability. Hyperstimulation of the HPA axis, such as occurs during stress, mobilizes energy stores and cardiovascular tone that is critical to insure survival. However, long-term exposure to elevated glucocorticoids leads to increased risk of cardiovascular disease, compromised immune responses, hypertension, and impairment of neural function. Some investigators believe that high physiological levels of glucocorticoids may lead to neuronal cell death. This may, in turn, affect the aging process in general because the HPA axis plays such a central role in the maintenance of homeostatic regulatory processes.

Many different factors may contribute to the

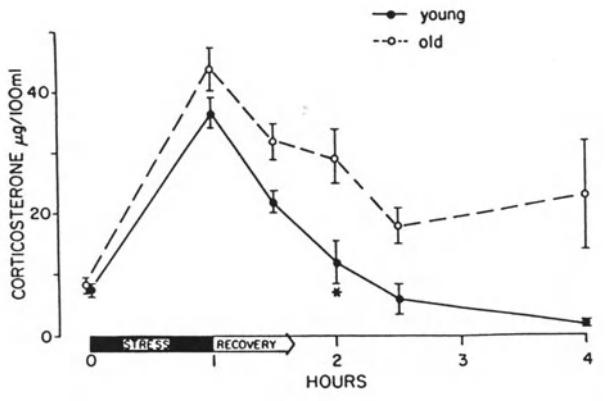

Fig. 14. Corticosterone levels in young (3–5 months) and aged (24–28 months) Fischer 344 rats during 1 h of immobilization stress, followed by 4 h of poststress recovery. Asterisk indicates time when titers are no longer significantly elevated above baseline (determined by two-tailed paired *t* test). In the case of young subjects, this was after 1 h of the recovery period; for aged subjects, such recovery did not occur within the monitored time period. (From Sapolsky R et al. *Proc Natl Acad Sci* 1984; 81:6174–6167).

decreasing ability of older animals to recover from stress. Most of the evidence points to the possibility that as animals age, the hippocampus' ability to inhibit CRH neurons diminishes and that this may be the primary cause for the prolonged elevations in glucocorticoids after a stressful stimulus. This concept is supported by several lines of evidence. First, responsiveness of the pituitary gland to CRH stimulation and of the adrenal cortex to ACTH administration does not change in older animals during stress-induced prolonged hypersecretion. This suggests that the prolonged elevation of glucocorticoids during stress results from hyperstimulation from higher centers. Second, damage to the hippocampus of young animals can cause a sluggish recovery from stress that is similar to that observed during normal aging. Therefore, it has been argued that normal aging involves deterioration of the hippocampus, making it unable to exert its normal negative-feedback action on CRH neurons. Third, in aging animals, the density of corticoid receptors in the hippocampus is considerably lower than in young animals, which could lead to decreased responsiveness to glucocorticoid negative feedback. When the number of corticoid receptors are experimentally suppressed in young animals, it causes a premature aging-like prolongation of the stress response. Likewise, experimental manipulations, which maintain receptors in the hippocampus of older animals, are associated with the maintenance of a youthful-like response to stress and delays in the age-related prolongation of glucocorticoid secretion following stress.

It is unclear whether the primary site of the aging-related decline is the hippocampus *per se* and whether it exhibits an inherent decreased ability to inhibit CRH or whether deterioration of the hippocampus results from the cumulative exposure to glucocorticoids. Evidence shows that decreasing the exposure to glucocorticoids by removing the adrenal in young animals can delay the age-related changes in the hippocampus. Conversely, treating young animals with elevated glucocorticoids can accelerate the rate of decline in hippocampal function and the decrease in glucocorticoid receptors normally observed during aging. Small elevations in glucocorticoids may not by themselves cause neuronal dysfunction or death. However, it appears that the cumulative effect of small increases in glucocorticoid levels predisposes or enhances the vulnerability of the hippocampus to damage from other insults.

5.3. Mechanisms of Alterations in the HPA Axis

5.3.1. Glucocorticoid Effects on Glucose Homeostasis

A classic catabolic action of glucocorticoids is to inhibit glucose uptake in virtually all peripheral tissues. In the brain, glucocorticoids decrease glucose transport and, therefore decrease the amount of glucose within neurons. Neurons utilize predominantly glucose as their metabolic fuel and adequate amounts of glucose are required for normal neuronal function. Neurons are even more dependent upon circulating glucose because they do not store large amounts of glycogen. Thus, a decrease in adequate glucose concentrations may make these neurons more vulnerable to any challenge associated with stressful stimuli. It has been suggested that as animals age and glucocorticoid concentrations rise or remain elevated for prolonged periods of time after a stress, neurons become metabolically depleted and therefore less able to withstand other insults, which would ordinarily not be injurious.

5.3.2. Role of Changes in Corticoid Receptors

There are two types of corticoid receptors: mineralocorticoid receptors (type I) receptors and glucocorticoid (type II) receptors. Each of these receptors has different affinities for corticoids and may function differentially to mediate the diverse actions in corticoids in various tissues. Aging is associated with

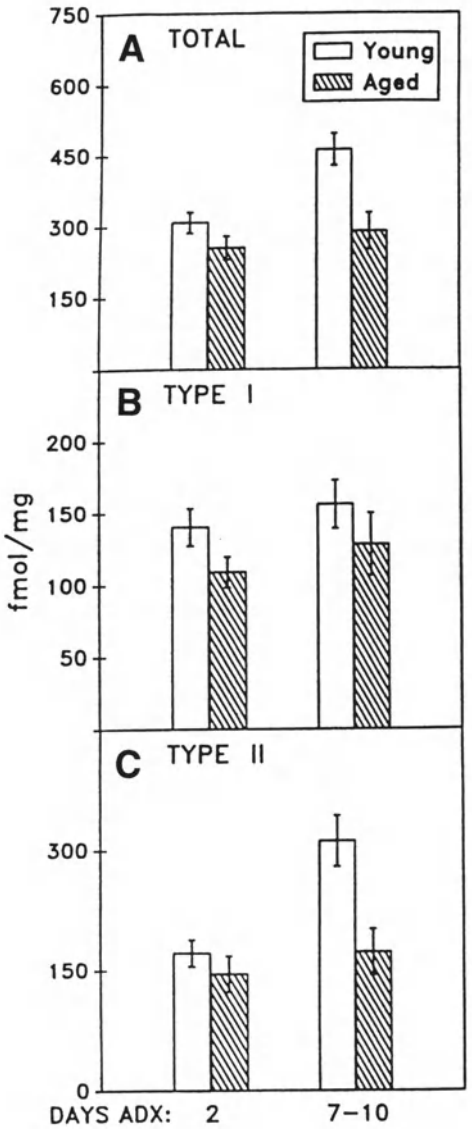

Fig. 15. Effects of age on adrenalectomy induced up-regulation of hippocampal corticosteriod receptors. Data represent mean ± S.E. These data shown that the ability of adrenalectomy to up-regulate total and type II receptors is attenuated in the aged rat. (From Eldridge, et al. *J. Neurosci.* 1989; 9:3237–3242).

decreases in binding to both types of receptors in the hippocampus (Fig. 15). Gene expression of each of these receptors decreases as well, suggesting a decrease in receptor biosynthesis at the level of gene transcription. RNA stability may account for the decrease in receptor protein. The loss in receptor density correlates with hyperresponsiveness to stress and memory impairment in older animals strongly suggesting that changes in receptor expression has functional repercussions.

The specific adrenocorticosteroid receptor most affected in aging is unclear. It appears that mineralocorticoid receptors seem to be most consistently affected and that changes in this receptor subtype correlate with both baseline hypersecretion and impaired glucocorticoid negative feedback. Studies suggest that there are adaptive changes in receptors during aging. It appears that the rate of up-regulation and down-regulation of both mineralocorticoid and glucocorticoid receptors decrease with age. This reduced plasticity may help to explain the decreased ability of glucocorticoids to feedback negatively at the level of the hippocampus.

Changes in the density of glucocorticoid and mineralocorticoid receptors in the hippocampus may influence many aspects of the subcellular actions of the hormone. For example, alterations in several aspects of Ca^{2+} homeostasis, from resting Ca^{2+} concentrations in cells or synapses, to influx, buffering, and extrusion, have been found in aged mammalian neurons. These actions seem to be mediated more by the glucocorticoid receptor rather than the mineralocorticoid receptor. Because it is well established that elevated and/or dysregulated cytosolic Ca^{2+} can be toxic to a variety of excitable cells, including neurons, there has been growing interest in the possibility that these glucocorticoid mediated alterations may play a critical role in brain aging.

In summary, the CRH/ACTH/glucocorticoid axis exhibits more subtle changes than the growth hormone or reproductive axis, yet changes in the ability to withstand stress appear to have cumulative effects on the entire axis. It appears that changes at the level of the hippocampus and its ability to negatively regulate CRH secretion decline with age. Whether these changes are inherent in the hippocampus, or whether the cumulative effects of glucocorticoids cause the hippocampal deterioration is unclear.

6. SUMMARY

We have used three neuroendocrine axes to illustrate the complex changes that occur with age. Each system exhibits unique changes in terms of when changes occur during the life-span, and the potential primary causes of the alterations. Some commonalities amongst the axes appear; however, it is apparent that different factors modulate the effects of age in each of these axes. One of the most apparent conclusions that can be drawn is that the temporal organization of pituitary hormone secretion and the relationships within each neuroendocrine axis and between them is profoundly affected during aging. Because

these relationships are thought to be of great importance in the health of organisms and their ability to respond to the environment, it is likely that deterioration of neuroendocrine systems with age lead to an increasing vulnerability to additional perturbations and increasing vulnerability to age-related diseases.

SELECTED READINGS

Corpas E, Harman SM, Blackman MR. Human growth hormone and human aging. Endocrine Reviews 1993; 14:20–39.

Masoro EJ. Animal models in aging research. In: Schneider EL, Rowe JW, eds. Handbook of the Biology of Aging. San Diego, CA: Academic, 1990: pp. 72–97.

Prior JC. Perimenopause: the complex endocrinology of the menopausal transition. Endocrine Rev 1998; 397–428.

Seeman TE, Robbins RJ. Aging and hypothalamic-pituitary-adrenal response to challenge in humans. Endocrine Rev 1994; 15:233–260.

Thorner MO, Chapman IA, Gaylinn BD, Pezzoli SS, Hartman ML. Growth hormone releasing hormone and growth hormone releasing peptide as therapeutic agents to enhance growth hormone secretion in disease and aging. Recent Progress in Hormone Res 1997; 52:215–246.

Wise PM, Kashon ML, Krajnak KM, Rosewell KL, Cai A, Scarbrough K, Harney JP, McShane T, Lloyd JM, Weiland NG. Aging of the female reproductive system: a window into the aging brain. Recent Progress in Hormone Res 1997; 52: 279–305.

Wise PM, Herman JP, Landfield PW. Neuroendocrine aspects of the aging brain. Advances in Cell Aging and Gerontol 1997; 2:193–241.

Xu X, Sonntag WE. Growth hormone and the biology of aging. In: Mobbs CV, Hof PR, eds. Functional Endocrinology of Aging. Basel, Switzerland: Karger, 1998: pp. 67–88.

PART IV NEUROENDOCRINE REGULATION OF BIOLOGICAL RHYTHMS

22 Chronobiology

Gene D. Block, PhD, Marie Kerbeshian, PhD and Erik D. Herzog, PhD

Contents

1. INTRODUCTION

Sleep-activity cycles in animals and man, daily movements in plant leaves, and seasonal cycles of flowering, growth, and reproduction have been observed and noted since antiquity. The first experimental evidence that such biological rhythms were endogenous, and not simply driven by environmental cycles, was provided in 1729 by a French geologist, Jean Ortous deMairan, in a report to "l'Academie Royale des Sciences." DeMarian observed that Mimosa plants retained their daily leaf movement pattern even when removed from their natural environment and transported to a continually dark cellar. Although some investigators later embraced a purely exogenous explanation for the generation of biological rhythms (the so-called "factor X" theories), by the late 1950s there was general agreement about the endogenous origins for near 24-h biological periodicities. Perhaps the single most compelling experimental result demonstrating the inherently endogenous nature of these rhythms was the observation that daily rhythms were not really "daily" when plants or animals were placed under constant environmental conditions. Rather, they deviated from the solar day; they were circadian, *circa* (about), *diem* (a day), consistently starting a little earlier or later than the day before. Moreover, different organisms measured at the same solar time, under the same experimental conditions, exhibited rhythms with periodicities that differed from one another. This observation rendered any purely exogenous explanation highly improbable.

There is presently a considerable body of knowledge about circadian rhythms. Perhaps most significantly, it is now recognized that biological clocks are ubiquitous, controlling a multitude of physiological and behavioral parameters in organisms ranging from primitive bacteria to humans. In addition to the control of obvious rhythmic behaviors, such as the sleep-activity cycle, biological clocks play a more covert, but critical role in both the recognition of local time, required for solar navigation and orientation in birds and bees, and in the measurement of elapsed time, required for time memory in bees (Zeitgedachtnis). Along with a detailed understanding of the adaptive significance of circadian timing, the physiology of the clock system is being revealed. Biological clocks have been localized within the tissues of higher animals and, more recently, the study of circadian rhythmicity has been addressed successfully at the molecular level. After nearly 40 yrs of study a very satisfying picture is emerging of a complex, yet experimentally

From: *Neuroendocrinology in Physiology and Medicine*, (P. M. Conn and M. E. Freeman, eds.),

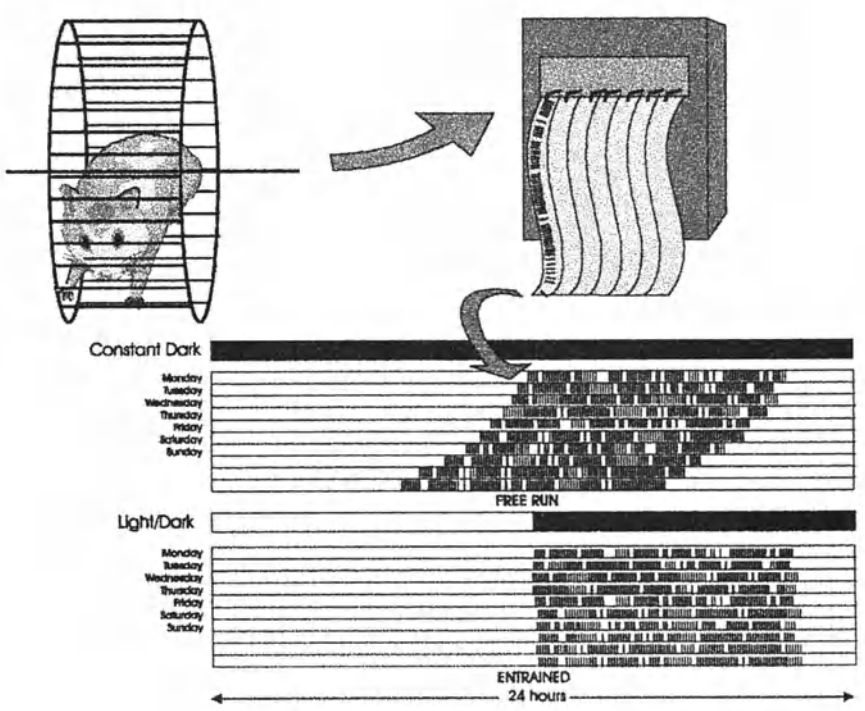

Fig. 1. Measurement of behavioral rhythmicity. Traditional "actogram" method for recording wheel running activity from rodents. With each revolution of the wheel, a pen deflects on an event recorder. The event recorder chart is cut into 24 h strips and pasted chronologically one below another. When most rodents are placed into constant darkness they generate a rhythm in locomotor activity that free-runs with a period less than 24 h. Thus, activity onsets occur at an earlier clock time each cycle and consequently, the record of pen deflections drifts to the left. If the animal is placed on a 24-h light cycle (and becomes entrained) locomotor onsets begin at the same time each day.

approachable system responsible for providing an appropriate internal temporal milieu throughout the animal and plant kingdoms.

2. BEHAVIORAL ANALYSIS OF CIRCADIAN RHYTHMICITY

2.1. Free-Running and Entrained Rhythms

Few physiological and behavioral processes escape modulation by the biological clock. One of the most robust and easiest rhythms to observe experimentally is the activity/rest cycle of higher animals. In rodents, this is typically accomplished by placing the animal in a cage containing a running wheel and measuring the temporal pattern of wheel running on an event recorder (Fig. 1). When maintained in the laboratory under constant conditions (i.e., no light or temperature cycles present), most animals will express the natural period of their biological clock. This is referred to as a free-running rhythm. In nocturnal animals, this periodicity is most often less than 24 h, in diurnal animals, it is most often greater than 24 h. Moreover, the precise periodicity expressed will vary depending upon whether an animal is maintained in constant darkness or constant light and will also depend upon the animal's previous light-cycle experience. Thus, there is no single free-running period for an organism. Rather there is a species-specific range of freerunning periods whose bandwidth is determined by both genetics and environmental conditions.

Few organisms spend extended periods of time under the constant environmental conditions that would allow for expression of sustained free-running rhythms. Rather, most organisms are exposed to the daily solar cycle that transforms the circadian cycle into an exact 24-h rhythm. This occurs through a process referred to as entrainment by which the biological clock is "corrected" by the daily light cycle. Importantly, through the process of entrainment, a stable and predictable phase relationship is established between the light cycle and behavior. Specifically, the clock is set to "local time" allowing the organism to time its behavior appropriately to the regional light cycle.

There has been extensive study of the process of entrainment. Broadly speaking, a biological clock running "too slow" or "too fast" can be corrected in one of two ways. Either the angular velocity of the rhythm can be altered or the angular position can be rapidly corrected each cycle. Altering the angular velocity is referred to as parametric entrainment. During the entire illuminated portion of the cycle, light acts to slow down or speed up the biological clock thereby regulating the period at 24-h. In this case, the angular velocity of the clock is altered over a substantial fraction of the circadian cycle. In the case

of entrainment by adjustment of angular position, the biological clock is allowed to move at its natural cadence during the cycle, but its angular position is corrected each cycle by a rapid phase adjustment at dawn or dusk. This process is referred to as nonparametric entrainment insofar as the parameters controlling the period of the oscillation are not modified. The oscillation proceeds at its natural velocity interrupted by one or more rapid phase adjustments. Such a process is familiar to many of us from our days in elementary school when we would hear "click, click click" as the centrally controlled wall clocks were readjusted at a certain time each day.

In the natural environment, entrainment turns out to be a fairly complex, but very interesting process involving both the parametric and nonparametric actions of light. In the laboratory, animals have been effectively entrained to very brief pulses of light and nonparametric models, derived from the study of the effects of these light pulses, have proven remarkably useful in understanding the entrainment process. Critical to understanding nonparametric entrainment is the phase response curve.

2.2. Phase Response Curve

When a single, brief light pulse is provided to an animal in constant darkness, the light pulse can effect a rapid change in the phase of the free-running rhythm (Fig. 2). Light pulses delivered at different phases of the cycle result in phase shifts of differing magnitude and sign. At some points in the cycle, light has little or no effect; at other phases, light can cause a phase advance or phase delay of the rhythm. A complete characterization of the phase shifting behavior of the rhythm probed by light at many phases in the cycle is referred to as a phase response curve or PRC. A typical PRC, in this case derived for the Syrian hamster, is shown in the inset of Fig. 2. The abscissa indicates the Circadian Time at which the light pulse was administered. Circadian Time is derived by dividing the free-running rhythm τ by 24. Thus, each Circadian Hour equals $\tau/24$ clock hours. This calculation allows one to identify comparable phases among different rhythms irrespective of free-running period. Circadian Time 0 is defined as the time at which dawn would have occurred on a Light:Dark cycle consisting of 12 h of light and 12 h of darkness. By convention, the ordinate plots the magnitude of the phase advance (+) or phase delay (−) in actual clock hours, not in circadian hours.

The light-generated PRCs of most organisms are similar in overall shape. They consist of a region where light has little or no effect on the rhythm, the so-called dead zone located in the middle of what would have been daytime (Circadian Time 0–12 is referred to as the Subjective Day); a region in the first half of the Subjective Night, in which light leads to phase delays, and a region during the last half of the Subjective Night where light generates phase advances. In general, phase response curves fall into two classes based on their slope. Type 1 PRCs, which have an average slope close to 1, are low amplitude with maximum phase shifts of typically less than 2 h. Most organisms, including mammals, exhibit such PRCs to single light pulses. Type 0 PRCs have an average slope close to 0 and reveal large amplitude phase shifts. The fruitfly *Drosophila* exhibits such a PRC in its eclosion behavior, with maximum phase shifts of up to 12 h to a single 1 min light pulse.

Although derived from single pulses of light, the PRC has proven useful for understanding entrainment by periodic light pulses. In the nonparametric entrainment model, periodic light pulses act on the biological clock to cause recurring phase shifts as described by the PRC to single pulses and entrain the circadian rhythm. Entrainment occurs when the free-running period (τ) is made equal to the period of the light cycle (T) through daily rapid corrective shifts in the phase of the rhythm ($\Delta\phi$) such that

$$\tau - T = \Delta\phi.$$

The fact that the PRC contains regions of negative slope ensures that wherever a light pulse first falls, eventually the rhythm will be successively shifted until light falls at a phase where the phase shift just equals the difference between the period of the biological rhythm and the period of the light cycle. Thus, for example, if the biological clock has a free-running period of 23 h, entrainment to a 24-h light cycle would require a 1 h delay each cycle. Using the hamster PRC shown in Fig. 2 as an example, no matter when initially the light pulse falls within the cycle, eventually the light will fall at approximately Circadian Time 12 (the beginning of the wheel running activity period), a phase on the PRC that leads to about a 1 h phase delay.

The phase response curve does a remarkably good job of predicting entrainment behavior of organisms to periodic light pulses. It has also been successfully applied with a nonparametric model of two pulses per cycle where seasonal daylength is taken into account by treating the photoperiod as a brief light

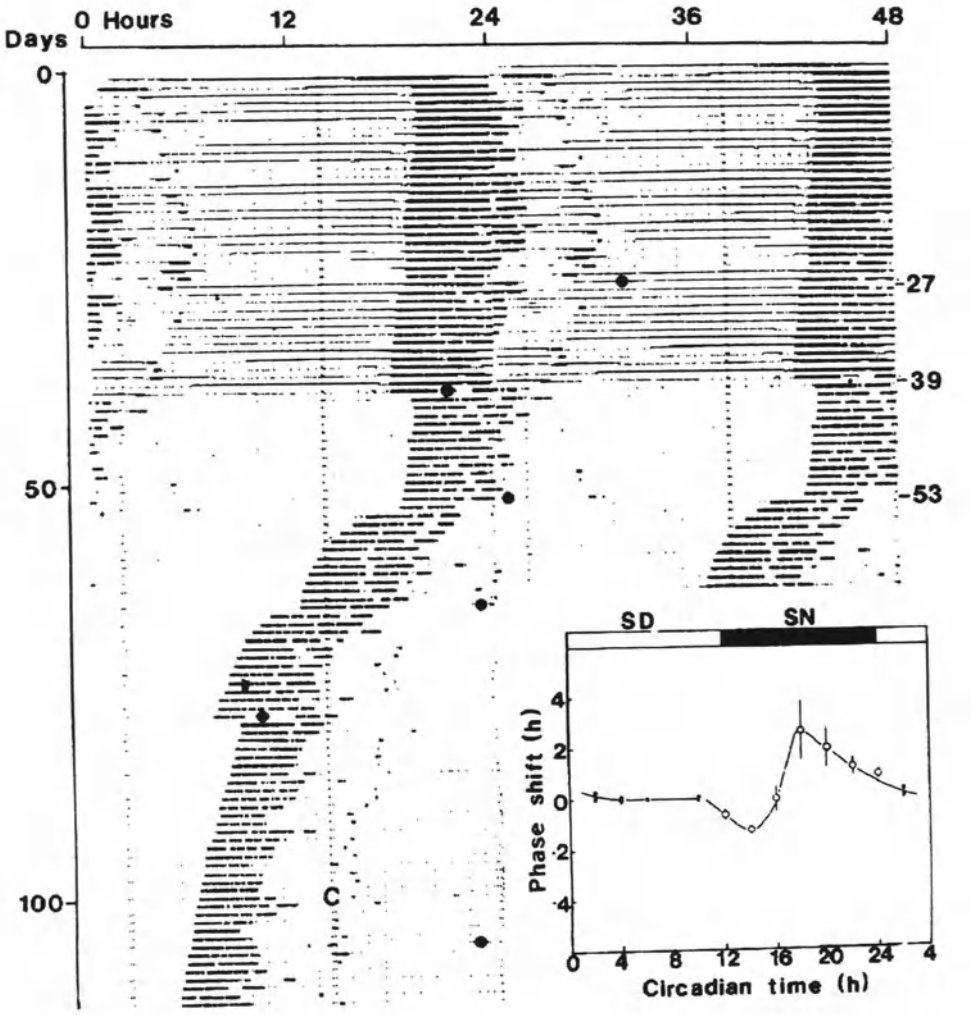

Fig. 2. Generation of a phase response curve (PRC). This record demonstrates the effects of single 1 h pulses of light on the phase of the freerunning rhythm. All light pulses are given while the hamster is in continual darkness. Each solid circle indicates the time of a single pulse of light given on days 27,39,53,65, 78, and 106 of a locomotor record of a Syrian hamster maintained otherwise in continual darkness. The first pulse on day 27 is given in the early Subjective Day and has little effect on the free-running rhythm. In contrast, the light pulse given during the Subjective Night on day 39 leads to a phase delay in the locomotor rhythm. The phase shift for each light pulse is calculated after the free-running period has again stabilized. Note that light pulses given in the early Subjective Night (during the first half of the activity period) generate phase delays that occur rapidly, whereas light pulses given late in the Subjective Night lead to phase advances that are not complete until several cycles. The resulting PRC is shown in the inset. Phase advances are plotted as positive values (above) and phase delays as negative values (below). The time of Subjective Day (SD) and Subjective Night (SN) are shown at the top of the PRC. (Elliott, 1981)

pulse at dawn and dusk, the so-called skeleton photoperiod. In this case, entrainment occurs when:

$$\tau - T = \Delta\phi_1 + \Delta\phi_2$$

where $\Delta\phi_1$ is the phase shift engendered by dawn and $\Delta\phi_2$ by dusk.

Although entrainment in the environment appears to involve a complex interplay of phase and period adjustments, simple laboratory-derived nonparametric entrainment models take us far in understanding and appreciating the role of the light cycle in synchronizing circadian rhythms. As Colin Pittendrigh, a founder of the field of biological timing, has so eloquently pointed out, the primary dividend of entrainment is phase control, thereby allowing an organism to initiate activity at an optimal time with respect to the prevailing light/dark cycle.

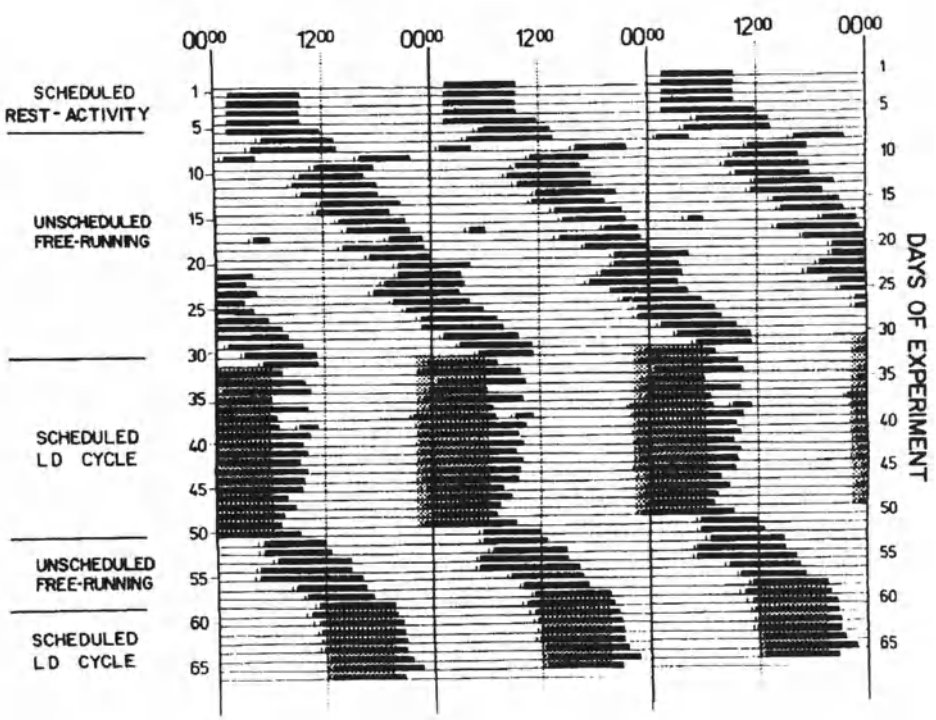

Fig. 3. Entrainment of human circadian sleep-activity rhythm. Triple-plotted sleep-activity cycle of a human subject exposed to a strict light-dark cycle. Horizontal black bars indicate sleep time. On days 1–5, the subject was entrained to normal day/night cues including scheduled rest and activity, light and dark, and mealtimes. On days 6–31 the subject was allowed to self-select times of sleep, meals, and lighting; the record reveals a free-running period of approximately 25 h. On days 32–50 a light/dark cycle was imposed (shaded area is darkness) with gradual transitions of light intensity at dawn and dusk. The subject's sleep-activity cycle entrained to this light/dark cycle. When the subject was released into unscheduled conditions on days 51–58, the rhythm free-ran. From day 59 onwards the light/dark cycle was provided again. (Czeisler et al., 1981)

2.3. Synchronization of Human Circadian Rhythms

Humans exhibit robust circadian rhythms in a large number of behavioral and physiological processes ranging from cognitive performance and sleep-activity cycles, body temperature, melatonin secretion, and corticosteroid levels to the secretion of inorganic ions in the urine. Humans have been most effectively studied in special living units in which they are isolated from all possible temporal clues, including social interactions. As with other animals, human circadian rhythms appear to be synchronized by light/dark cycles (Fig. 3), although, interestingly, when subjects are given access to supplemental, voluntarily controlled lighting (e.g., a reading lamp), the light cycle can be insufficient to entrain the human biological clock without the concomitant exposure to rhythmic social cues. Recently, an exhaustive set of studies have addressed the effects of single and multiple light pulses on the human biological clock. What has emerged is an intriguing finding. Because exposure to single light pulses generates modest phase shifts, leading to a Type 1 PRC, 5 h light pulses on three successive days generate large phase shifts (up to 12 h) and a Type 0 PRC. Thus, it appears that humans can experience large readjustments in the phase of their biological clocks after a relatively short time. This observation challenges conventional wisdom that Type 0 PRCs are only observed in lower organisms, such as algae, plants, and insects.

2.4. Hands of the Clock Versus the Clock Oscillator

It is an empirical generality that phase shifts produced by light pulses delivered in the early Subjective Night vs late Subjective Night differ not only in their sign (i.e., phase delays in the early Subjective Night, phase advances in the late Subjective Night), but also in the speed at which they occur. Light-induced phase delays occur rapidly, certainly within a single circadian cycle, whereas phase advances to a single light pulse develop over several days. These differences

in the speed of phase shifts are readily apparent in the response of the Syrian hamster to single light pulses (Fig. 2). Prima facie, this observation presents a paradox. The adequacy of the phase response curve in predicting attributes of entrained biological rhythms is based on the assumption that phase shifts to single light pulses are completed within the cycle in which the pulse is given. As PRCs are generated for steady-state phase shifts (i.e., calculated after waiting several cycles to insure that the phase shift is stable), it is unclear how the PRC could accurately predict entrainment behavior to repetitive light pulses that do not result in complete phase advances prior to the light pulse on each succeeding day.

In a set of experiments using *Drosophila,* the apparent paradox was resolved. It was discovered that the central component of the biological clock (the so-called light-sensitive "A" oscillator) phase shifts immediately to light pulses, rapidly generating either phase delays or advances. Unexpectedly, the actual behavior shifts more slowly. It appears that the behavior is not controlled directly by the "A" oscillator, but rather by a secondary "B" oscillator. The "A" oscillator is reset immediately by light cycles, but the behavior that is controlled by the "B" oscillator phase advances only after several cycles. Thus, immediately after a light pulse the "hands of the clock" may not accurately reflect the actual "phase of the clock." The asymmetry in time to completion of phase advances and delays appears to be owing to the nature of the coupling of the "A" oscillator to the "B" oscillator and is also a function of the the free-running periods of each oscillator. A detailed discussion of the dynamics of this multioscillator ensemble is outside the scope of the present review. The asymmetry in the speed of phase advances and phase delays in behavior is a very general observation and may underlay the difficulties some individuals experience in readjusting to eastward, but not westward air travel. Eastward travelers would need to phase advance their clocks, which would occur more slowly than the phase delays associated with westward travel.

2.5. Multioscillatory Complexity and Its Effects on the Clock System

In addition to the differences in the speed at which phase advances and delays are effected, other observations likewise support the view that the circadian systems are comprised of several oscillators, more like clock shops than clocks. Perhaps one of the most dramatic behavioral demonstrations of multioscillator organization is the phenomenon of splitting that is observed in several rodent species. Splitting is the dissociation of the circadian locomotor rhythm into two components, typically under constant light. In the split condition, the two freerunning components can exhibit different periods from one another and both will most often differ from the free-running period of the unsplit rhythm (Fig. 4). The phenomenon has provided the basis for a two oscillator model for rodents in which locomotor rhythmicity is proposed to emerge from the interactions of two mutually coupled circadian oscillators, the evening or "E" oscillator and the morning or "M" oscillator.

The coupled oscillator model has been useful in providing a possible explanation for several important circadian phenomena including the history-dependence of the freerunning period. During entrainment both the period of the previous light cycle and the length of the photoperiod (the fraction of time the light is on) can influence the subsequent free-running period. It has been proposed that this phenomenon, commonly referred to as after effects, is because of a change in the phase relationship of the two mutually coupled oscillators (E and M) brought about through entrainment to the previous light cycles. In darkness, the two oscillators eventually regain their natural phase relationship, but this can take many cycles.

Not surprisingly, the human circadian system also appears to be composed of multiple oscillators. The multioscillator complexity is revealed in the phenomenon of internal desynchronization in which the sleep-activity cycle and body temperature rhythm exhibit different periodicities (Fig. 5). Under laboratory conditions the rhythms in body temperature and sleep-activity can spontaneously dissociate or can be driven apart by very long-period light cycles (greater than 30 h periods). Under these exceptional light cycles, some humans will entrain their sleep-activity rhythm to the long cycle, whereas the temperature rhythm, incapable of this entrainment, free-runs. Extensive study of the behavior of the human temperature and sleep-activity cycles indicates that the two oscillators controlling these behaviors are normally coupled, keeping the two cycles synchronized to one another.

2.6. Photoperiodism and Circadian Rhythms

The E/M oscillator model proposed for mammals has also been suggested as a possible basis for the seasonal time measurement system controlling annual reproductive and growth cycles. This issue highlights the important and complex link between the circadian system and seasonal time measurement. As with circadian rhythms, seasonal cycles are widespread.

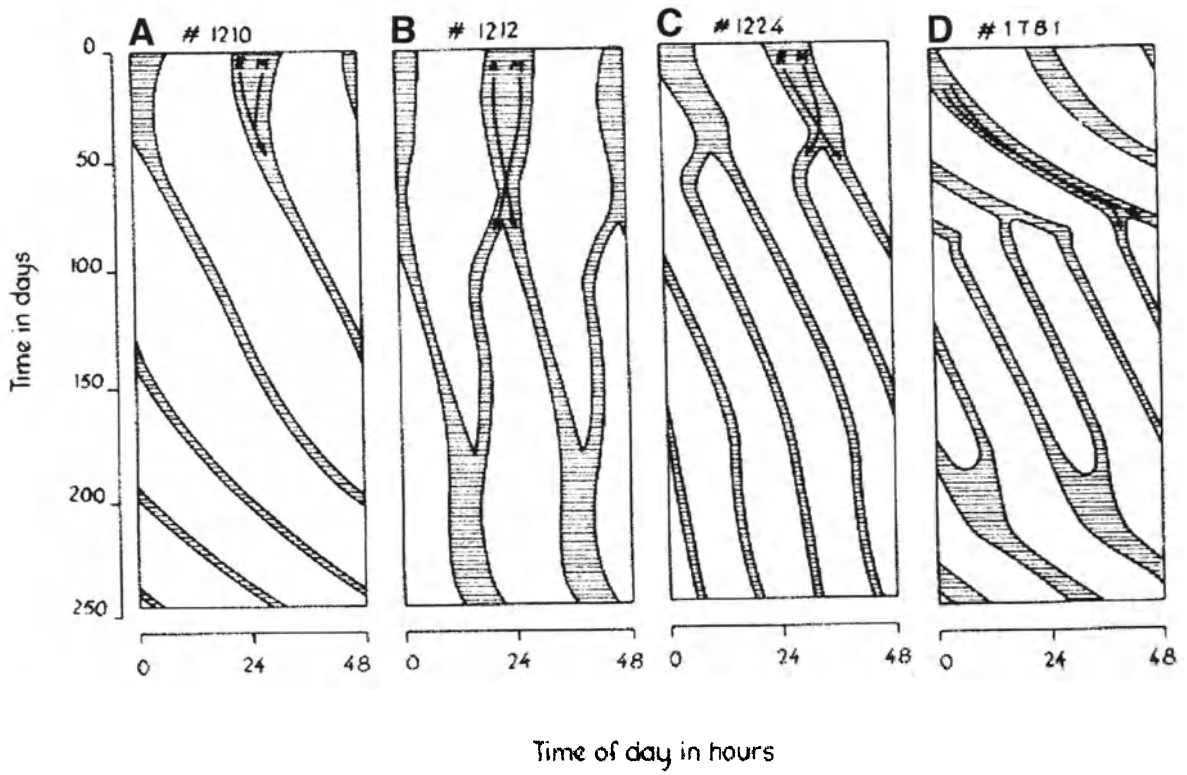

Fig. 4. Evidence for multioscillator organization in hamsters. Locomotor records from four hamsters (*Mesocricetus*) exposed to constant light. For ease in viewing locomotor activity records, the original actograms are represented schematically by horizontal lines indicating the duration of activity for each cycle. In constant light, the circadian activity rhythms most often "split" into two components. Hamsters shown in Panels B, C, and D exhibit splitting behavior, whereas the hamster in Panel A exhibited a compression of the activity period, but did not split. In the split condition, the two activity bands move apart with different periods and usually reentrain to each other with a 180-degree phase difference. They are thought to be controlled by two separate, but mutually coupled oscillators. (Pittendrigh, 1976b)

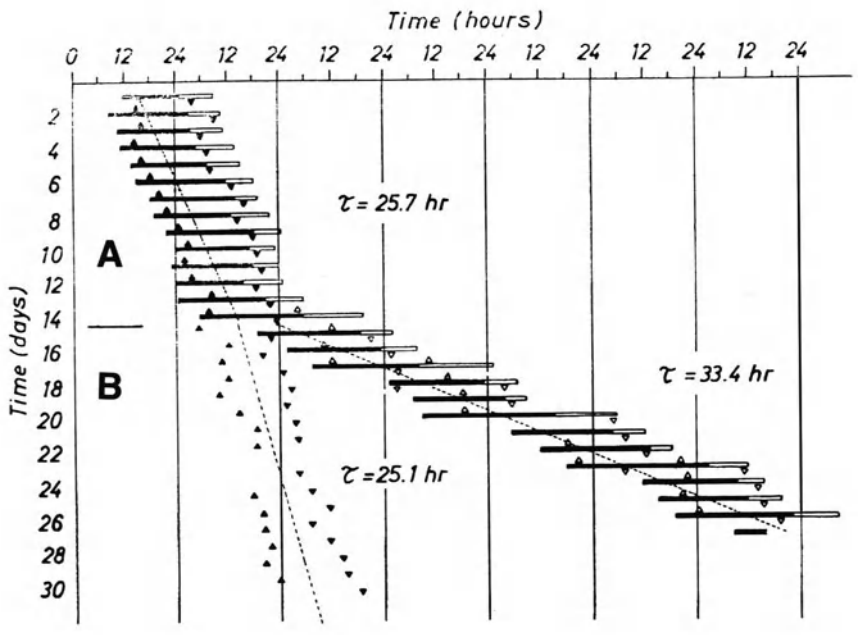

Fig. 5. Spontaneous dissociation of sleep-activity and temperature rhythms. Sleep-activity and temperature record of a 24-year-old female living under constant conditions without any time cues. About day 14, internal desynchronization occurs: the sleep-activity cycle begins to free-run with a long period, whereas the rhythm in body temperature shortens slightly. Temporal course of the rhythm is shown by successive bars. The activity rhythm is represented by bars (black = activity, white = rest). The rectal temperature rhythm is represented by triangles, indicating the temporal position of maximum (upward) and minimum (downward) values. The ordinate is the sequence of the Subjective Days. (Wever 1979)

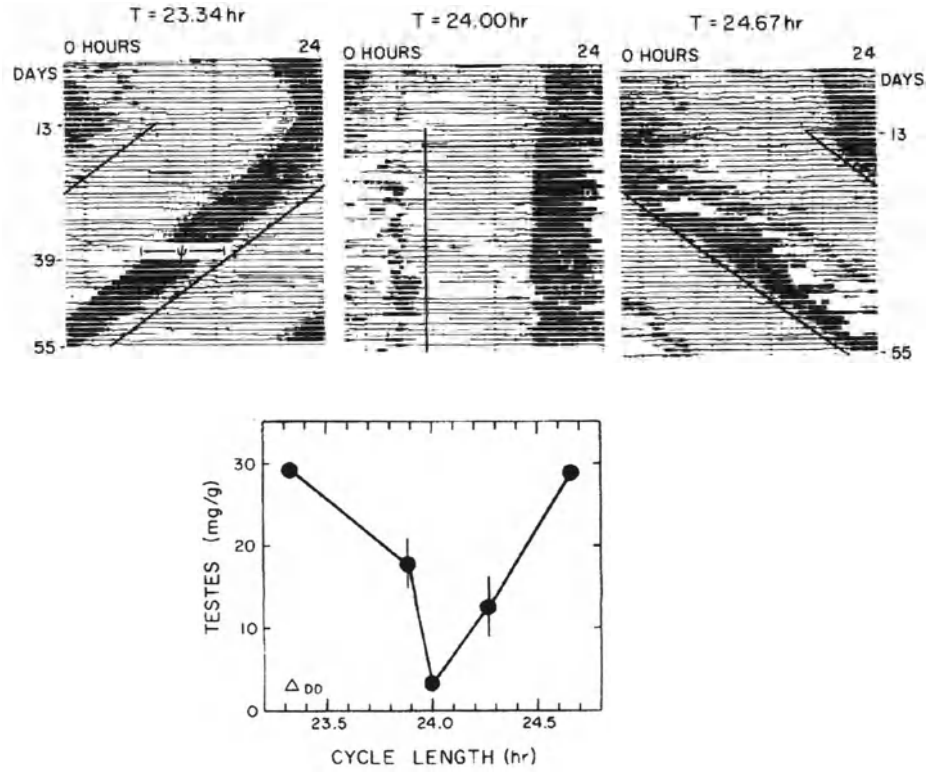

Fig. 6. Circadian timing and photoperiodism in hamsters. Upper panels: By entraining the wheel-running rhythm to periodic light pulses with different periodicites (T's), it is possible to "place" the light pulse at different phases within the circadian cycle. Three examples are given of entrainment by different period light cycles. When $T = 23.34$, the period of the light cycle is less than τ and entrainment occurs via periodic phase advances. Because phase advances occur in the late subjective night, the light pulses (heavy black line that connects the midpoints of the 1 h pulses) fall at the end of the subjective night. When $T = 24.67$, the period of the light cycle is greater than τ and entrainment occurs via periodic phase delays. Because phase delays occur in the early subjective night, the light pulses, indicated by the solid line, occur at the beginning of the nocturnal activity period. When $T = 24$ it is very close to τ and the light pulses fall in or near the dead zone during the subjective day. Lower panel: Testicular response as a function of T. Each point represents the mean testes weight of a group of 10–12 hamsters exposed to one of five different T's. The testes regress when the light pulse falls at a phase that would normally be illuminated by a "short day" light cycle. (Elliott, 1981)

Examples include reproduction, hibernation, diapause in insects, growth, flowering in plants, and animal migration. The issue of seasonal rhythmicity is especially interesting from an evolutionary point of view insofar as many different strategies have evolved to measure seasonal time. Some organisms, including some birds and mammals, rely on circannual clocks that are entrained by the prevailing photoperiod. Others animals, notably some insect species, have developed mechanisms for measuring the actual duration of the photoperiod. In other cases, photoperiodic time measurement uses the circadian clock in seasonal time measurement.

In experiments carried out in several laboratories, investigators were able to determine that it is not the actual day length or night length of the light cycle that is measured, but rather the phases at which light occurs within the circadian cycle. This concept, first elaborated as the Bunning Hypothesis, was later confirmed by showing that even brief periodic pulses of light can mimic a long photoperiod if provided at the appropriate phase of the circadian cycle. A particularly instructive example of this occurs in hamsters which normally reproduce on long days. In an experiment employing 1 h light pulses, it was possible to either induce or not induce a decrease in testes size, characteristic of short winter days, by illuminating the animal at the appropriate time of day (Fig. 6). By taking advantage of the fact that the phase of the light pulse within the cycle is a function of the difference in period between τ and T and by selecting different T's, it was possible to position the light pulse at different phases within the circadian cycle. When the light pulse fell at a phase normally illuminated only

by a short winter day, there was gonadal regression, similar to what was observed in continual darkness. On the other hand, if the light pulse fell at other phases within the cycle, the testes remained large. This is a very remarkable result in that the same duration light pulse can either be stimulatory or not stimulatory depending upon the phase at which it is given.

The Bunning Hypothesis has also been referred to as the external coincidence model in which light plays a dual role. First, light cycles entrain an underlying circadian rhythm. Second, light induces a photoperiodic response if there is coincidence between an external light signal and a particular phase within the circadian cycle. A second model has been proposed, the internal coincidence model, in which light plays a single role in entrainment. In this model, light causes internal oscillators to change their phase relationships and when the two internal rhythms are appropriately coincident a photoperiodic response occurs. In the case of the E and M oscillators in rodents, the hypothesis is that the M oscillator is entrained by dawn and the E oscillator by dusk. Consequently, different length photoperiods lead to differing phase relationships between the E and M oscillators with some phase relationships being stimulatory and some not. These are provocative hypotheses and it is not yet certain which of these two models best describes the photoperiodic time measurement process in mammals. Indeed, both mechanisms may be utilized in different organisms or integrated together within individual time measurement systems.

3. PHYSIOLOGY OF CIRCADIAN SYSTEMS

3.1. Elements of the Circadian System

As discussed, phenomenological models have provided great insight into the functional and adaptive significance of biological clocks. At the same time, animal physiologists have attempted to identify the actual tissue and cellular substrates of the timing system. The experimental strategy that has guided this work initially was straightforward insofar as the system was viewed as a simple cascade consisting of an endogenous oscillator responsible for generating the circadian rhythm, a photoreceptor structure entraining the oscillator and coupling pathways between these elements and to the motor system or other effector circuits responsible for generating the observed behavior (Fig. 7). At the outset, the issue was primarily whether or not the circadian oscillator was a discrete process that was localizable to a specific portion of the nervous system or whether, similar to memory storage mechanisms, the oscillator was diffusely organized throughout large portions the nervous system. Good fortune prevailed and from the study of a number of systems it is clear that circadian oscillators can be localized within restricted portions of the nervous and endocrine systems. There are a number of model systems that have provided complementary insights into circadian physiology. In mammals, a central circadian oscillator resides within the hypothalamus.

3.2. The Suprachiasmatic Nucleus is a Mammalian Circadian Pacemaker

The paired suprachiasmatic nuclei (SCN) of the hypothalamus act as circadian oscillators in controlling a host of physiological and behavioral processes in mammals. The rat SCN contains about 8000 neurons on each side. The cells are small about 8–12 μm in diameter and they appear to make extensive connections among themselves. The evidence that these nuclei govern circadian rhythmicity is substantial and diverse. SCN ablation results in behavioral and physiological arrhythmicity. Isolating the SCN within the brain yields similar results: SCN neurons continue to fire in a circadian pattern, but all other processes outside the SCN become arrhythmic. SCN isolated in vitro also exhibit circadian rhythms of neuronal electrical activity. Finally, implants of fetal SCN tissue restore rhythmicity to SCN-lesioned, arrhythmic animals.

3.2.1. Individual Neurons Express Circadian Rhythms

Despite this strong evidence implicating the SCN as a circadian pacemaker, less is known about the underlying cellular processes generating the circadian periodicity. As mentioned above, cellular investigations employing an acute brain slice preparation reveal a circadian rhythm in single and multiunit neuronal activity that persists for at least three cycles in vitro. Recordings from SCN explants maintained under culture conditions reveal circadian rhythms that persist for several weeks (Fig. 8). SCN explants secrete vasopressin rhythmically for up to four cycles, and the timing of vasopressin release matches the pattern of neuronal activity. It is not certain which cell types within the SCN generate these circadian oscillations.

Recent evidence suggests that individual neurons within the SCN can generate circadian periodicities. SCN neurons from rat, mouse, or hamster that are dispersed in culture exhibit circadian rhythms in electrical activity for up to 4 wk after dispersal. Although

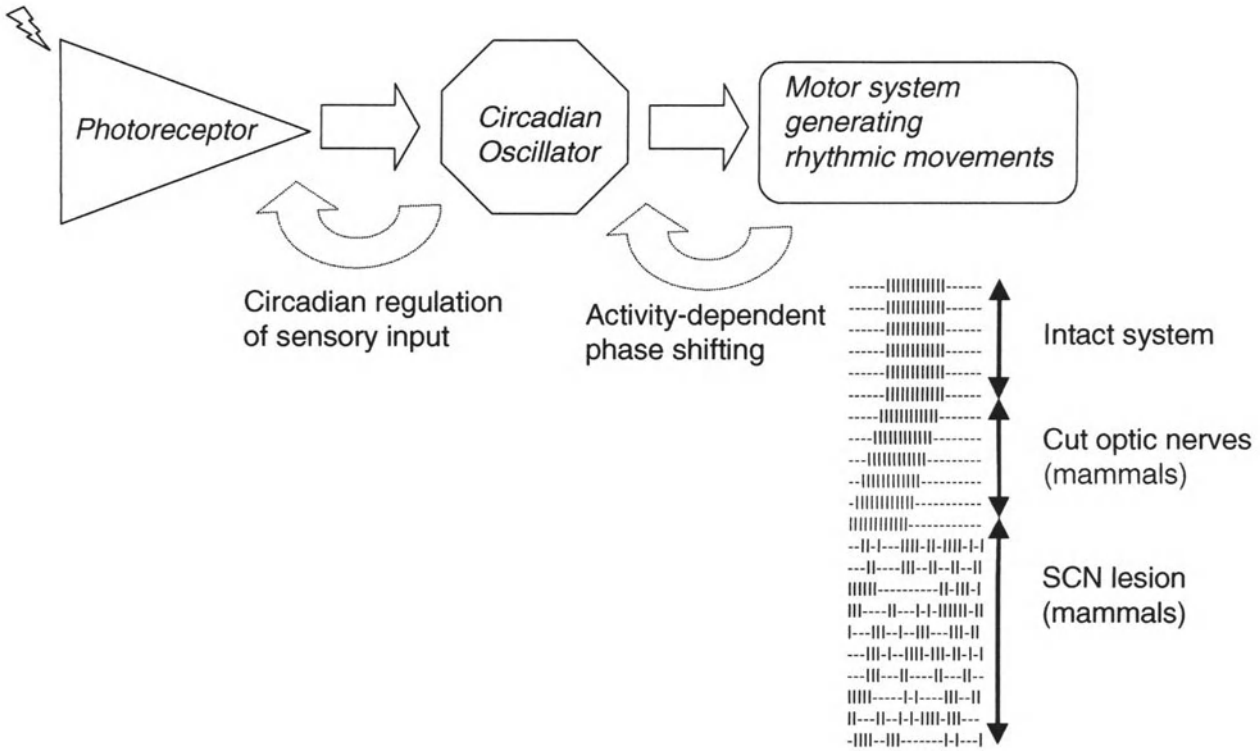

Fig. 7. Conceptual model of the circadian system. The circadian system is conceived as consisting of three components: 1) a photoreceptor that provides photic information about local time, 2) a circadian oscillator that generates the near 24 hour cyclicity, and 3) the motor system circuitry responsible for generating the overt rhythmic behavior. Disrupting the photoreceptor or destroying the pathway to the oscillator will result in free-running behavior in the presence of light cycles. Disruption of the oscillator or its pathway (such as an SCN lesion in mammals) results in behavioral aperiodicity. Although, at one time, the system was seen strictly as a unidirectional cascade, more recently it has become clear that locomotor activity may feedback to influence the oscillator. In some organisms, the oscillator rhythmically controls the sensitivity of its own photoreceptive input.

recordings from physically isolated SCN neurons are needed for definitive proof, it seems most likely that individual neurons are circadian oscillators.

3.2.2. Neurotransmitters Entrain the SCN

In addition to generating a circadian oscillation, the SCN must be able to entrain to the environment in order to act as a pacemaker. The SCN receives specialized neuronal inputs required for entrainment, including input from the eyes via the retinohypothalamic tract. The in vitro slice preparation has proven useful for studying the processes by which the SCN is entrained by light cycles and modulated by regulatory pathways within the central nervous system. Various treatments can be applied to the SCN at discrete time points, and any shift in the phase of the circadian oscillation can be measured by monitoring neuronal electrical activity.

Just as light can phase shift circadian rhythms only during the Subjective Night, the SCN is sensitive to the phase-shifting effects of neurotransmitters at specific times of the cycle. Many neurotransmitters and neurohormones can phase shift the SCN's rhythm of neuronal activity (*see* Chapter 24 for a discussion of melatonin's role in entrainment), but only a few can mimic the effects of light. Glutamate is one of these neurotransmitters: it delays the rhythm in neuronal activity during the early subjective night, advances the rhythm during the late subjective night, and has no effect during the subjective day. A current model of glutamate's action (Fig. 9) involves the stimulation of NMDA receptors, increases in intracellular calcium, activation of nitric oxide synthase, and extracellular movement of nitric oxide. Both glutamate and nitric oxide induce the phosphorylation of the $Ca2^{+}$/ cAMP response element binding protein (CREB); phosphorylation of CREB stimulates its positive regulation of immediate early-gene transcription. An understanding of how glutamate and nitric oxide regulate the action of CREB in a clock-dependent manner will be crucial for an understanding of the biochemical basis of SCN entrainment.

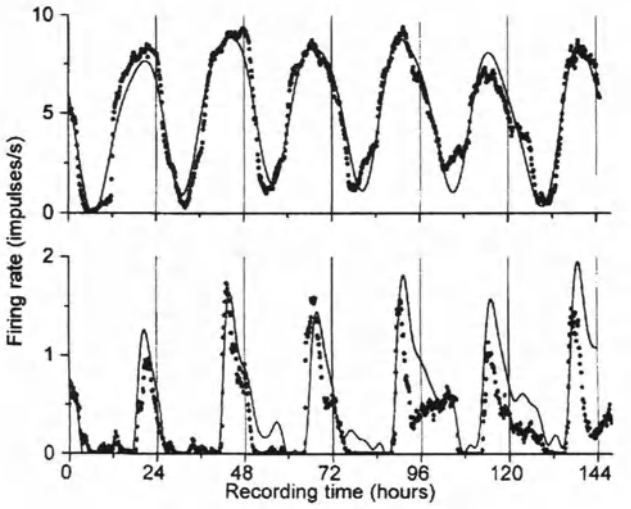

Fig. 8. Circadian rhythms in electrical activity in SCN explants. Impulse frequency from two neurons recorded from separate SCN explants. Explants were prepared from 5 to 6 d old mice. The 300 μm slice was placed on a multimicroelectrode array that allows for long duration recording of spontaneous neuronal impulses. The recordings were initiated after 23 d (top) or 19 d (bottom) in vitro. Both cells displayed a circadian rhythm in firing rate with a period close to 24 h. (Herzog et al., 1997)

3.2.3. Pacemakers Outside of the SCN

Although the SCN appears to be the dominant circadian pacemaker structure in the mammal, there are other circadian pacemakers. The isolated retina of rodents expresses a robust circadian rhythm in melatonin release. In fact, retinal pacemakers are relatively common in both vertebrate and invertebrate phyla. Circadian rhythms have been identified in the isolated retinas of fish, birds, amphibians, reptiles, and mollusks. In lower vertebrates and invertebrates, a number of other structures continue to express circadian periodicities in a dish. Techniques are now available to measure molecular rhythmicity within a number of isolated tissues in *Drosophila* revealing rhythms in at least five different tissues. It seems almost certain that other mammalian tissues are also rhythmic. Recent evidence shows that immortalized mammalian fibroblasts express circadian rhythms in vitro. These findings raise important issues about the organization of circadian systems. Clearly, structures such as the SCN play dominant roles in controlling a host of circadian functions within the mammal. Whether oscillations in other tissues play localized roles in regulating specific tissues or contribute more globally within a multioscillator ensemble remains to be determined.

4. MOLECULAR BASIS OF CIRCADIAN RHYTHMS

It is becoming apparent that circadian pacemakers reside within specialized cells in the organism. Indeed, the mechanism that generates daily rhythms appears to involve primarily the intracellular processes of transcription and translation and, therefore, is distinct from the generation of shorter periodicities expressed by neurons that rely on ionic fluxes and metabolic pumps.

Most of our understanding about the molecular basis of biological timing is derived from studies on *Drosophila*. In the 1970s, screening experiments identified mutant flies with slow, fast and aperiodic behavioral phenotypes. Cloning of the gene responsible for the altered circadian behavior produced the first bonafide clock gene, *Period* or *Per*. Subsequently, three other clock genes (*Timeless, dClock*, and *Cycle*) have been characterized. Although the

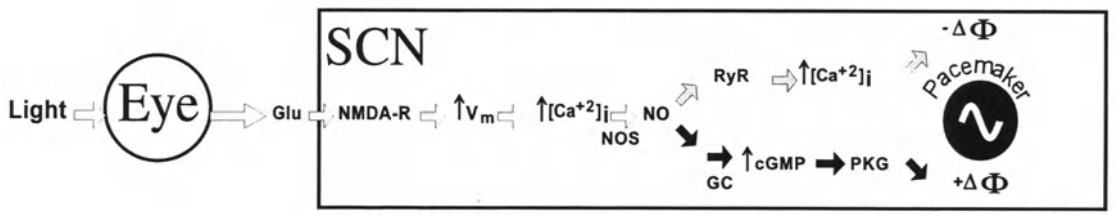

Fig. 9. A schematic of the mammalian photic entrainment pathway. Light entering through the eyes acts to change the phase of the mammalian circadian pacemaker by causing the release of glutamate (Glu) from the terminals of retinal projections to the SCN. Glu depolarizes cells in the SCN by binding NMDA receptors (NMDA-R), causing an influx of calcium (Ca^{+2}). Elevated levels of intracellular calcium activate nitric oxide synthase (NOS), which produces the gaseous neurotransmitter, nitric oxide (NO), which has differing effects on the pacemaker depending upon the time of day. In the early evening, NO leads to a phase delay through an intracellular release of calcium mediated by ryanodine receptors. In the late evening, NO leads to a phase advance through a separate pathway involving activation of cGMP-dependent protein kinase (PKG). It is not well understood how these biochemical events affect the molecular events that comprise the pacemaker mechanism. (Ding et al., 1998)

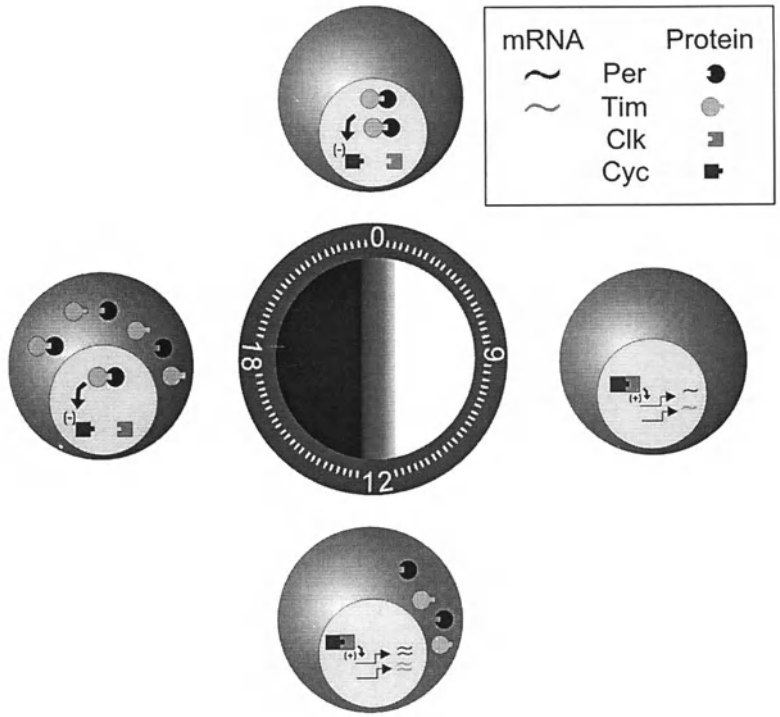

Fig. 10. A molecular model for circadian rhythm generation. Near dusk (12:00), transcription of two clock genes, *Per* and *Tim*, produces high levels of their respective mRNAs. Translation of the proteins from these mRNAs lags by a few hours so that PER and TIM proteins reach their peak levels around midnight (18:00). PER and TIM bind to each other, enter the nucleus, and inhibit the binding of two transcriptional activators, CLK and CYC. The breakup of the CLK–CYC dimer leads to a rapid reduction in the transcription of *Per* and *Tim*. Over the next few hours, the PER–TIM complex degrades. As PER–TIM levels fall (6:00), CLK–CYC complexes begin to form. The new CLK–CYC dimers begin the next circadian cycle by activating transcription of *Per* and *Tim* again.

mechanism is not complete in all of its details, a very satisfying picture is emerging of how these genes and their products work together in an autoregulatory feedback loop to produce circadian periodicity.

Figure 10 illustrates how the daily rhythm appears to be generated in constant darkness. The *dClock (Clk)* and *Cycle (Cyc)* genes are constituitively expressed, producing a pair of proteins that bind to each other in the cell's nucleus. This protein dimer activates transcription of the *Per* and *Timeless (Tim)* genes. At about noon (six hours after dawn) the mRNA levels of *Per* and *Tim* rise slowly over several hours. The amounts of PER and TIM proteins follow about 4 h later. As the proteins accumulate, they bind to each other and enter the cell's nucleus beginning near dusk and peaking about four hours before dawn. Elevated levels of the PER-TIM dimer in the nucleus inhibit the CLK-CYC dimer's ability to activate transcription. As the repression increases, transcription of *Per* and *Tim* decreases and, over the next few hours the *Per* and *Tim* mRNA levels begin to fall and their respective protein levels fall at dawn. With reduced PER-TIM dimers, the cycle begins again with CLK-CYC dimers driving transcription of the *Per* and *Tim* messages. Thus, the circadian cycle consists of a feedback loop where proteins negatively regulate the transcription of their genes with a period close to 24 h.

Importantly, this molecular oscillator synchronizes to the local light cycle. Light acts to degrade the TIM protein at all times. Because both PER and TIM must dimerize to enter the nucleus, light acts to block nuclear entry of both proteins. Thus, while mRNA levels are rising after noon, light prevents TIM protein from accumulating until after dusk. As the light dims, TIM accumulates binds to PER and the PER–TIM dimer enters the nucleus to shut down transcription of both *Per* and *Tim*. In this way, light directly determines the amount of a central clock component (TIM) which determines when the clock can advance to the next step in its daily cycle.

Evidence from another well-studied biological clock in a bread mold, *Neurospora*, suggests that, although the details may differ, the elements of all circadian pacemakers share specific structural similarities. Three genes have been identified in the autoregulatory loop that generate circadian rhythms in asexual spore formation in this fungus: *Frequency* (*Frq*), *White Collar 1* (*WC1*), and *White Collar 2* (*WC2*). Highly conserved amino acid sequences within these proteins and the clock proteins in animal circadian clocks are important for specific protein–protein dimerization, DNA binding and transactivation of transcription. Like the *Drosophila* clock, the *Neurospora* clock is also comprised of a 24-h, auto-

regulatory negative feedback loop between translation and transcription where the FRQ protein inhibits the WC1-WC2 dimer s ability to drive *Frq* expression.

Researchers are now finding similar transcription/translation negative feedback loops across all phyla. Homologues to the *Period*, *Clock*, *Timeless*, and *Cycle* genes have been found in mammalian circadian pacemakers and specific functional domains within these genes appear to be highly conserved. These shared features have led researchers to believe that the principles that generate circadian rhythms are ancient and many of the molecular elements are identical.

5. CONCLUSIONS

Few physiological or behavioral processes escape regulation by the biological clock. The profound influence of this cyclic modulator on bodily homeostasis makes it important that we understand the origins and pathways of the circadian clock system. Although much has been learned recently about the molecular and cellular structure of this system, much remains to be revealed. For example, it is not known with certainty which cell types within the SCN are capable of expressing autonomous rhythms and whether all cells producing such rhythms provide identical or complementary signals. It is also uncertain how many mammalian tissues can generate circadian rhythms and what role those rhythms serve. For example, retinal rhythms are now well documented in rodents, leaving us with a suspicion that other tissues, as in *Drosophila*, are capable of autonomous oscillations. The existence of a large number of oscillating sites raises many intriguing issues about internal coordination and hierarchical control. Observations of behavior such as splitting and after effects in rodents, speed of phase shifts in *Drosophila*, and internal desynchronization in humans have led to postulated phenomenological models based on multiple oscillators. It will be of substantial interest to determine whether the models correspond in any way with the multiple oscillator organization that is being revealed through physiological study. For example, will "E" and "M" oscillators be revealed with anatomical specificity? These are among many important issues that await experimental resolution.

SELECTED READINGS

Aschoff J. Exogenous and endogenous components in circadian rhythms. Cold Spring Harbor Symp Quant Biol 1960; 25:11.

Block GD, Khalsa S, Michel S, Geusz M, McMahon D. Cellular basis of biological timekeeping. Int Rev Cytol 1993; 146:83.

Czeisler CA. The effect of light on the human circadian pacemaker. In: Circadian Clocks and Their Adjustment, Chadwick & Ackrill, West Sussex, England: CIBA Foundation John Wiley & Sons, Ltd., 1993; 254.

Elliott J. Circadian rhythms, entrainment and photoperiodism in the Syrian hamster. In: BK Follett, DE Follett, eds. Biological Clocks in Seasonal Reproductive Cycles. Bristol, England: Colston Papers No 32 Colston Research Society, John Wright & Sons, Ltd. 1981; 203.

Gillette MU. Cellular and biochemical mechanisms underlying circadian rhythms in vertebrates. Current Opinion Neurobio 1997; 7:797.

Klein DC, Moore RY, Reppert SM (eds). Suprachiasmatic Nucleus: The Mind's Clock. New York: Oxford University Press, 1991.

Czeisler CA. The effect of light on the human circadian pacemaker. In: Circadian clocks and their adjustment. Chadwick & Ackrill, West Sussex, England: CIBA Foundation John Wiley & Sons, Ltd, 1993; 254.

Ding JM, Buchanan GF, Tischkau SA, Chen D, Kuriashkina L, Faiman LE, Alster JM, Mcpherson PS, Campbell KP, Gillette MU. A neuronal ryanodine receptor mediates light-induced phase delays of the circadian clock Nature 1998; 394:381.

Dunlap JC. Circadian Rhythms: An End in the Beginning. Science 1998; 280:1548.

Pittendrigh CS. Circadian rhythms and the circadian organization of living systems. Cold Spring Harbor Symp Quant Biol 1960; 25:159.

Pittendrigh, CS. Circadian organization and the photoperiodic phenomena In: BK Follett, DE Follett, eds. Biological Clocks in Seasonal Reproductive Cycles. Bristol, England: Colston Papers No 32 Colston Research Society, John Wright & Sons, Ltd., 1981; 1.

Pittendrigh CS, Daan S. A functional analysis of circadian pacemakers in nocturnal rodents. IV. Entrainment: pacemaker as clock. J Comp Physiol 1976a; 106:291.

Pittendrigh CS, Daan S. A functional analysis of circadian pacemakers in nocturnal rodents.V. Pacemaker structure: a clock for all seasons. J Comp Physiol 1976b; 106:333.

Weaver R. The Circadian System of Man. Results of Experiments under Temporal Isolation. New York: Springer-Verlag, 1979.

23 Influence of Light and the Pineal Gland on Biological Rhythms

Henryk F. Urbanski, PHD

CONTENTS

1. INTRODUCTION

"Let us then conceive here that the soul has its principal seat in the little gland which exists in the middle of the brain, from whence it radiates forth through all the remainder of the body by means of the animal spirits, nerves, and even the blood, which, participating in the impressions of the spirits, can carry them by the arteries into all the members. . . ."

More than 300 years ago, the French philosopher René Descartes (1596–1650 AD) was well aware of the existence of the *pineal gland* and in his treatise "The Passions of the Soul" he described it as playing a crucial role in the interaction between the soul and the body. This 17th century Cartesian concept of the pineal gland as the "seat of the human soul" was probably developed from the much earlier postmortem studies of Herophilus (325–280 BC), an Alexandrian anatomist, who according to the chronicles of Galen (130–200 AD) referred to the pineal gland as a valve regulating the flow of "spirits" between the *3rd ventricle* and *4th ventricle* of the brain. Looking back at these ancient ideas, it is all too easy to dismiss them as being erroneous, too simplistic, or even superstitious. With the surge of scientific knowledge in recent decades, especially in the fields of biochemistry, neuroanatomy, and molecular biology, we now know that the pineal gland plays an important role as a circadian photoneuroendocrine transducer. And yet, even today many questions remain unanswered about this mysterious "little gland" and speculations regarding the physiological role of *melatonin*, its principal hormone, are likely to persist well into the 21st century.

From: *Neuroendocrinology in Physiology and Medicine*, (P. M. Conn and M. E. Freeman, eds.), © Humana Press Inc., Totowa, NJ.

2. THE PINEAL GLAND

2.1. Anatomy

The pineal gland, also called the *epiphysis cerebri*, is a small reddish-brown structure, which in humans, is shaped somewhat like a pine cone (*pinea* in Latin). Even in adults, it is only 5–10 mm long and has a mass of only 100–150 mg. Why then is it so intriguing to anatomists and philosophers? First, the pineal gland is unusual for a brain structure because it is unpaired.

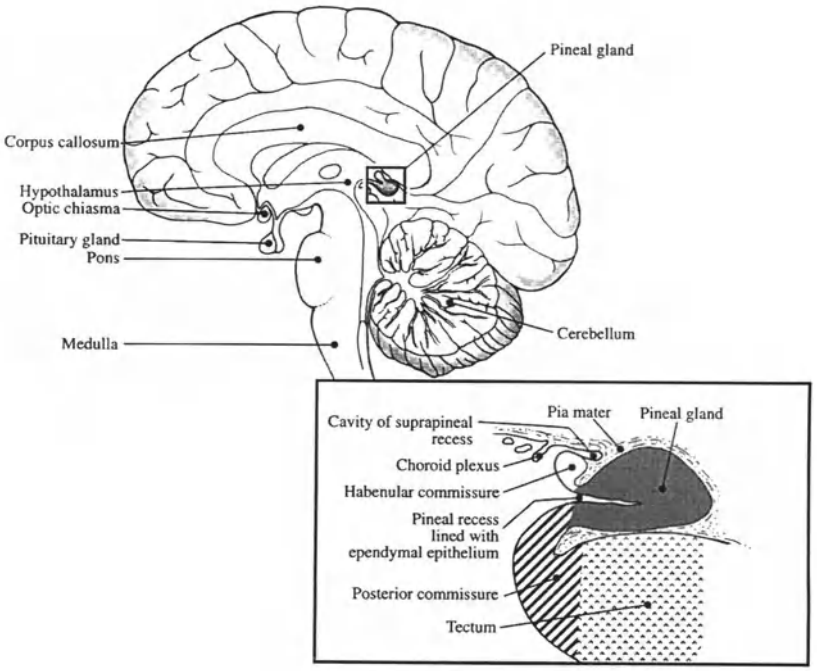

Fig. 1. Diagram of midsagittal section through the human brain emphasizing the central location of the pineal gland and a close-up view of its attachments.

Second, it is situated almost at the center of the brain, just below and slightly to the rear (Fig. 1). Third, although it is a *forebrain* structure it is situated in very close proximity to the upper part of the *midbrain*, overlying the cerebral aqueduct (*aqueduct of Sylvius*) and extending posteriorly above and between the paired *superior colliculi* of the *tectum* (Fig. 2). Embryologically, the pineal gland arises as an evagination of modified *ependymal cells* from the roof of the dorsocaudal third ventricle, becoming one of many circumventricular secretory organs in which the *blood-brain barrier* is absent. In most mammals the pineal gland retains a stalk-like connection which in humans is composed of two laminae, separated by the pineal recess of the third ventricle (Fig. 1). The superior (rostral) lamina is continuous with the *habenular commissure* and the inferior (caudal) lamina with the *posterior commissure*. Overall, there is considerable variation in the relative location, size, and shape of the pineal gland in mammals. In laboratory rodents such as rats and hamsters the bulk of the gland is situated much more superficially than in humans, lying against the inner cranium and well away from the third ventricle; it is securely attached to the meninges and usually remains stuck to the inner cranial surface when the brain is removed during routine dissection. In mammals that are indigenous to the temperate zones and polar regions of the world the pineal gland is often very large (e.g., hares and seals), whereas in mammals that live closer to the equator it is usually very small (e.g., elephants and rhinoceros) or may even be absent entirely (e.g., anteaters and sloths). To some extent, therefore, the size of the pineal gland may reflect the degree to which a species is *photoperiodic* (i.e., relying on the annual change in day length or *photoperiod* to control its breeding season; *see* Section 6.1). In some fish, amphibians, and lacertilian reptiles the pineal gland has an extracranial parietal component with lens-like and retina-

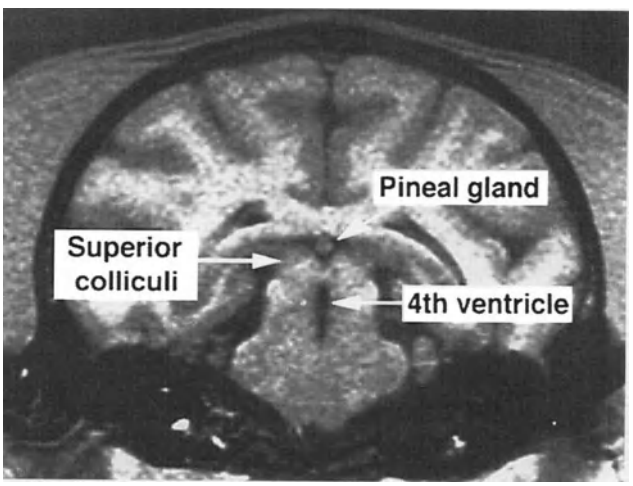

Fig. 2. Coronal MRI section through the brainstem of a rhesus monkey showing the anatomical relationship between the pineal gland and midbrain structures. In the rhesus monkey, as in the human, the pineal gland is located almost at the center of the brain.

like structures, which can act as a *photoreceptor* or "third eye." However, in mammals the pineal gland is not directly responsive to photic stimuli. Instead, it receives this environmental information via a complex multisynaptic neural pathway that originates in the retina (*see* Section 4.3.).

2.2. Innervation and Vascular Supply

The pineal gland of mammals has a peculiar innervation for a brain structure. In most species, including humans, the pineal gland receives little afferent innervation directly from the brain itself, despite its close proximity (Fig. 1 and Fig. 2). Instead, its most significant afferents come from postganglionic *sympathetic* fibers that arise from the paired *superior cervical ganglia* (SCG) in the neck. In humans, these fibers traverse along the arterial supply to the head before forming the bilateral *nervi conarii*. They then course rostrally beneath the *great cerebral vein of Galen* and enter the pineal gland at its posterior pole. Although there is some evidence for *parasympathetic*, commissural and peptidergic innervation of the pineal gland, the function of these neural inputs is unclear. The pineal gland is highly vascularized and has a blood flow rate of about 4 mL/min/g, which is considered to be second only to that of the kidney. Blood is supplied to the pineal gland by vessels that originate in the adjacent *choroid plexus* (Fig. 1), whereas venous drainage from its capillary network ultimately empties into the great cerebral vein of Galen. In most mammals, including humans, the pineal gland lacks a blood-brain barrier and so is susceptible to the influence of peripherally acting drugs.

2.3. Structure

The pineal gland is divided into lobules by richly vascular connective tissue septa. These lobules contain some glial cells, many of which are *astrocytes*. However, the bulk of the gland is composed of secretory parenchymal cells called *pinealocytes*, which have a distinctive shape. Their bulbous cell body contains a large round nucleus and granular cytoplasm. It also has characteristic club-like processes closely apposed to fenestrated capillaries (typical of endocrine glands). From adolescence onwards calcareous deposits (called *acervuli*) become prominent in the pineal gland. They contain $Ca_3(PO_4)_2$ and *hydroxyapatite* and are secreted by the pinealocytes. It is currently unknown whether this calcification of the pineal gland has any physiological significance, either during the onset of puberty or during old age. The degree of calcification varies widely between individual adults and does not show an obvious relationship with the pineal gland's endocrine capacity. On the other hand, the calcareous deposits make the adult human pineal gland clearly visible in brain CAT scans. Indeed, because of its near-central location in the brain the calcified pineal gland is commonly used by radiologists to determine whether there has been a shift in midline brain structures.

3. THE PINEAL GLAND AS AN ENDOCRINE ORGAN

3.1. Discovery of Melatonin

The pineal gland contains many bioactive compounds including several peptides (e.g., *vasopressin*, *renin-angiotensin*, *ACTH*, and *POMC*-related peptides). However, by far the most extensively studied pineal compound is a highly potent *indole* called melatonin, discovered in 1958 by Aaron Lerner et al. Although it was known for many years that pineal gland extracts could lighten the skin color of amphibians, it was not until extracts from thousands of bovine pineal glands were processed chromatographically that a bioactive compound, *5-methoxyindole acetic acid*, was identified. Because this novel compound could affect *melanin* pigmentation in frog skin and because it was chemically related to *serotonin* (5-hydroxytryptamine; 5-HT), it was named melatonin. Despite its name, however, it should be emphasized that melatonin has no effect on melanophores in mammals.

3.2. Biosynthesis of Melatonin

Because of the efforts of many researchers, including Julius Axelrod, David Klein, and Richard Wurtman, the biosynthetic pathway of melatonin in the pineal gland is now well established (Fig. 3). The precursor of melatonin is the essential amino acid *L-tryptophan* (L-TRP), which is taken up from the blood by pinealocytes and converted to serotonin through hydroxylation and decarboxylation. This two step process involves the enzymes *tryptophan-5-hydroxylase* and *5-hydroxytryptophan decarboxylase*, respectively. Concentrations of serotonin in the pineal gland, which are generally much higher than in the rest of the brain, are especially elevated during the day (*photophase*) but then fall markedly during the night (*scotophase*) as a result of its conversion to melatonin. This nocturnal conversion involves a two-

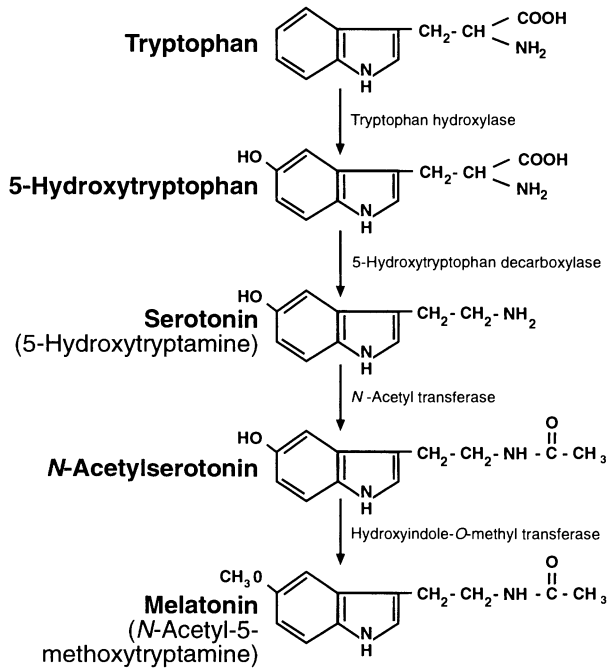

Fig. 3. The chemical structure of melatonin and its biosynthetic pathway. Note that the *N*-acetylation of serotonin and its subsequent conversion to melatonin occurs almost exclusively at night.

step enzymatic process. Initially, serotonin is converted to *N-acetyl serotonin* by the enzyme *N-acetyl transferase* (NAT), which shows a 30- to 70-fold increase in activity during the night. *N-acetyl* serotonin is then methylated by the enzyme *hydroxyindole-O-methyl transferase* (HIOMT) to produce *N-acetyl-5-methoxytryptamine*, more commonly known as melatonin.

3.3. Metabolism of Melatonin

Between 50 and 70% of circulating melatonin is thought to be bound to plasma albumin but it is unclear whether or not this has any physiological significance. Melatonin is primarily metabolized in the liver, and also in the kidney. Its clearance from the peripheral circulation is biphasic, with half-lives of about 3 and 45 min. (Note, its metabolism in the cerebral spinal fluid is probably different). Although some variation exists in melatonin's metabolic pathway between species, in the human and rodent melatonin is inactivated by 6-hydroxylation followed by sulfate and glucuronide conjugation, with *6-sulfatoxymelatonin* (aMT6s) being the main urinary metabolite. Both plasma and urinary concentrations of aMT6 closely correlate with plasma melatonin concentrations, providing a useful clinical measure of melatonin secretion in humans. In the central nervous system (CNS), some melatonin is thought to undergo ring splitting to produce *methoxykynurenamines*, which may be neuroactive.

3.4. Melatonin Binding Sites

Based on the pharmacology and kinetics of 2-[^{125}I]melatonin binding, melatonin binding sites are generally classified as being either high-affinity receptors (ML_1) or low-affinity receptors (ML_2), with equilibrium dissociation constants (K_d) of <200 pM and >1 nM, respectively. The low-affinity melatonin receptors do not show a specific distribution pattern in the brain and their physiological role is unclear. In contrast, high-affinity melatonin receptors have been studied extensively through the use of radioligand binding and quantitative autoradiography, and have been identified in a wide range of vertebrate species, including humans. The affinity of these receptors is sensitive to *guanine* (G) nucleotides and their activation causes an inhibition of *adenylate cyclase* via a pertussis toxin-sensitive pathway. They therefore resemble receptors belonging to the superfamily of G-protein-coupled receptors. Although high-affinity melatonin receptors are concentrated in specific brain nuclei, they are also localized in a few non-neuronal sites. In humans and most other mammals, the sheep being a notable exception, these receptors are abundant in the *suprachiasmatic nucleus* (SCN) and much of melatonin's influence on circadian rhythms is probably mediated through this population of receptors (*see* Section 5). In many photoperiodic rodents high-affinity melatonin receptors are also highly expressed in the *mediobasal hypothalamus* where they are likely to be involved in mediating photoperiodic information to the reproductive axis (*see* Section 6). High-affinity melatonin receptors located in the *inner plexiform layer* of the retina are thought to play a role in retinal physiology, whereas those located in the *preoptic area*, *cerebral cortex*, and *thalamus* may be involved in mediating the sleep-inducing effect of melatonin in some species (e.g., humans).

High-affinity melatonin receptors have also been identified in cerebral and caudal arteries and possibly play a role in the regulation of cardiovascular and thermoregulatory function. However, the most prominent nonneuronal expression of high-affinity melatonin receptors in most mammals is in the *pars tuberalis*

(PT) of the *pituitary gland*. Although the exact function of this receptor population is unknown there is evidence to suggest that it might play a role in the photoperiodic regulation of *prolactin* secretion, especially in the sheep (a seasonally breeding mammal). Studies in the rat have shown that the density of melatonin binding sites in the PT, as well as the SCN, shows an inverse relationship with circulating melatonin concentrations, being high in the daytime and low at night. This diurnal rhythm in PT melatonin binding site density appears to be abolished immediately after pinealectomy suggesting that it may be driven by the diurnal secretion of the endogenous ligand itself. Although PT melatonin receptors have been observed in nonhuman primates they have not been obvious in most of the human postmortem tissue that has been studied. Therefore, it is unclear whether melatonin can exert significant central effects on the neuroendocrine reproductive axis of humans. Indeed, the expression of high-affinity melatonin receptors in peripheral human reproductive tissues, such as *granulosa cells of preovulatory follicles* and *prostate epithelial cells*, suggests that melatonin's primary influence on human reproductive function might be exerted peripherally rather than centrally.

3.5. Cloning of Melatonin Receptors

The first high-affinity melatonin receptor was cloned in 1994 by Takashi Ebisawa et al., who relied on expression cloning from *Xenopus laevis* dermal melanophores. Many different investigators have since used a *polymerase chain reaction* (PCR) approach to clone three different subtypes of vertebrate melatonin receptor genes, commonly referred to as Mel_{1a}, Mel_{1b}, and Mel_{1c}. All three of these receptor subtypes have a similar gene structure. The receptor protein-encoding region is composed of two *exons* with a large (>8 kb) *intron* in between. Based on their deduced amino acid sequence these receptors are presumed to have seven transmembrane domains, which is a characteristic feature of G-protein-coupled receptors. Mel_{1a}, Mel_{1b}, and Mel_{1c} have been cloned in several lower vertebrates species, including the zebrafish, *Xenopus laevis* and the chicken. So far, however, only the Mel_{1a} and Mel_{1b} subtypes have been cloned in mammals. The human homologues of these receptor subtypes show >80% amino acid similarity with those of other mammals and the two genes appear to reside on two different chromosomes; the human Mel_{1a} gene, which encodes 350 amino acids, has been mapped to chromosome 4q35.1 and Mel_{1b}, which encodes 362 amino acids to chromosome 11q21-22. The Mel_{1a} receptor is expressed in the rodent and human SCN and PT (i.e., two regions that show abundant 2-[^{125}I]melatonin binding in autoradiography studies). Also, there are limited data from human postmortem observations to suggest that the expression of Mel_{1a} in the SCN may decrease after puberty. In humans, the Mel_{1b} receptor is 60% identical to the Mel_{1a} receptor at the amino acid sequence level and it is clearly expressed in the retina, where it probably plays a role in retinal physiology. However, its expression in the human SCN or PT is not obvious and its overall function in the human brain is unclear. In the Siberian hamster (*Phodopus sungorus*), the Mel_{1b} receptor gene contains two nonsense mutations within the coding region, implying an inability to code for a functional receptor, and yet its circadian and reproductive responses to melatonin are typical of nocturnal photoperiodic species. This finding suggests that both of these responses are mediated primarily through the Mel_{1a} receptor, which is functionally expressed in this species of hamster. In mice, the relative importance of Mel_{1a} and Mel_{1b} receptors in the control of circadian function appears to be more complicated. Targeted disruption of exon 1 in the Mel_{1a} receptor has been shown to block high-affinity 2-[^{125}I]melatonin binding in the mouse brain (Fig. 4) and to inhibit the acute suppressive effect of melatonin on SCN function (assessed by monitoring multiunit electrical activity). On the other hand, it did not eliminate the *phase shifting* effect of melatonin on the SCN suggesting that the Mel_{1b} receptor, which is known to be expressed in the mouse SCN, might also play a role in controlling some aspects of circadian function. In the chicken, the Mel_{1c} receptor shows 60% amino acid sequence identity with the related Mel_{1a} and Mel_{1b} receptors. Currently, however, very little is known regarding its function and it has not yet been cloned in mammals. A novel G-protein-coupled receptor, H9, has been cloned from the human pituitary gland. This gene encodes a protein of 613 amino acids that is 45% identical at the amino acid level to the human Mel_{1a} and Mel_{1b} melatonin receptors. It is expressed in the hypothalamus and pituitary gland suggesting that it might be involved in controlling neuroendocrine function. However, because it does not bind 2-[^{125}I]melatonin or [^{3}H]melatonin when expressed in COS-1 cells it is referred to as an "orphan" receptor (i.e., its natural ligand is unknown).

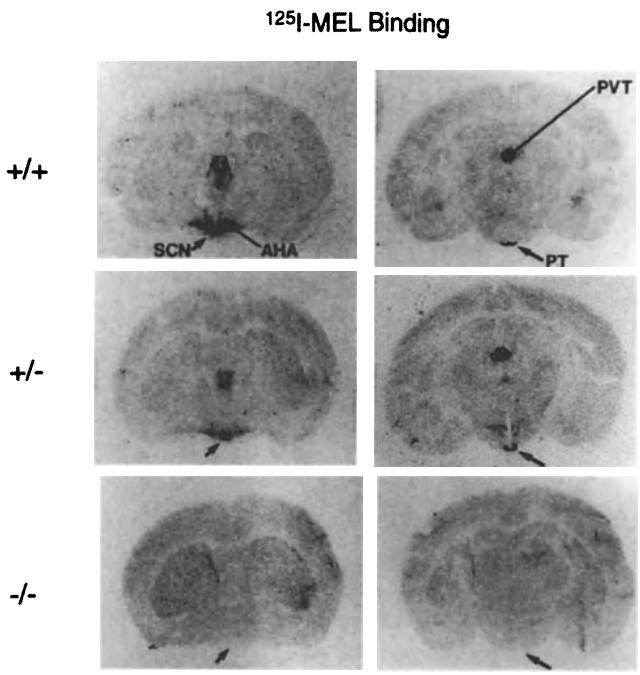

Fig. 4. Targeted disruption of the Mel_{1a} receptor eliminates detectable 2-[^{125}I]melatonin binding from mouse brain. +/+, +/–, and –/– denote 2-[^{125}I]melatonin autoradiograms from wild-type, heterozygous, and homozygous litter mates, respectively. The two representative brain sections from each genotype (shown in the left and right columns) represent the major sites of specific 2-[^{125}I]melatonin binding in the mouse brain. SCN = suprachiasmatic nucleus, AHA = anterior hypothalamic area, PT = pars tuberalis, PVT = paraventricular nucleus of the thalamus (Liu C, Weaver DR, Jin X, Shearman LP, Pieschi RL, Gribkoff VK, Reppert SM. Molecular dissection of two distinct actions of melatonin on the suprachiasmatic circadian clock. *Neuron* 1997;19:91).

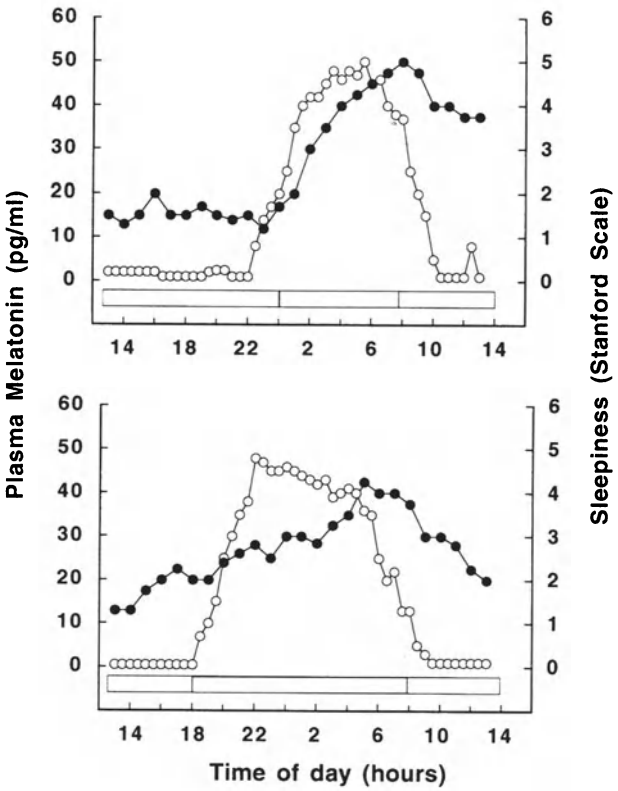

Fig. 5. Melatonin secretion (open circles) and index of sleepiness (closed circles) in humans exposed to 24 h of continuous dim light. In the days preceding the experiment, the individuals were maintained either in long photoperiods (16 h light:8 h dark per day; upper panel) or short photoperiods (10 h light:14 h dark per day; lower panel). Note that the circadian rhythm of melatonin secretion persists even in the absence of external photoperiodic cues and that the duration of melatonin secretion is proportional to the duration of the dark period (indicated by the horizontal shaded bars) to which the subjects were most recently exposed. Each point represents the mean (± SEM) of six individuals (Redrawn from Wehr TA. The duration of human melatonin secretion and sleep respond to changes in day length (photoperiod). *J Clin Endocrinol Metab*, 1991;73:1276; courtesy of The Endocrine Society).

4. NEUROENDOCRINE TRANSDUCTION OF PHOTOPERIODIC SIGNALS

4.1. Circadian Pattern of Melatonin Secretion

In mammals, the concentration of melatonin in the peripheral circulation becomes undetectable after pinealectomy, indicating that the principal source of the hormone is the pineal gland. In some mammals, melatonin is also produced by the retina, *Harderian gland* (in the eye orbit), gut, and blood platelets, but these nonpineal sources of melatonin are thought to be of local importance only. The pineal is a highly vascular gland and newly synthesized melatonin is rapidly released into the peripheral circulation. There is no evidence for storage of melatonin within the pineal gland (unusual for an endocrine gland) and little if any melatonin is thought to be released directly into the cerebrospinal fluid. Consequently, there is a very close association between the concentration of melatonin in the peripheral circulation and the rate of its synthesis in the pineal gland.

In humans, as in other vertebrates, serum melatonin profiles show a clear-cut circadian pattern, with melatonin secretion occurring almost exclusively during the night (Fig. 5). This is true for almost all species regardless of whether they have a *diurnal*, *nocturnal*, or *crepuscular* activity pattern. Accordingly, in many species (including rats and hamsters) melatonin secretion is at its peak while the animals are awake, whereas in others (including rhesus monkeys and humans)

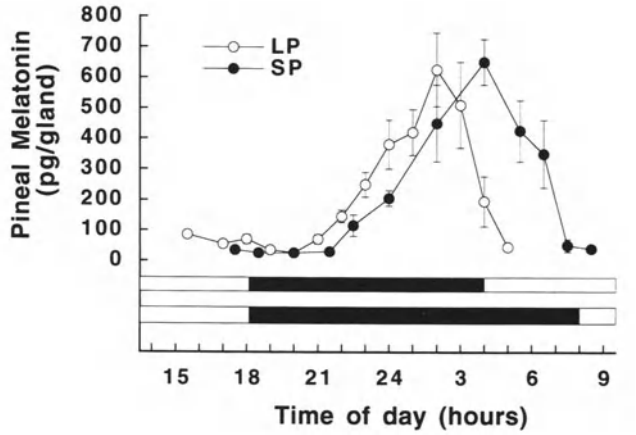

Fig. 6. Melatonin content in the pineal gland of adult male Syrian (golden) hamsters maintained either in long photoperiods (LP; 14 h light:10 h dark) or short photoperiods (SP; 10 h light:14 h dark) for 8 wk; the duration of darkness is indicated by a horizontal black bar. Note the longer duration of melatonin production in animals maintained in SP (i.e., long nights). Each point represents the mean (± SEM) of five to seven animals (Adapted from Pitrosky B, Kirsch R, Vivien-Roels B, Georg-Bentz I, Canguilhem B, Pévet P. The photoperiodic response in Syrian hamsters depends upon a melatonin-driven circadian rhythm of sensitivity to melatonin. *J Neuroendocrinol* 1995;7:889; courtesy of Blackwell Science Ltd.).

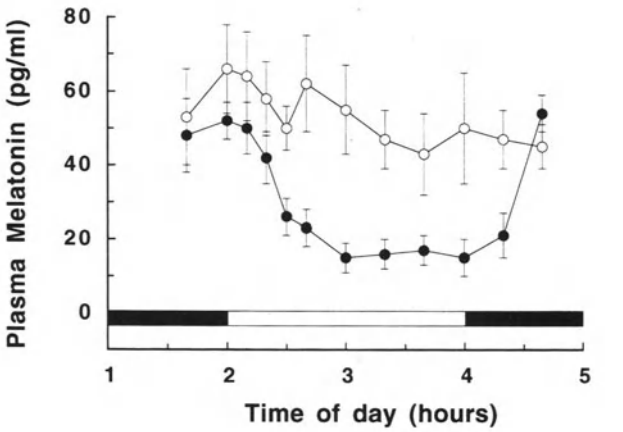

Fig. 7. Suppressive effect of light on human melatonin secretion. The open circles represent the effects of light pulses of 500 lux intensity; this level of illumination is typical of that found in an office or brightly lit room. Closed circles represent light pulses of 2500 lux, which are much more effective in suppressing melatonin secretion. Each point represents the mean plasma melatonin concentration (± SEM) for six individuals (Data from Lewy AJ, Wehr TA, Goodwin FK, Newsome DA, Markey SP. Light suppresses melatonin secretion in humans. *Science* 1980;210:1267).

melatonin secretion is at its peak during sleep. Depending on the species, three subtly different nocturnal melatonin profiles are evident. A *type A* pattern of melatonin secretion, as seen in the Syrian (golden) hamster (*Mesocricetus auratus*), is characterized by a delay of several hours after lights out before a nocturnal increase in melatonin synthesis occurs (Fig. 6). A *type B* pattern, as seen in the laboratory rat and human, is more common (Fig. 5). This shows a gradual increase in melatonin synthesis from the time when lights are switched off with a peak occurring approximately in the middle of the dark phase. A *type C* pattern, as seen in sheep, is also common. This shows a rapid increase in melatonin synthesis after lights are switched off and a high-plateau level is then maintained throughout most of the night.

How are these circadian patterns of melatonin secretion generated and how are they kept in phase, or in synchrony, with the external light-dark cycle? It is well established that bright light can acutely suppress melatonin secretion in humans (Fig. 7). Although this response to light is not as intense as that seen in rodents, it is qualitatively similar and has provided a basis for the treatment of human circadian rhythm disorders (*see* Section 5). An important consequence of altering day length, either naturally at different times of the year or experimentally using artificial photoperiods, is that a corresponding change in the circadian pattern of melatonin secretion occurs. Moreover, this occurs regardless of which type of melatonin secretion profile a species normally exhibits. Thus, even though Syrian hamsters show a *type A* profile and humans show a *type B* profile, in both cases the exposure to short days (i.e., long nights) results in an increases in the duration of nocturnal melatonin secretion (Fig. 5 and Fig. 6).

It is important to note that the melatonin rhythm is endogenous or self-sustaining. Under normal environmental conditions, light not only acutely inhibits melatonin secretion but also acts as a *zeitgeber* ("time-giver" in German) to synchronize the endogenous melatonin rhythm with the external day–night cycle. In normal light–dark cycles, the melatonin rhythm is said to be entrained; it shows a periodicity of 24 h with a peak of secretion occurring in the middle of the night. On the other hand, when bright light inputs are absent, and the influence of other zeitgebers (e.g., social cues and physical arousal) is kept to a minimum, the melatonin rhythm becomes uncoupled from the external environment. The melatonin rhythm is said to be *circadian* (from the Latin *circa dies,* meaning "about a day") because it is self-sustaining and has an intrinsic period of about, though not exactly, 24 h long. Consequently, in the absence of strong

zeitgebers, such as bright light, the melatonin rhythm will begin to drift or "free-run" and at times will become markedly out of phase with the external day–night cycle. This situation frequently occurs in people who are blind, or in sighted individuals who spend extended periods of time in continuous dim illumination, and is symptomatic of circadian dysfunction. Therefore, by modulating its output of melatonin in response to light, the pineal gland provides an effective way of ensuring that an organism's internal physiology remains in synchrony with the changing external environment.

4.2. Development and Aging of the Melatonin Rhythm

Although a circadian rhythm of melatonin secretion is evident throughout most of our lives it does not develop until the ninth to twelfth week after birth. Before this time, the human fetus and infant must rely on their mother's melatonin rhythm; this can be transmitted both via the placenta and via the milk. To what extent this maternal source of melatonin is crucial for normal early development of the offspring is unknown, but given that melatonin has well-established sleep-inducing properties in humans, one might expect the sleep–wake cycle of mothers to be more in synchrony with that of breast-fed babies than with that of bottle-fed babies. By the end of the first year of life, total melatonin production rapidly increases, with peak circulating nocturnal levels occurring between the ages of 1 and 3 yr. During puberty, blood melatonin concentrations clearly decline, but then remain more or less stable during early adulthood. Between the ages of 45 and 65 yr, nighttime melatonin secretion markedly decreases, although in some individuals it remains elevated (Fig. 8). The physiological significance of this decline remains to be elucidated, but may be linked to the increased incidence of circadian sleep disorders in the elderly. At all ages, there is wide variation in the amplitude of the melatonin rhythm between individuals, although it is consistent within individuals from day to day.

4.3. Circadian Oscillators

The pineal gland of some birds has an intrinsic capacity to secrete melatonin in a circadian manner, without depending upon innervation. For example, when dispersed chick pinealocytes are cultured in vitro, they show a 24 h rhythm of melatonin secretion that persists even in continuous darkness (Fig. 9). In contrast, the functional integrity of the mammalian

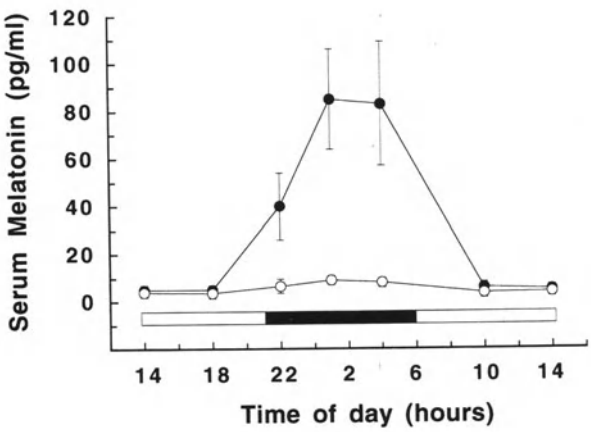

Fig. 8. Serum melatonin concentrations in young (26.4 ± 2.3 years; solid circles) and aged (84.0 ± 1.8 years; open circles) over a 24-h period. Each point represents the mean (± SEM) of five individual values. Note the marked attenuation of the serum melatonin peak in the aged group (Redrawn from Iguchi H, Kato K-I, Ibayashi H. Age-dependent reduction in serum melatonin concentrations in healthy human subjects. *J Clin Endocrinol Metab*, 1982;55:27; courtesy of The Endocrine Society).

pineal gland is absolutely dependent on its sympathetic innervation. For example, when it is transplanted to a site beneath the kidney capsule, it will continue to grow but produce very little melatonin, and its rhythmic pattern of secretion will disappear.

How then is the circadian rhythm of melatonin

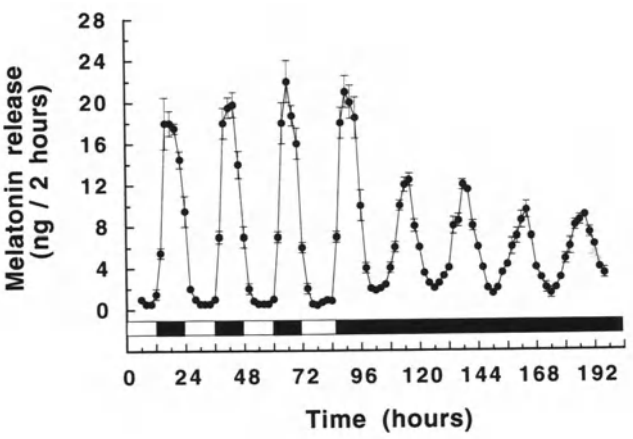

Fig. 9. Melatonin secretion from dispersed chick pinealocytes cultured at 37°C. Exposure to light or dark is indicated by the horizontal white and black bars, respectively. Note that the circadian rhythm of melatonin secretion persists even after the cells are exposed to continuous darkness, although the amplitude of the melatonin peak is diminished. Each point represents the mean measurement (± SEM) of four replicate cultures (Data from Barrett RK, Takahashi JS. Temperature compensation and temperature entrainment of the chick pineal cell circadian clock. *J Neurosci* 1995;15:568).

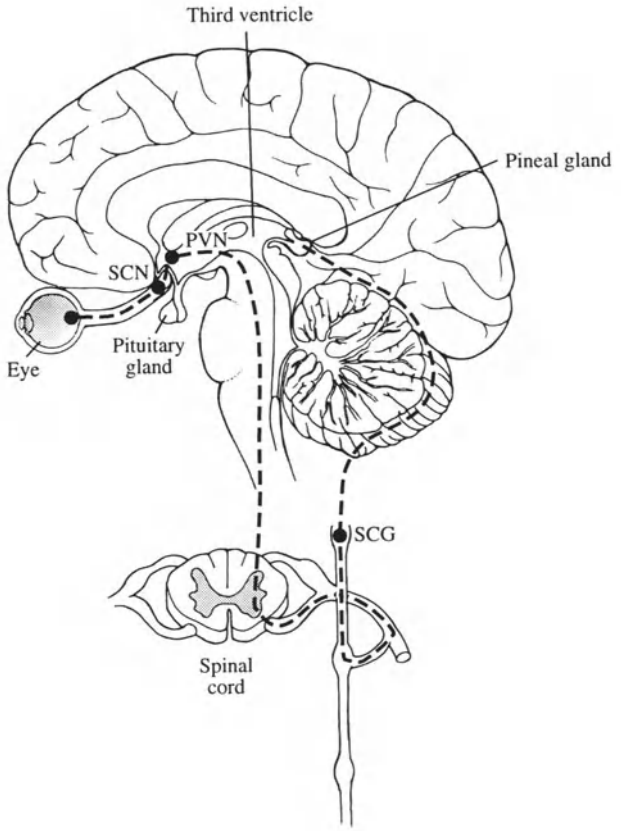

Fig. 10. Diagram of the neural pathway that connects the eyes to the pineal gland in humans. In contrast to the pineal gland of some lower vertebrates, the pineal gland of mammals is not directly responsive to light. Instead, it relies on a multisynaptic neuronal pathway that originates in the retina and involves the suprachiasmatic nucleus (SCN), paraventricular nucleus (PVN) and superior cervical ganglion (SCG) (Data from Tamarkin L, Baird CJ, Almeida OFX. Melatonin: a coordinating signal for mammalian reproduction? *Science* 1985; 227:714).

synthesis and secretion established in the mammalian pineal gland and how is it entrained to the external light–dark cycle? Numerous studies, involving lesion and knife-cut experiments, have demonstrated the existence of a multisynaptic neural pathway connecting the mammalian pineal gland to the eye. Although these studies were performed using animals, a similar pathway is presumed to exist in humans (Fig. 10). Retinal ganglion cell fibers project to the SCN in the hypothalamus via a monosynaptic glutamatergic pathway (the *retino-hypothalamic tract);* this is a non-visual optic pathway that shows 70% crossing in the optic chiasm. In mammals, the paired SCN function as the primary circadian oscillator or biological clock (see Chapter 22) and it is the SCN that ultimately controls the circadian pattern of melatonin secretion in mammals. Although the retino-hypothalamic tract represents the primary neural link between the external environment and the internal circadian clock, there is evidence that some environmental cues may also be transmitted via the *intergeniculate leaflet* of the *thalamus* and the *median raphé*. The photic signals are relayed from the SCN to the *paraventricular nucleus* (PVN) of the hypothalamus and then pass along nerve fibers that traverse the *medial forebrain bundle* and *reticular formation* to the *intermediolateral cell column* of the spinal cord. They are then transmitted to preganglionic adrenergic fibers and pass to the *superior cervical ganglion* (SCG). Finally, postganglionic sympathetic fibers innervate the pineal gland. These release norepinephrine, which binds to β-adrenergic receptors on the pinealocyte membrane, causing an activation of *cyclic adenosine monophosphate* (cAMP); the resulting increase in *N*-acetyl transferase (NAT) enzymatic activity during the night ultimately enhances melatonin synthesis (Fig. 3). Noradrenergic stimulation of β-adrenergic receptors is initiated during the first half of the night and ultimately results in down regulation of the β-adrenergic receptor during the second half. These receptors thus show a 24 h rhythm with their highest density occurring during darkness. α-adrenergic receptor binding is less significant in the pineal gland, but it does potentiate the β-adrenergic stimulation of nocturnal melatonin synthesis.

To summarize, the mechanism that regulates melatonin secretion and ensures that it accurately reflects external photoperiodic cycles has three essential components: (a) a photoreceptor, (b) an endogenous circadian pacemaker, and (c) an endocrine organ. In mammals, these components are primarily represented by separate structures (i.e., the retina, SCN, and pineal gland, respectively), which are functionally integrated by a complex multi-synaptic neural pathway. Moreover, if any of these components is disrupted (e.g., in individuals who are blind) the normal pattern of elevated nocturnal melatonin secretion will become severely perturbed or completely abolished.

Although melatonin of retinal origin is thought to be only of local physiological significance in mammals, it has been shown to make a significant contribution to the concentration of melatonin in the peripheral circulation in birds and lower vertebrates. Nevertheless, the production of melatonin by the mammalian retina is interesting because the underlying mechanism is probably different from the one involved in controlling melatonin production by the mammalian pineal gland. For example, when neural retinas of

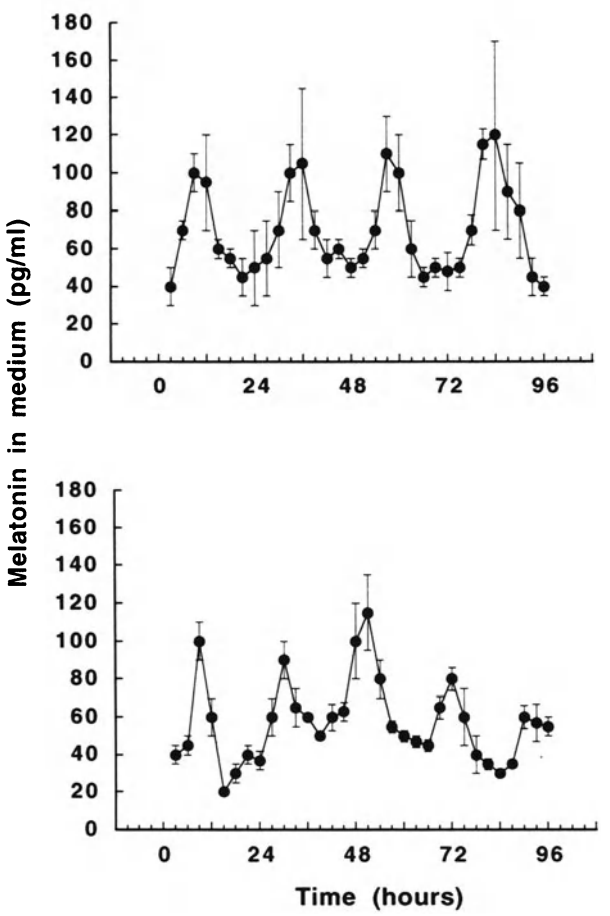

Fig. 11. Free-running rhythms of melatonin synthesis in retinas from wild-type and *tau* mutant Syrian (golden) hamsters (upper and lower panels, respectively) maintained in culture at 27°C. The natural periods of these rhythms is approximately 24 h and 20 h, respectively. In contrast to the mammalian pineal melatonin rhythm, the mammalian retinal melatonin rhythm does not depend on circadian inputs from the suprachiasmatic nucleus (the central clock) to be self-sustaining. Each point represents the mean (± SEM) for four animals (Data from Tosini G, Menaker M. Circadian rhythms in cultured mammalian retina. *Science* 1996; 272:419).

Syrian hamsters were cultured at a constant 27°C (but not at 37°C) they showed a circadian rhythm of melatonin synthesis that persisted for at least 5 d (Fig. 11). Interestingly, the retinas from a mutant strain of hamster, which is known for its short circadian cycles, showed a much shorter free-run period of retinal melatonin synthesis. This ability of the mammalian retina to synthesize melatonin in a sustained rhythmic manner even in the absence of environmental zeitgebers indicates that it contains an endogenous circadian oscillator, and in this regard, it resembles the pineal gland of some birds (Fig. 9).

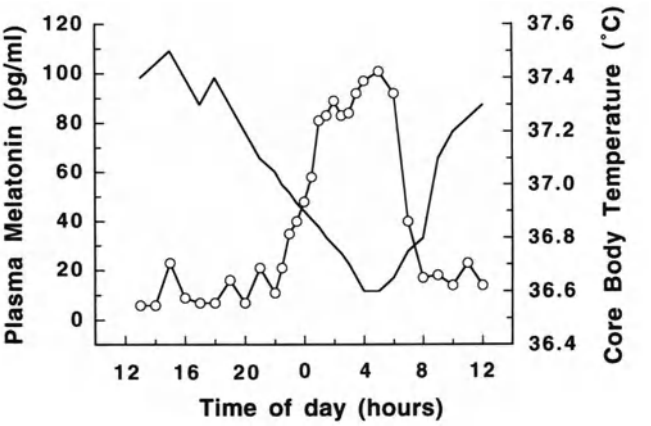

Fig. 12. Plasma melatonin and core body temperature data from a 22-yr-old man. Because core body temperature is known to be affected by variations in activity, posture, and the sleep–wake state, the measurements were made with the subject awake and performing a constant routine. Note the close inverse relationship between plasma melatonin concentrations and core body temperature (Adapted from Shanahan TL, Czeisler CA. Light exposure induces equivalent phase shifts of the endogenous circadian rhythms of circulating plasma melatonin and core body temperature in men. *J Clin Endocrinol Metab*, 1991;73:227; courtesy of The Endocrine Society).

5. PRACTICAL ASPECTS OF THERAPY USING LIGHT AND MELATONIN

5.1. Human Circadian Rhythms

Like most animals, humans have adapted to life in an environment where periods of light and dark alternate each day. Much of our physiology is thus rhythmic with peaks and nadirs occurring regularly at specific times of the day. One of the most obvious circadian rhythms in humans is the sleep–wake cycle which is closely associated with the circadian rhythm of melatonin secretion (Fig. 5). Both of these rhythms show a peak during the dark phase (*scotophase*) of the day–night cycle. In contrast, the circadian rhythm of core body temperature is inversely related to the melatonin rhythm (Fig. 12) and normally shows a peak during the light phase (*photophase).* These endogenous rhythms are all "self-sustaining." That is, they continue to be expressed even in the absence of external zeitgebers (e.g., when exposed to continuous dim illumination). Many hormones besides melatonin also show a circadian pattern of release. The secretion of *cortisol* from the adrenal gland shows a peak around the time of dawn and a nadir around the time of dusk. The secretion of *growth hormone* and *prolactin* from the *anterior pituitary gland* also shows a 24-h

rhythmic pattern, but these two hormonal rhythms are not directly driven by the day–night cycle *per se*; the nocturnal peak of growth hormone secretion is primarily dependent on sleep whereas the nocturnal rise in prolactin secretion is dependent on refraining from activity.

Most human circadian rhythms are ultimately driven by the oscillator circuits that reside in the SCN. When entrained to the external day–night cycle the activity of the central pacemaker displays a 24 h period. However, the natural period (*tau*) of this pacemaker is rarely exactly 24 h long; indeed, in some animals such as the *tau* mutant hamster (Fig. 11) it is several hours shorter. Consequently, when these rhythms are allowed to free-run (i.e., by depriving the SCN of external zeitgebers) they begin to drift out of phase with the environmental rhythms and thus ultimately lead to the development of circadian rhythm disorders.

5.2. Circadian Rhythm Disorders

Disruption of the circadian system produces a variety of ailments, but typically includes perturbed sleep–wake cycles, indigestion, general malaise, and poor performance at work. One of the most common underlying causes of this disruption is rapid travel across several time zones (i.e., in an east–west or west–east direction). This is referred to as jet lag, and is a frequent complaint amongst today's cosmopolitan airplane travelers. It develops when an individual does not allow adequate time for his or her endogenous rhythms to entrain to the local day–night cycle; it usually takes about 1 d for our internal rhythms to phase adjust for each time zone crossed. For example, when traveling from Los Angeles to London (i.e., 8 h time difference) it may take more than a week to feel completely in synchrony with the external environment. Jet lag should not be confused with "travel fatigue," which can occur after a prolonged period of flight in any direction. Thus, a flight from New York to Santiago in Chile may well cause travel fatigue because of its long duration. However, because the two cities are located on approximately the same meridian the flight should not cause jet lag. Because the free-run period of the human central pacemaker is usually slightly longer than 24 h, most people find it easier to phase delay their sleep–wake rhythm (i.e., to wake up later on consecutive mornings). Consequently, most people find it easier to reentrain their rhythms after flight in an east–west direction, rather than in a west–east direction.

Another common cause of circadian rhythm disorders is shift work. This occurs in individuals whose work schedule demands that they rapidly alter the phase of their sleep–wake rhythm. As with jet lag, the time period during which a shift-worker's internal rhythms are out of synchrony with the socially imposed activity–rest rhythm is associated with internal disharmony. A more severe and chronic manifestation of circadian rhythm disfunction can occur in individuals who are blind. Despite the absence of major photic input to their SCN some blind individuals can use other zeitgebers, such as social cues and physical arousal, to keep their biological clock entrained with the day-night cycle. More commonly, however, their central biological clock free-runs causing circadian endogenous rhythms to periodically become 180° out of phase with the external environmental rhythms. During these periods of desynchronization, the individual my feel excessively sleepy during midday and suffer from insomnia at night. Perturbed sleep patterns also occur frequently in sighted individuals, especially in the elderly. Because aging is generally associated with a marked decline in the amplitude of melatonin production (Fig. 8) it has been suggested that impaired pineal endocrine function might play a significant causative role. Moreover, because many people spend most of their active hours indoors, it is possible that they receive inadequate exposure to bright light for maintenance of circadian rhythm synchrony, which in turn could predispose them to delayed and advanced sleep phase insomnia.

5.3. Potential Therapy for Circadian Disorders

In order to treat human circadian disorders it is necessary to determine the degree to which the internal clock is out of phase with the external circadian cycle and then to cause an appropriate phase delay or phase advance. Because it is not easy to directly measure circadian activity in the SCN of humans, it must be inferred by observing a more overt rhythm that is driven by the central pacemaker. A commonly used overt indicator in animal studies is the rhythm of locomotor activity (e.g., wheel running activity in rodents; see Chapter 22) which can be considered as being analogous to the digital display of a modern clock. Just as the digits on the clock reveal information about the workings of the internal oscillator circuit without being an integral part of that circuit, the locomotor activity rhythm reveals information about the

workings of the SCN without being an integral part of the central pacemaker. In humans, however, locomotor activity rhythms are difficult to measure and are not reliable indicators of SCN activity because they can easily be masked by various external influences, especially social cues. In contrast, the circadian rhythm of melatonin secretion is masked only by bright light and so is generally considered to be the most accurate and reliable overt indicator of SCN function in humans.

Agents that can phase shift the activity of the SCN pacemaker have therapeutic potential in the treatment of circadian rhythm disorders. Currently, the two most effective means of achieving this are bright light and melatonin. It is well established that bright light can entrain circadian rhythms in humans but until recently, it was unclear whether melatonin itself also has this capacity. Melatonin has now been shown to elicit two effects on SCN function. First, it can acutely inhibit the in vitro electrical activity of rat SCN neurons in vitro, and second, it can synchronize the developing circadian clocks of hamsters *in utero*. Furthermore, studies in humans have demonstrated that as little as 0.5 mg of exogenous melatonin given orally can phase shift the endogenous rhythm of melatonin secretion, as long as it is administered at an appropriate time of day. To reveal such an effect, the subjects need to be maintained in constant environmental conditions; for example, they are kept awake in a semirecumbent posture and in dim continuous illumination. Under these conditions, exposure to either a pulse of bright light or to melatonin causes the magnitude and direction of the endogenous melatonin rhythm to change, depending on the time of administration relative to the phase of the internal rhythm. The results from these studies can be used to construct *phase response curves* (PCRs), as depicted in Fig. 13, and these can be used to design therapies for specific circadian rhythm disorders. By convention, the free-run period of the internal clock is divided into 24 *circadian hours*. In the case of the melatonin rhythm, which is assumed to accurately reflect the activity of the central pacemaker, the start of the circadian increase in melatonin secretion occurs at *circadian time* (CT) 14. The key aspect of the light and melatonin PCRs is that they are almost exactly 12 h out of phase with each other. Bright light pulses will thus cause a phase advance of the circadian clock (i.e., aspects of the rhythm will occur earlier on the next day) if administered between CT 18 and CT 6, but will cause a phase delay if administered between CT 6 and CT 18. In contrast, exogenous melatonin

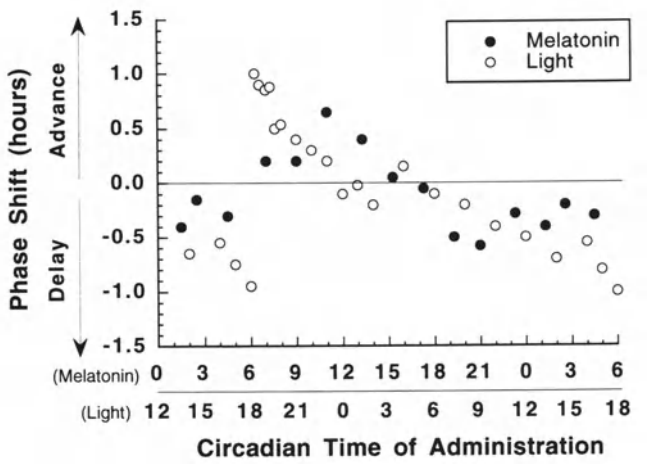

Fig. 13. Phase response curves (PCR) of light (open circles) and melatonin (solid circles) plotted together. To facilitate examination of the relationship between the two PCRs the phase shift magnitudes for the light PCR (Czeisler et al., 1989) have been divided by 10 and the scale on the abscissa has been shifted by 12 h. The onset of melatonin secretion in evening dim light was used as a phase marker for the melatonin rhythm and was designated as circadian time (CT) 14. Note that the two PCRs are similar but approximately 12 h out of phase with each other (Redrawn from Lewy AJ, Bauer VK, Ahmed S, Thomas KH, Cutler NL, Singer CM, Moffit MT, Sack RL. The human phase response curve (PRC) to melatonin is about 12 hours out of phase with the PRC to light. *Chronobiol Int*, 1998;15:71; courtesy of Marcel Dekker Inc.).

will cause a phase advance if administered between CT 6 and CT 18 but will cause a phase delay if administered between CT 18 and CT 6. An example of how information from these PCRs can be used to manipulate the phase of the central pacemaker is depicted in Fig. 14. So far, bright light therapy has been demonstrated to be the most effective approach, but it does require dedication and a commitment of time. Successful shifting of phase typically requires exposure to bright light at specific times of the day and avoidance of light at other times. Because of the accumulating evidence that exogenous melatonin can phase shift human circadian rhythms, therapies involving the administration of melatonin may also prove to be effective in the treatment of circadian disorders. Indeed, it may be the only choice for treatment of such disorders in individuals who are blind. Melatonin is generally considered to be very safe. It has very low toxicity and is not mutagenic in standard in vitro tests. The principal advantage of a melatonin-based therapy is that taking oral melatonin at a specific time of the day is more likely to have a higher rate of compliance than therapy involving an hour or more

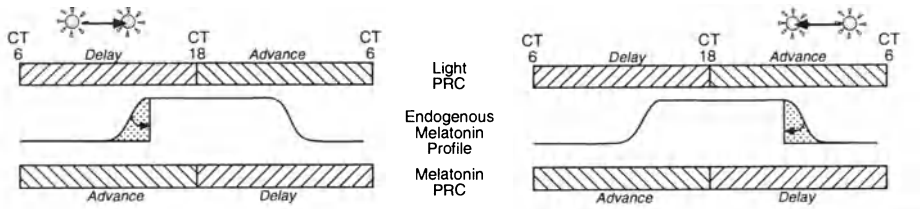

Fig. 14. Schematic diagram showing how the acute suppressive effect of light on melatonin production may indirectly, as well as directly, entrain the endogenous circadian pacemaker. Endogenous melatonin release extends from about circadian time (CT) 14 to CT 1. Between about CT 6 and CT 18 exposure to light causes a phase delay of this endogenous rhythm, whereas exposure to melatonin itself causes a phase advancement. Between about CT 18 and CT 6 exposure to light causes a phase advancement, whereas exposure to melatonin causes a phase delay. In the example shown on the left, exposure to light later in the evening than usual stimulates more of the *delay zone* of the light PCR (direct effect). It also acutely delays the onset of melatonin production, which in turn reduces the stimulatory effect of melatoninin the *advance zone* of its own PRC; consequently, the phase-delaying effect of evening light will be enhanced even further (indirect effect). In the example shown on the right, exposure to light earlier in the morning than usual stimulates more of the *advance zone* of the light PCR (direct effect). It also acutely terminates melatonin production at an earlier time, which in turn reduces the stimulatory effect of melatonin on the *delay zone* of its own PRC; consequently, the phase-advancing effect of morning light will be enhanced even further (indirect effect). Thus, endogenous melatonin may augment entrainment of the endogenous circadian pacemaker by the light-dark cycle (Adapted from Lewy AJ, Bauer VK, Ahmed S, Thomas KH, Cutler NL, Singer CM, Moffit MT, Sack RL. The human phase response curve (PRC) to melatonin is about 12 h out of phase with the PRC to light. *Chronobiol Int*, 1998;15:71; courtesy of Marcel Dekker Inc.).

of early morning exposure to bright light. On the other hand, because the PCRs for bright light and melatonin are almost exactly 180° out-of-phase with each other, perhaps the most effective therapeutical approach to circadian rhythm dysfunction would exploit the combined phase shifting properties of both.

6. MEDIATION OF SEASONAL ENVIRONMENTAL SIGNALS

By modulating its circadian pattern of melatonin secretion the pineal gland not only provides us with information about the time of day but also about the time of year. The pronounced difference between summer and winter day lengths in polar regions of the world produces seasonal differences in the pattern of melatonin secretion, with more melatonin being produced during the long winter nights. At lower latitudes such differences are less apparent but can be induced by maintaining individuals under either long or short artificial photoperiods (Figs. 5 and 6). Photoperiodically driven changes in the pattern of melatonin secretion are used extensively by animals to coordinate a wide range of physiological events that vary with the season. These include reproduction, body weight, behavior, and growth of winter or summer coat. Although seasonal changes in melatonin secretion in humans are likely to be more subtle, because they receive less direct exposure to natural photoperiods, such changes may contribute to the cause of seasonal changes in mood, eating and sleeping habits, and also to the development of *seasonal affective disorder* (SAD), the most common form of which is *winter depression.* Whether SAD results from a seasonal phase delay in the circadian system with respect to the light-dark cycle, or from some other cause, is unclear.

6.1. Seasonal Breeding

Seasonal breeding is a common feature of animals that are indigenous to nonequatorial regions of the world. By restricting their reproductive activity to a specific time of the year, these animals ensure that their offspring are born only when environmental conditions are optimal for survival. Most seasonally breeding mammals that live at high latitudes are photoperiodic. That is, they exploit the annual changes in day length, or photoperiod, to synchronize the timing of their breeding season. Small mammals, such as hamsters and voles, are reproductively active and produce offspring in the spring and summer, but stop breeding in the fall when day length becomes shorter than about 12 h of light per day (i.e,. when nights become long). They are thus said to be "long-day breeders." Larger mammals, such as sheep and deer, also produce offspring in the spring and summer. However, because their gestation period is almost half a year long their breeding season is half a year out of phase with that of smaller mammals (i.e., it is confined to the fall and winter rather than to the spring and summer); they are thus said to be "short-day

breeders." Nevertheless, both long-day and short-day breeders rely on the same elaborate photoneuroendocrine circuitry to transduce photoperiodic information into a particular pattern of melatonin secretion (Fig. 10). The bottom line is that in all photoperiodic mammals exposure to short photoperiods, either naturally during the fall and winter or artificially in the laboratory, prolongs the duration of the nocturnal melatonin pulse and markedly changes its circadian profile (e.g., Fig. 6). It should be emphasized, however, that both long-day and short-day breeders show enhanced melatonin production when exposed to short photoperiods and that the primary difference between them lies in how they exploit this endocrine information to modulate the activity of their reproductive system. It should also be emphasized that seasonal breeding does not simply involve a change in reproductive behavior but also involves a profound alteration in the secretion of reproductive hormones including the gonadotropins [*follicle-stimulating hormone* (FSH) and *luteinizing hormone* (LH)] and *sex steroids*. In males, termination of the breeding season is generally also associated with a profound regression of the testes. For example, exposure of adult male Syrian hamsters to short photoperiods causes the combined testicular mass to decrease from >3.5 g to <0.5 g, and spermatogenesis to cease completely. Interestingly, the suppressive effect of short photoperiods on the reproductive axis of hamsters can be mimicked using exogenous melatonin, as long as it is administered at the appropriate time of day. This can be readily demonstrated by an experiment in which male hamsters are exposed to long photoperiods to stimulate development of their reproductive axis (Fig. 15). When these animals are also given a daily subcutaneous injection of melatonin (25 μg) every afternoon, the usual stimulatory action of long photoperiods becomes inhibited. The exact mechanism is unclear, but it is generally assumed that the afternoon administration of exogenous melatonin effectively combines with the animal's endogenous nocturnal melatonin to produce a sustained elevation of circulating melatonin concentrations (i.e., similar to that occurring in hamsters exposed to short photoperiods). In contrast, an identical dose of melatonin injected each morning is completely ineffective, probably because it is cleared from the circulation too far in advance of the endogenous melatonin peak.

In sheep, exogenous melatonin exerts a markedly different effect on the reproductive axis. Unlike hamsters, sheep are short-day breeders (i.e., they mate in the fall and winter and produce lambs the following

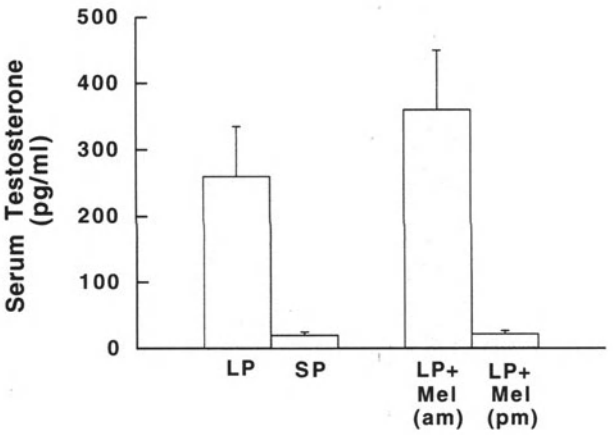

Fig. 15. The influence of melatonin on the reproductive axis of male Syrian (golden) hamsters. All of the animals were maintained in short photoperiods (SP) (i.e., 6 h light:18 h dark per day) for 7 wk before use in the study. For the next 4 wk, they were then either exposed to long photoperiods (LP) (i.e., 14 h light: 10 h dark per day) or maintained in SP. During this period, some of the LP animals received a daily subcutaneous injection of melatonin (25 μg) either in the morning (am) or in the afternoon (pm). Note that the normal rise in serum testosterone concentrations that occurs when the animals are exposed to LP is completely blocked by exogenous melatonin, as long as is administered in the afternoon; morning injections of melatonin are ineffective. Each bar represents the mean serum testosterone measurement (± SEM) of five to eight animals (Data from Urbanski HF, Fahy MM, Collins PM. Influence of *N*-methyl-*D*-aspartate on the reproductive axis of male Syrian hamsters. *J Endocrinol*, 1993;137:247).

spring and summer). Thus, when exogenous melatonin is administered to ewes in the summer (to mimic winter circulating melatonin concentrations) it precociously activates the reproductive axis and induces lambing several months earlier than usual. Taken together, these findings emphasize that in seasonal breeding mammals, melatonin is not antigonadotropic or progonadotropic *per se*. Instead, its role is simply to convey circadian and seasonal information to the reproductive neuroendocrine circuits.

6.2. Human Reproductive Physiology

Humans appear capable of reproducing at any time of the year and by definition, therefore, they are not seasonal breeders. On the other hand, Till Roenneberg and Jürgen Aschoff have performed a comprehensive analysis of monthly birth rates from 166 regions of the world and have concluded that humans, as a whole, do show an annual rhythm of reproduction. There is a general tendency for maximal conception rates to begin close to the vernal (spring) equinox; that is, at a time of year when days and nights are equal in

length. Whether melatonin plays a role in this circannual rhythm is unknown, but is likely given that it does so in most other mammals. In general, however, the influence of melatonin on the human reproductive axis appears to be marginal and the available evidence supporting its physiological role is largely circumstantial. For example, a significant reduction of nocturnal melatonin secretion is associated with the onset of puberty, but so far there is no direct evidence that it is a cause; indeed, it might simply reflect increased dilution of the hormone as a result of increased vascular volume. Similarly, although abnormally high-circulating melatonin concentrations have been reported in women with *functional hypothalamic amenorrhoea* and in men with *hypogonadotropic hypogonadism*, it is unclear whether they contribute to the development of these reproductive disorders or whether they are a consequence. Currently, there is no clear consensus as to whether melatonin levels change during the menstrual cycle, although there is some evidence to suggest that it might be lowest at the time of expected ovulation. Large doses of melatonin (30–75 mg) in conjunction with progestin have been proposed as a novel approach to human contraception, but it remains to be convincingly demonstrated that endogenous melatonin plays a physiological role in regulating reproductive function in humans.

7. CONCLUSION

Through its circadian pattern of melatonin secretion, the pineal gland helps to keep the internal physiology of animals in synchrony with the day–night cycle and with the annually changing seasons. Although the melatonin rhythm is assumed to accurately and reliably reflect the activity of the central pacemaker in the SCN, the extent to which melatonin itself can directly influence human biological rhythms, such as the sleep–wake cycle, remains to be elucidated. Exogenous melatonin has been shown to be effective at synchronizing circadian rhythms in blind individuals and also to help phase adjust circadian rhythms in sighted individuals prior to transmeridian flights. With the cloning of different melatonin receptor subtypes and the prospect of producing specific agonists and antagonists, it may soon be possible to develop more effective therapies for these circadian rhythm disorders as well as for seasonal affective disorder.

In recent years, numerous books aimed at the lay public have espoused the virtues of using melatonin to treat, alleviate, or prevent a wide range of human ailments. Based mainly on results from animal studies, claims have been made that melatonin can combat aging, boost the immune system, and reduce the risk of cancer. Although the extent to which melatonin can exert these beneficial effects in humans remains to be determined, the sleep-inducing property of exogenous melatonin in humans is already well established. In the United States, melatonin is currently classified as a food supplement and so is readily available to the public without a physician's prescription. Consequently, the use of melatonin as a "self-help" remedy for insomnia is already prevalent, especially among the elderly who commonly have problems getting a good night's sleep. Because the popular image of melatonin as a "wonder drug" will inevitably lead to its increased use in the future, more scientific studies are needed to substantiate its potential therapeutic effects and, more importantly, to show that its long-term indiscriminate use does not adversely affect the circadian system.

SELECTED READINGS

Arendt J. Melatonin and the Mammalian Pineal Gland, 1st ed. London: Chapman and Hall, 1995.

Arendt J. Melatonin and the pineal gland: influence on mammalian seasonal and circadian physiology. Rev Reproduction, 1998; 3:13.

Bronson FH. Seasonal variation in human reproduction: environmental factors. The Quart Rev Biol, 1995; 70:141.

Brown GM. Melatonin in psychiatric and sleep disorders: therapeutic implications. CNS Drugs, 1995; 3:209.

Cagnacci A. Melatonin in relation to physiology in adult humans. J Pineal Res, 1996; 21:200.

Cassone VM. Effects of melatonin on vertebrate circadian systems. Trends in Neurosci, 1990; 11:457.

Dawson D, Encel N. Melatonin and sleep in humans. J Pineal Res, 1993; 15:1.

Lewy AJ, Sack RL. Exogenous melatonin's phase-shifting effects on the endogenous melatonin profile in sighted humans: a brief review and critique of the literature. J Biol Rhythms, 1997; 12:588.

Lincoln GA. The pineal gland. In: Austin CR, Short RV, eds. Reproduction in Mammals: Book 3, Hormonal Control of Reproduction. 2nd ed. Cambridge, UK: Cambridge University Press, 1987, chapter 3, p 52.

McArthur AJ, Hunt AE, Gillette MU. Melatonin action and signal transduction in the rat suprachiasmatic circadian clock: activation of protein kinase C at dusk and dawn. Endocrinol, 1997; 138:627.

Pang SF, Pang CS, Poon AMS, Shiu SYW, Song Y, Lee PPN, Zhao Y, Liu ZM. Recent development of pineal melatonin and its receptors in humans. In: Tang PL, Pang SF, Reiter RJ, eds. Melatonin: A Universal Photoperiodic Signal with Diverse Action. Front Horm Res. Basel: Karger, 1996; 21:133.

Quay WB, Kachi T. Amine-secreting endocrines. In Timaras PS, Quay WB, Vernadakis A, eds. Hormones and Aging. Boca Raton, FL: CRC Press, 1995; 67.

Reiter RJ. Melatonin and human reproduction. Ann Med, 1998; 30:103.

Roenneberg T, Aschoff J. Annual rhythms in human reproduction: I. Biology, sociology or both? J Biol Rhythms, 1990; 5:195.

Wehr TA. A 'clock for all seasons' in the human brain. In Buijs RM, Kalsbeek A, Romijn HJ, Pennartz CMA, Mirmiran M, eds. Progress in Brain Research. 1996; 111:321.

Wirz-Justice A, Graw P, Kräuchi K, Gisin B, Jochum A, Arendt J, Fisch H, Buddeberg C, Pöldinger W. Light therapy in seasonal affective disorder is independent of time of day or circadian phase. Arch Gen Psychiatry, 1993; 50:929.

Zhdanova IV, Wurtman RJ. The pineal hormone—melatonin. In: Conn PM, Melmed S, eds. Endocrinology: Basic Clinical Principles. Totowa: Humana, 1997; 279.

24 The Neuroendocrine Control of Circadian Rhythms

Nancy L. Wayne, PHD

CONTENTS

1. INTRODUCTION

The bilateral suprachiasmatic nuclei (SCN) of the hypothalamus is the location of the primary circadian clock in mammals. Humoral and/or neuronal outputs from the SCN drive rhythms in behavioral, physiological, and biochemical functions. Other chapters in this textbook have discussed circadian rhythmicity of hormone secretion, reflecting the output of the SCN to various neuroendocrine systems. The focus of this chapter will consider evidence that neurohormones can feed back on the SCN and modulate circadian clock function.

Feedback loops between tissues or organs are a hallmark of homeostasis and maintenance of optimal physiological function. For endocrine systems, a typical feedback loop includes an Endocrine Organ A that secretes Hormone A and a second Endocrine Organ B that secretes Hormone B (*see* Fig. 1). Hormone A stimulates Endocrine Organ B to secrete Hormone B. Hormone B enters the circulation, and when it binds to its specific receptors on Endocrine Organ A, it acts to inhibit the secretion of Hormone A. Lowered secretion of Hormone A is no longer sufficient to stimulate secretion of Hormone B, thus relaxing negative feedback on Endocrine Organ A. This negative-feedback mechanism prevents secretion of Hormone A from rising out of control. It appears that various feedback mechanisms between the SCN and neuroendocrine systems could also play a role in the modulation of circadian rhythmicity. The SCN drives rhythmic secretion of various neurohormones in the brain, and there is growing evidence that many of these neurohormones can feed back on the SCN and cause phase shifts in circadian-pacemaker function (*see* Fig. 1).

This chapter will consider neurohormones that have been shown to modulate function of the circadian system. Studies investigating the potential feedback effects of neurohormones on circadian-pacemaker function have employed a variety of experimental approaches: (1) analysis of the circadian behavioral responses to treatment of animals with neurohormones; (2) analysis of the circadian rhythm of SCN membrane excitability in response to treatment of tissue slices in culture with neurohormones; (3) identification of neurohormones or their receptors in SCN neurons. Because there is more information on the effects of melatonin on circadian rhythmicity than any other neurohormone, this chapter will place particular

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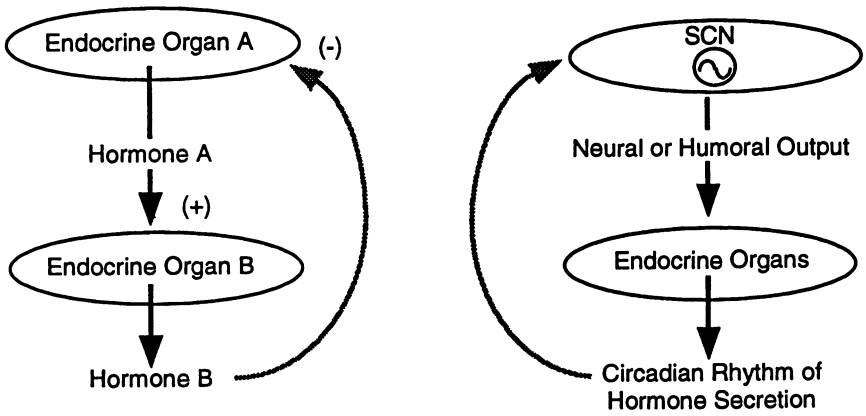

Fig. 1. Principle of homeostasis involving feedback control systems. *Left:* model of negative feedback control of a typical endocrine organ. A primary endocrine organ **(A)** secretes a hormone that stimulates secretion from a second endocrine organ **(B)**. Hormone B then negatively regulates secretion of Hormone A such that its circulating levels do not fluctuate outside of optimal levels. *Right:* model for endocrine feedback regulation of circadian-pacemaker cells. Output from circadian-pacemaker cells in the SCN drives rhythmic secretion of hormones that can then feed back on the SCN to effect the circadian system.

emphasis on the effects of this monoamine hormone on circadian pacemaker activity in mammalian species.

2. MELATONIN ALTERS CIRCADIAN-PACEMAKER FUNCTION

2.1. Melatonin and Its Role During Development

The circadian system in mammals is not fully developed until some time after birth. In the rat, neurogenesis of the SCN occurs between days 13 and 16 of gestation, and synaptic innervation of the SCN starts to occur at around day-19 with the majority of innervation occuring postnatally. There is evidence that the fetal SCN generates endogenous circadian activities, and that fetal rhythmicity is synchronized or entrained by the mother through humoral signals. There is a day-night difference in activity of the day-19 fetal rat SCN, indicating that the fetal SCN, which is practically devoid of synaptic input from the retina, endogenously generates circadian activities that are entrained to the light:dark cycle. There are clear daily rhythms in metabolic and electrophysiological activities of fetal SCN maintained in vitro, supporting the idea that circadian-pacemaker activity develops during fetal life prior to retinal innervation.

How does the fetal circadian system tell ambient time (i.e., distinguish day from night)? Does light illuminate the fetus in its womb and entrain the pacemaker directly? Or is the mother providing the fetus with environmental timekeeping information? A wide variety of evidence supports the latter possibility; that is, entraining signals originate from the mother. Blinded pregnant rats have circadian rhythms that are not synchronized with the light:dark cycle and provided an early test of whether or not the fetal circadian system entrains to ambient light or to maternal SCN signals. The fetal rhythm was synchronized with that of the mother and not ambient light:dark cycles, supporting the idea that output from maternal SCN entrains the fetal circadian system. Further work supporting a maternal entraining signal showed that destruction of the maternal SCN led to desynchronization of circadian rhythmicity of the individual fetuses within a litter. This influence of the maternal SCN is not permanent; postnatal rat pups born to mothers with SCN lesions do eventually entrain to light:dark cycles and have circadian period lengths that are no different from pups born to sham-lesioned control mothers. Likewise, the influence of melatonin on SCN activity is not necessarily permanent. In the case of the Syrian hamster (*Microtus auratus*), administration of melatonin to pregnant mothers can entrain circadian rhythmicity in the fetal hamster, an effect that is extended into early postnatal life. But the circadian system of the adult Syrian hamster seems to be unresponsive to phase-shifting effects of melatonin treatment. Nevertheless, there is likely to be an adaptive advantage to having fetal/neonatal circadian rhythms in synchrony within a litter and between offspring and mother, at least early on in life.

What maternal signal(s) entrain the fetal SCN? The

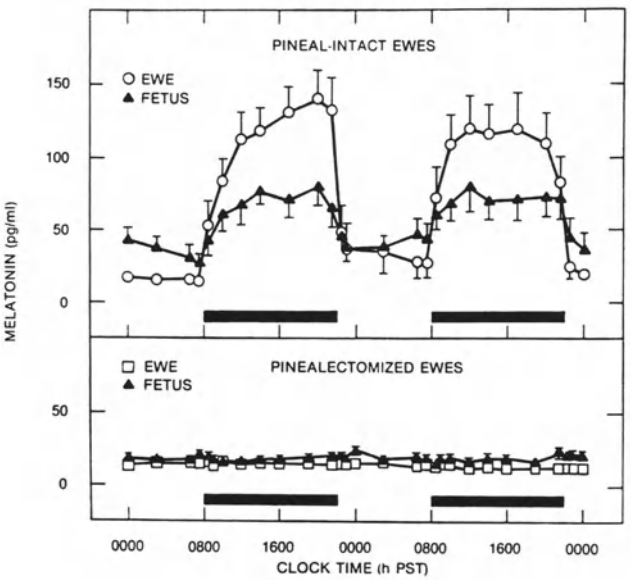

Fig. 2. Circulating levels of melatonin (mean ± SEM) in pregnant ewes and their fetuses. *Top:* pattern of melatonin secretion from six pineal-intact ewes (open circles) and their fetuses (closed triangles). *Bottom:* pattern of melatonin secretion from six pinealectomized ewes (open squares) and their fetuses (closed triangles). Dark bar indicates 14-h of dark (0800–2200 h, PST). In the pineal-intact ewes, levels of circulating melatonin are elevated during the dark period in both mother and fetus. Pinealectomy abolished the nocturnal rise in melatonin secretion in both the ewe and her pineal-intact fetus. Reprinted from *Biology of Reproduction, Vol 39*, Yellon SM, Longo LD. Effect of maternal pinealectomy and reverse photoperiod on the circadian melatonin rhythm in the sheep and fetus during the last trimester of pregnancy, 1988: 1093–1099. With permission from the Soc Study of Reproduc.

hormone melatonin is a reasonable putative entraining agent. Its secretion from the pineal gland is controlled by the SCN and light, such that circulating levels are elevated at night and suppressed during the day (*see* Fig. 2, upper panel). Melatonin is a small molecule derived from the amino acid tryptophan (*see* Fig. 10 in Chap. 23). It is lipid soluble, and so readily crosses the placenta and is also incorporated into milk. Melatonin measured in fetal circulation is entirely of maternal origin; normal pregnant ewes show elevations in levels of circulating melatonin during the night, as do their fetuses. Pinealectomizing the mother reduces circulating melatonin to low to undetectable levels in both mother and fetus (*see* Fig. 2). In the normal animal, changes in the light:dark cycle would alter the pattern of maternal melatonin, which would then be transferred to the fetus (via placenta) or neonate (via milk) and could act as an entraining agent. Furthermore, fetal and neonatal SCN contain an abundance of melatonin receptors, so the anatomical target for melatonin's actions is in place during early life. Maternal melatonin that crosses the placenta and is present in milk could then provide a way to entrain fetal/neonatal circadian rhythms to those of the mother prior to innervation of the SCN by the retinohypothalamic tract (RHT). Like in other mammals, the SCN of fetal/neonatal Syrian hamsters contain abundant melatonin binding sites. However, during postnatal development, a significant portion of these binding sites disappear. This reduction in SCN melatonin-binding sites parallels the change in response of the Syrian-hamster SCN to melatonin treatment, as discussed above. This dramatic reduction in melatonin binding sites during postnatal development is not observed in Siberian hamster, rat, or human SCN.

Although pinealectomy of the mother does not appear to have a significant effect on the fetal circadian system, injecting SCN-lesioned pregnant Syrian hamsters with melatonin is sufficient to entrain fetal circadian rhythmicity (*see* Fig. 3). These apparent discrepant results suggest the involvement of redundant signals to the fetal circadian clock. In other words, melatonin is sufficient to entrain fetal rhythmicity, but in its absence another maternal signal is capable of acting as a synchronizing agent. Another potential maternal entraining signal is rhythmic feeding. Rhythmic feeding would lead to rhythmic changes in maternal blood nutrient levels (e.g., glucose, amino acids, fatty acids), which in turn could generate a nutrient-induced signal to the fetus. SCN lesions disrupt the normal circadian rhythm in feeding. Also, as mentioned earlier, SCN-lesioned pregnant rats have litters that show initial desynchronization of circadian rhythms between individual pups. However, if the SCN-lesioned pregnant rat is forced into a rhythmic feeding regime by restricting access to food at certain times of the day, this provides a sufficient signal to entrain the fetal SCN.

What mediates the effects of rhythmic food consumption on fetal SCN entrainment? There are numerous possible nutrient signals that could be involved. One intriguing candidate is the amino acid tryrosine, through its conversion to the neurotransmitter dopamine. Maternal tyrosine would pass through the placenta and could then be converted to dopamine in the fetal brain. Thus, rhythmic maternal feeding could lead to rhythmic dopamine levels in the fetal brain.

Studies support a role for dopamine in fetal entrainment. There are high levels of D_1 dopamine receptor mRNA expression in fetal rat SCN, providing a sub-

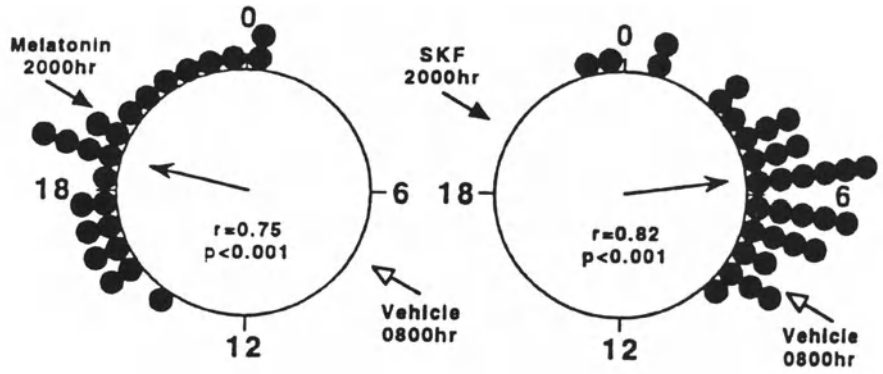

Fig. 3. Entrainment by single prenatal injections to pregnant Syrian hamsters of either melatonin (left) or the D1-dopamine receptor agonist SKF 38393 (right) given at the same time of day on day 15 of gestation. The large circles represent 24 h on the day of weaning. Small filled circles represent the phases of activity onsets of individual pups on the day of weaning. Mothers were SCN-lesioned and maintained in constant dim light. Both treatments established synchrony among the pups' phases compared to untreated controls. Single prenatal injections of melatonin and SKF 38393 at the same time (2000 h) established phases of entrainment on the day of weaning that differed by approximately 12 h. Subjective day is from 0-12 h, subjective night is from 12-0 h. Reprinted from *J Comparative Physiol A, Vol 180*, Viswanathan N, Davis FC. Single prenatal injections of melatonin or the D1-dopamine receptor agonist SKF 38393 to pregnant hamsters sets the offsprings' circadian rhythms to phases 180 degrees apart, 1997: 339–346. With permission from Springer-Verlag.

strate for dopamine's actions in early life. Activation of this receptor stimulates expression of the immediate-early gene *c-fos*, a marker of cellular activity. Importantly, *c-fos* activation has been implicated in a number of studies to be an important molecular component of the photic entrainment pathway. Perhaps this molecular element in the entrainment pathway can be activated by a number of inputs to the SCN, providing a system of redundant signaling to the circadian clock (an important feature of most every physiological system). In addition, prenatal activation of D_1 dopamine receptors can entrain fetuses from SCN-lesioned hamsters, similar to what is seen with rhythmic food restriction. Interestingly, a single injection of either melatonin or the D_1 dopamine receptor agonist SKF 38393 to SCN-lesioned Syrian hamsters on day 15 of gestation entrains fetal rhythms that were 180° out-of-phase (*see* Fig. 3). The melatonin treatment established a fetal phase of entrainment consistent with a "nighttime" signal, whereas the SKF 38393 treatment established a phase of entrainment consistent with a "daytime" signal. Furthermore, melatonin has been shown to inhibit dopamine release from chick and rabbit retina and rat hypothalamus *in vitro*. And so, not only is the SCN response to the D_1 dopamine receptor at its lowest during the night, nocturnal elevation in melatonin would potentially inhibit release of dopamine. Perhaps in the absence of a melatonin signal, additional signals such as D_1 dopamine receptor activation are sufficient to entrain the fetal circadian pacemaker.

2.2. Melatonin Affects Behavioral Rhythms in Adults

2.2.1. Rodents

In adult birds and lizards, the pineal gland and its rhythmic secretion of melatonin is an important circadian pacemaker controlling a wide variety of behavioral and physiological daily rhythms. In those species, the pinealocytes are directly photoresponsive and generate circadian rhythms in melatonin synthesis and secretion without input from the eyes or SCN. This is not the case with mammals in which the pineal gland does not generate endogenous pacemaker activity and is not directly photoresponsive. The rhythmic secretion of melatonin is well known to mediate the effects of day length on reproductive function in seasonally breeding mammals (discussed in Chap. 25). But does the nocturnal rhythm in melatonin regulate circadian rhythmicity in adult mammals, as it does in the lower vertebrates and fetal/neonatal rodents? Removal of the main source of circulating melatonin, the pineal gland, has little effect on circadian rhythms of locomotor activity in the mammalian species investigated. This negative finding suggests that pineal melatonin does not play a significant role in regulating the circadian system. However, this conclusion is complicated by the many studies showing an effect of melatonin on circadian rhythmicity and the existence of melatonin receptors in the SCN of many adult mammals.

Animals that are placed in constant environmental

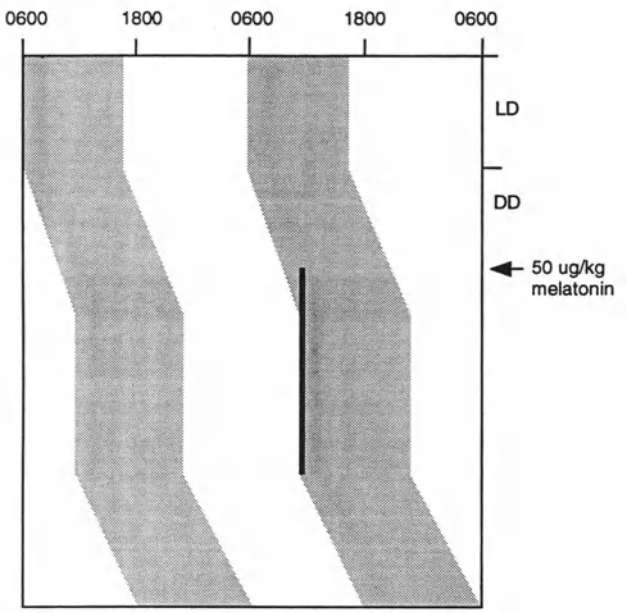

Fig. 4. Effects of daily melatonin injection on the circadian rhythm of wheel-running activity in adult rats. Diagramatic representation of an actogram is double plotted (two 24-h periods of data plotted side-by-side). Shaded areas represent the timing and duration of wheel-running activity. In a light:dark (LD) cycle, rhythmicity is entrained to the 24-h light cycle (Zeitgeber time on X-axis). However, this rhythm free-runs in constant darkness (DD); in this case, with an endogenous period that is greater than 24 h. Daily injections with 50 μg/kg of melatonin leads to entrainment in DD. Entrainment does not begin until activity onset is coincident with the time of injection (vertical line on right plot). Onset of activity is phase-locked to the time of injection until the injections are stopped, then the rhythm free-runs again with a period that is greater than 24 h. Redrawn from *J Biolog Rhythms, Vol 1*, Cassone VM, Chesworth MJ, Armstrong SM. Dose-dependent entrainment of rat circadian rhythms by daily injections of melatonin, 1986: 219–229.

conditions (i.e., absence of all entraining cues; constant darkness, constant temperature, constant food availability) exhibit endogenous rhythms in behavior, physiology, and biochemistry. For example, rats maintained in constant darkness will exhibit a rhythm of locomotor activity that is approximately, but not equal to, 24 h (*see* Fig. 4). This rhythm is said to be "free-running" because the rhythm persists in the absence of external timing cues, and the period of the rhythm is fairly stable over a prolonged duration of time. However, injection of melatonin at the same time every day can entrain this rhythm in both pineal-intact and pinealectomized rats free-running in constant darkness, supporting a role of melatonin in regulating at least one aspect of circadian rhythmicity, entrainment. Interestingly, entrainment with melatonin is not accomplished until the onset of locomotor activity nearly coincides with melatonin injection (*see* Fig. 4). This finding suggests that there is a sensitive phase to the entraining effects of melatonin. This entraining effect of melatonin requires an intact SCN. Rats that have had their SCN lesioned show a complete disruption of circadian rhythms in locomotor activity and drinking; these animals exhibit short bouts of activity that are uniformly distributed throughout day and night. These animals are not entrained with light or melatonin injections. Given that light is an extremely potent entraining agent and that pinealectomy has no effect on circadian rhythmicity, melatonin would act at best as a secondary timing cue in the adult mammal.

2.2.2. Humans

The indiscriminate use of melatonin in countries, including the United States, in which its sale is unregulated has instigated much scientific debate regarding its effectiveness in humans as a therapeutic agent and the long-term consequences of its use. At this point, there is no information from formal studies investigating melatonin toxicity in humans following chronic use. However, there is evidence that melatonin treatment (using the appropriate timing and dose) can alter circadian rhythmicity in humans. This has implications for treatment of sleep disorders associated with jet lag, shift work, and blindness.

Studies conducted in sighted humans exposed to light:dark cycles show that melatonin causes phase advances of the circadian system when it is administered in the midmorning to early afternoon, and phase delays occur when melatonin is given in the late night to early morning. A phase response curve (PRC) of the effects of melatonin on phase shifts of the circadian system has been constructed (*see* Fig. 5), and is very different from the PRC to light. The magnitude of the phase shifts increases with increasing doses of melatonin. And a significant phase shift can be detected just one day after a single dose. Because of the dramatic dependence of the time of melatonin administration and dose on the sign and magnitude of the phase shift, these two variables must be taken into account when designing melatonin treatment for humans.

Two populations who might benefit from nonphotic entraining cues, such as exogenous melatonin treatment, are the totally blind and night-shift workers. The totally blind do not respond to light and night-shift workers are often not exposed to light or social entrainment cues. Therefore, it is not surprising

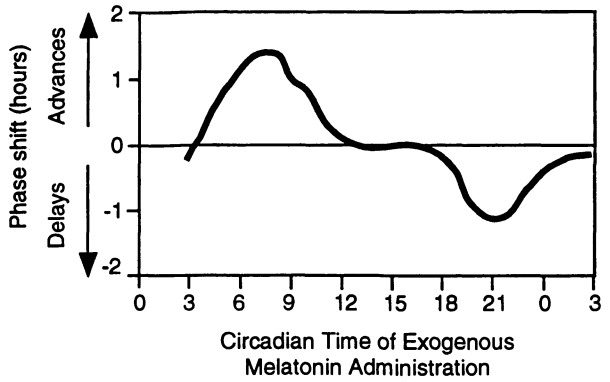

Fig. 5. Phase shifts in the rise of endogenous circulating melatonin in response to exogenous melatonin treatment in adult humans. Here, the rhythm of melatonin secretion is used as an indicator of circadian-pacemaker function. There were nine subjects involved in a total of 30 trials. Normal rise in circulating melatonin occurs about 14 h after lights on (defined in these experiments as CT 14). Thick black line represents a phase-response curve for melatonin. Melatonin treatment during the middle of the subjective day caused phase advances, whereas treatment during the late subjective night to early day caused phase delays in the endogenous melatonin rhythm. Redrawn from *J Biolog Rhythms, Vol 12*, Lewy AJ, Sack RL. Exogenous melatonin's phase-shifting effects on the endogenous melatonin profile in sighted humans: a brief review and critique of the literature. 1997: 588–594.

that these two populations experience a high incidence of circadian desynchrony in which circadian-pacemaker activity is not entrained to a 24-h day. The most common circadian abnormality in blind people is a nonentrained, free-running rhythm of just over 24 h. Insomnia and daytime sleepiness occur when the endogenous free-running rhythm is out of phase with the desired sleep time. Studies performed in blind people with a free-running circadian system showed that melatonin treatment can cause significant phase advances of endocrine rhythms. However, even though melatonin treatment can cause phase shifts, it has not been demonstrated to be an effective entraining agent in the blind. Studies in night-shift workers showed that the effect of melatonin treatment on phase shifts of the circadian system was highly variable. In one study, about one-third of the subjects showed phase delays to melatonin, the rest were determined not to respond to treatment. This type of work is very difficult in humans. There are problems with studying a population that is much more variable than the typical laboratory rodent. There are also problems with patient compliance to a rigid experimental protocol. Clearly, more work is required to determine the parameters of melatonin treatment that can cause, not just acute phase shifts, but true entrainment. In many mammals, including humans, melatonin has direct sleep-promoting effects that are independent from its effects on the circadian system. And so patients that suffer from sleep disorders can benefit from melatonin treatment at night due to its soporific effects.

2.3. Melatonin Causes Phase Shifts in SCN Membrane Excitability

2.3.1. SCN Firing Rate

The work discussed in this chapter has, so far, focused on experiments using a highly complex preparation: the whole animal. A complimentary experimental approach is to use a simpler preparation: a slice of hypothalamic tissue containing the SCN and maintained alive in vitro. Why do this work in vitro? The main reason is that the investigator has better control over the physical and chemical environment of the tissue slice than that of the whole animal. Because the SCN are visible in the living slice preparation, the investigator has tremendous control over the amount of extra-SCN tissue included in the experiments. This allows strong inferences about SCN activities being endogenously generated, rather than imposed upon by afferent signals. Importantly, SCN tissue maintained in vitro showed spontaneous rhythms in electrical and secretory activities with periods of approximately 24 h (circadian). This is consistent with other types of data (from SCN lesion and SCN transplant experiments) indicating that the SCN contains circadian-pacemaker cells. Application of melatonin to the solution surrounding the slice preparation causes a significant phase advance of the action-potential firing rate of SCN neurons from rats, by up to 4.5 h (or about 20% of the circadian cycle). This effect of melatonin on firing rate is not uniform throughout the circadian cycle, but occurs at discrete phases: (1) around the time of subjective lights off (if the SCN were still in the animal); (2) again just before the time of subjective lights on, with no shifts occuring during the subjective day (*see* Fig. 6). This phase shift persists for more than two circadian cycles following treatment. Interestingly, the first sensitive period to melatonin in vitro (around the time of 'lights off') coincides with the time in which melatonin injections entrain the circadian rhythm of locomotor activity in rats that were housed in constant environmental conditions (i.e., constant darkness, constant ambient temperature). It therefore appears that these periods of sensitivity to melatonin are endogenously generated within the SCN and do not require afferent inputs.

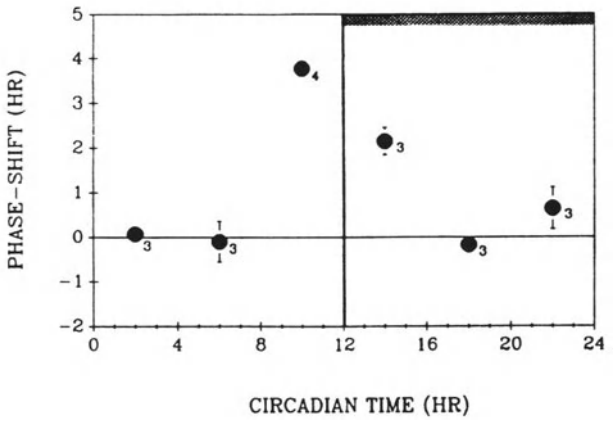

Fig. 6. Phase shifts in the circadian rhythm of rat SCN electrical activity in response to melatonin treatment. Each time-point tested represents averaged data (± SEM) from at least three experiments performed on SCN tissue slices maintained in vitro. CT 0 and CT 12 represent the time of lights on and lights off in the rat colony, respectively. Treatment of SCN slices with melatonin near the lights off (CT 10 to CT 14) and lights on (CT 22) transitions caused significant phase advances compared to untreated controls. Reprinted from *Brain Res, Vol 565*, McArthur AJ, Gillett MU, Prosser RA. Melatonin directly resets the rat suprachiasmatic circadian clock *in vitro*, 1991: 158–161. With permission from Elsevier Science.

Melatonin treatment also has acute effects on SCN firing rate, causing a rapid and reversible decrease in spontaneous firing rate of SCN neurons from rat tissue slices. This acute inhibitory effect of melatonin occurs during the late subjective day (about CT 9-14), with no response to treatment at other times of the circadian day.

2.3.2. Second-Messenger Regulation

Melatonin binds to high-affinity receptors in the SCN, which presumably activates cellular events to cause phase shifts in the circadian pacemaker. In an effort to determine the signal-transduction pathway that mediates the effects of melatonin-receptor activation (via binding of melatonin to high-affinity receptors) on target-cell function, studies have investigated the role of guanine-nucleotide binding proteins (G proteins). G-protein activation is a common mechanism by which membrane receptors initiate a cascade of events that ultimately alter cell functions (*see* Fig. 7). Briefly, binding of a ligand to its membrane receptor is thought to lead to a conformational change in the receptor such that the neighboring G protein becomes activated. This activation leads to exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP) on the G protein. Binding of GTP activates the G protein, which can then interact with effector enzymes (such as adenylate cyclase or guanylate cyclase) inserted in plasma membrane. G proteins can either stimulate effector enzymes or inhibit their activity. In the case of melatonin, binding to its high-affinity receptor inhibits adenylate cyclase activity. These enzymes (when activated) then stimulate production of second messengers (e.g., cAMP) that activate protein kinases. Protein kinases phosphorylate specific proteins, thus altering cell functions. In some cases, G proteins can interact with plasmaion channels directly, altering membrane excitability and cell function. Importantly, the receptor-G-protein complex has a high affinity for ligand, whereas membrane receptors uncoupled from G proteins have a low affinity for ligand. Pharmacological agents that uncouple G proteins from receptors (e.g., the nonhydolyzable GTP analog GTPγS) are therefore useful tools to investigate the role of G proteins in mediating effects of receptor activation on cell function. Studies have shown that the number of high-affinity melatonin receptors in brain tissue is reduced following treatment with GTPγS and other uncoupling agents. Further work has demonstrated that melatonin inhibition of adenylate cyclase activity occurs through a pertussis toxin-sensitive G protein.

What second-messenger pathways play a role in mediating the effects of melatonin on SCN firing rate? One candidate is the cAMP second-messenger system. Melatonin inhibits the cAMP second-messenger pathway in cultured SCN neurons from early neonatal Syrian hamsters. And as discussed above, binding of melatonin to its receptor inhibits adenylate cyclase activity through an inhibitory G-protein, thus blocking the cAMP second-messenger cascade. Application of either cAMP analogs or activators of endogenous cAMP to SCN tissue slices during the subjective day causes a significant phase advance of the circadian rhythm of action-potential firing rate of SCN neurons from rats. A PRC to 1-h treatments with the cAMP analog 8-benzyl amino cAMP (BA-cAMP) shows that peak sensitivity to treatment occurs at circadian time (CT) 4-6, with phase advances of up to 6 h (*see* Fig. 8). Smaller (but yet biologically significant) phase advances occur around the time of dusk. Treatment during the late subjective night had comparatively little effect. These studies suggest that cAMP is an important second messenger for activation of daytime phase advances. Can these data be reconciled with that of the effects of melatonin on phase shifting and the cAMP second messenger pathway? The relationship is not obvious. Both melatonin

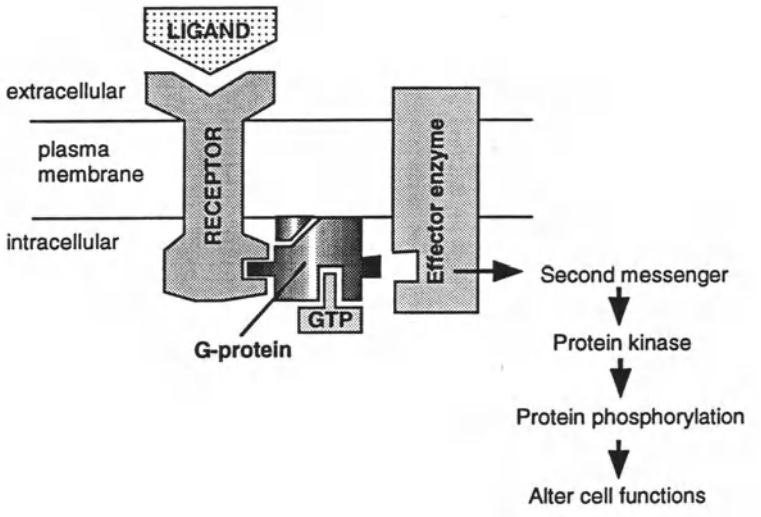

Fig. 7. Model of receptor-activated G-protein signal-transduction cascade. Details are given in the text.

and cAMP cause phase advances around dusk. But melatonin inhibits cAMP, and so is most likely not acting on that second-messenger system for its effects on phase advances near dusk.

Another candidate second-messenger pathway is diacylglycerol. Melatonin causes SCN phase advances near dusk and dawn through a pertussis toxin-sensitive G protein that stimulates phospholipase C and the diacylglycerol second-messenger pathway that activates protein kinase C (PKC). Specifically, pertussis toxin blocks the ability of melatonin to cause phase advances near dusk at CT 10. Treatment of SCN tissue slices with the phorbol ester TPA causes phase advances in firing rate near dusk at CT 10 and near dawn at CT 23, but not during the subjective day at CT 6. This effect of TPA shows similar time dependence and magnitude of phase shifts as seen with melatonin (*see* Fig. 6). The phase-shifting effects of melatonin and TPA are both blocked by the PKC inhibitors calphostin C and chelerythrine chloride. Melatonin treatment stimulates PKC phosphotransferase activity in SCN tissue slice at CT 10 and CT 23, but not CT 6. This effect was blocked by the PKC inhibitors. These findings support a role for PKC in mediating the effects of melatonin on phase advances at dusk and dawn.

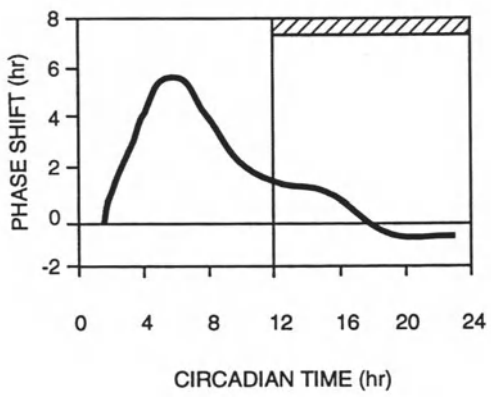

Fig. 8. Phase shifts in the circadian rhythm of rat SCN electrical activity in response to treatment with 50 x 10^{-4} *M* of the cAMP analog 8-benzyl amino cAMP. Hatched bar indicates time of subjective night. Compare this phase response curve (PRC) with that to melatonin shown in Fig. 6. Redrawn from *J Neurosci, Vol 9*, Prosser RA, Gillette ME. The mammalian circadian clock in the suprachiasmatic nuclei is reset *in vitro* by cAMP, 1989: 1073–1081.

2.4. Circadian Clock in the Mammalian Eye: Rhythmic Secretion of Retinal Melatonin

It has long been known that there are circadian oscillators in extra-SCN structures of nonmammalian vertebrates that play important roles in controlling circadian rhythms of physiology and behavior. These structures include the pineal gland and retina of birds, lizards, and amphibians. Although it is widely accepted that the SCN is the home of the primary circadian pacemaker in mammals, there has been great debate regarding the existence of other circadian pacemakers in mammals. Recent work has unequivocally demonstrated the existence of a circadian clock in the retina of the hamster. In addition to the pineal gland, the retina of mammals also synthesizes and secretes melatonin (though this retinal melatonin does not contribute significantly to circulating levels of the hormone). Cultured hamster retinas continuously perfused with medium at relatively cool temperatures of 27°C show robust rhythms of melatonin secretion in a light:dark cycle and in constant darkness. Melatonin secretion is suppressed during the day and elevated

at night. This rhythm of melatonin secretion can be entrained by light in vitro, supporting the existence of a direct light-input pathway to a circadian pacemaker within the retina. Furthermore, the period of the retinal circadian pacemaker reflects the period of the SCN pacemaker. The wild-type hamster SCN exhibits a period of rhythmicity of approximately 24 h, whereas that of the homozygous tau-mutant hamster shows a period of about 20 h. Likewise, retinas from wild-type hamsters show a melatonin rhythm of about 24 h, whereas those from homozygous tau-mutants show a melatonin rhythm of about 20 h. This finding indicates that the *tau* gene influences the period of both the retinal and SCN pacemaker cells. Why there exists a separate circadian pacemaker in the retina is not clear. Perhaps this is an evolutionary remnant that serves no important purpose in mammals. On the other hand, local rhythmic production of melatonin might play an important role in retinal function.

3. MELATONIN RECEPTORS IN THE SCN

3.1. Autoradiographic Localization of Melatonin Binding Sites in SCN

Development of radiolabeled di-iodomelatonin (2-[^{125}I]-iodomelatonin, ^{125}I-MEL) as a specific and sensitive ligand for melatonin receptors in the late 1980s allowed, for the first time, reliable detection of specific binding sites for melatonin. High-affinity binding sites for melatonin have been identified and characterized using this radiolabeled probe in the brains of a wide variety of vertebrates (from birds and lizards, to rodents, to humans) and are referred to as ML-1 type melatonin receptors. The equilibrium dissociation constant (K_d) for high-affinity melatonin binding sites is approximately 40 p*M*. Specific binding of these high-affinity sites is seen with melatonin concentrations in the physiological range (<100 p*M*). These melatonin binding sites are highly specific to melatonin, with little to negligible binding of related amines (2- iodomelatonin > melatonin ≥ 6-chloromelatonin > 6-hydroxymelatonin > N-acetyl-5-hydroxytryptamine >> serotonin). Furthermore, these binding sites activate cellular responses via G proteins. These characteristics are consistent with the high-affinity melatonin binding sites representing functional melatonin receptors (that were later cloned in a variety of animals, see below). Lower affinity melatonin binding sites have also been described, having a K_d value of about 2 n*M* and pharmacological specificity that are quite different from the ML-1 sites, and are referred to as ML-2 melatonin binding sites. Other low-affinity melatonin binding sites have been described that have binding, density, and pharmacological-specificity characteristics that differ from ML-2 sites. Notably, it remains unclear if any of the low-affinity melatonin binding sites are physiologically relevant.

Chapters 4.2 and 4.4 in this text have discussed localization of high-affinity melatonin binding sites (ML-1 receptors) to various brain tissues. This section will focus on localization of these receptors to SCN and the implications this has on circadian rhythmicity. High-affinity melatonin binding sites have been identified in abundance in the SCN of a wide variety of mammals including rat, hamster, mouse, and human; little to no binding is seen in the SCN of sheep and ferret. There is broad consensus that if a high-affinity receptor is located in relatively great abundance at some discrete anatomical location, then it must be playing a significant role in the function of that tissue. Much of the data showing that melatonin treatment caused phase shifts or entrained circadian rhythmicity in behaving animals and in SCN tissue in vitro were collected prior to or around the time that high-affinity melatonin binding sites in SCN were identified.

3.2. Cloning and Characterization of Melatonin Receptors

Cloning of the first melatonin receptor (the high-affinity G-protein-coupled Mel_{1a} receptor) was accomplished using a cDNA library constructed from an immortalized cell-line of *Xenopus laevus* dermal melanophores and a mammalian cell-expression cloning strategy. The rationale for using frog dermal melanophores is that they contain high concentrations of melatonin-receptor mRNA. In fact, the first assay for melatonin took advantage of its actions as a melanin-aggregating agent in tadpole melanophores, causing bleaching of skin pigmentation (melatonin has no effect on mammalian melanophores). The expressed recombinant *Xenopus* Mel_{1a} receptor exhibited pharmacological and functional characteristics that were similar to those of endogenous receptors in frog dermal melanophores. The Mel_{1a} receptor has now been cloned from several mammals (including rat, mouse, hamster, sheep, and human), and is expressed in regions of the brain that have shown high-affinity 2-[^{125}I]iodomelatonin binding, including SCN. The mammalian Mel_{1a} receptor shows 60% amino-acid sequence homology with the frog melatonin receptor, and the mammalian melatonin receptors show 80% homology with each other. Two other melatonin receptors have been cloned that are both 60% homolo-

gous with the Mel_{1a} receptor, the Mel_{1b} and Mel_{1c} receptors. The Mel_{1b} and Mel_{1c} receptors have pharmacological and functional properties that are similar to those of the Mel_{1a}. For example, all three receptor subtypes are coupled to inhibition of adenylate cyclase. Unlike the Mel_{1a} receptor, Mel_{1b} is not detectable by *in situ* hybridization techniques in SCN of rat. On the other hand, Mel_{1b} receptor was detected in mouse SCN using a reverse transcriptase-polymerase chain reaction (RT-PCR) assay on discrete samples of SCN tissue (a similar approach had not been reported with rat SCN at the time of this review). The Mel_{1c} receptor has been cloned in frog, chicken, and zebrafish, but not yet in a mammal.

Does the Mel_{1a} receptor mediate the effects of melatonin on circadian functions in mammals? *In situ* hybridization studies have shown expression of Mel_{1a} receptor mRNA in SCN of several mammalian species, including Siberian hamster, rat, mouse, and human. These findings suggest that the Mel_{1a} receptor plays a role in circadian actions of melatonin in at least these species. However, Mel_{1a} receptor-knockout studies have been inconclusive. Targeted disruption of the Mel_{1a} receptor eliminated 2-[^{125}I]iodomelatonin binding in the brain of homozygous mutant mice, confirming the efficacy and completeness of the knockout procedure. These mutant mice exhibited normal circadian rhythmicity compared to wild-type controls. Targeted disruption of the Mel_{1a} receptor eliminated the acute inhibitory effect of melatonin on SCN firing rate, but had no effect on the phase-shifting response to melatonin. This finding suggests that the Mel_{1a} receptor is not playing an important role in the circadian response to melatonin in the mouse. Given that the Mel_{1b} receptor is expressed in mouse SCN, including the mouse mutant that is homozygous for the Mel_{1a} receptor-knockout mutation, it is possible that melatonin's phase-shifting effect is acting through this receptor subtype. Interestingly, studies have shown that the Mel_{1b} receptor is nonfunctional in Siberian hamsters (two nonsense mutations are present within the coding region of the gene). And yet, melatonin has a robust phase-shifting effect on SCN firing rate in these animals. This finding suggests that the Mel_{1b} receptor is not playing an important role in the phase-shifting response to melatonin, at least in the Siberian hamster. At the time of this study, Mel_{1b} receptor-knockout mutations have not been performed in mouse. These overall findings are therefore inconclusive as to the role of known melatonin receptor-subtypes in mediating effects of melatonin on phase shifting in mammals. This area of research is still in its infancy, and clearly more work is required to understand the functional relationship between known physiological and behavioral effects of melatonin on circadian rhythmicity and the receptors that mediate this response.

4. NEUROENDOCRINE CELLS OF THE SCN

4.1. Rhythms in Neuropeptides in the SCN

A variety of neurotransmitters and neuromodulators (including glutamate, acetylcholine, and NPY) are thought to mediate the effects of light on the circadian system through RHT or geniculohypothalamic tract (GHT) synaptic connections to the SCN. In addition to this afferent input to the SCN, there is evidence that SCN neurons themselves synthesize and release neuropeptides that could play an important role in regulating circadian rhythmicity. Is synthesis and secretion of these SCN neuropeptides rhythmic? Do these neuropeptides regulate circadian rhythmicity in other tissues? Do they act as autocrine or paracrine factors and regulate SCN neuronal function?

Rhythmic properties of the neuropeptides arginine vasopressin (AVP), somatostatin (SS), vasoactive intestinal peptide (VIP), gastrin releasing peptide (GRP), and peptide histidine isoleucine (PHI) in SCN tissue are summarized in Table 1. Levels of both AVP peptide and AVP mRNA showed diurnal (under 12-h light: 12-h dark light:dark cycle, LD) and circadian (under DD) fluctuations in the SCN. AVP peptide and mRNA levels were maximal during mid-day in the light:dark cycle and mid-subjective day in DD. SS peptide reached maximal levels during mid-day in both LD cycle and DD. SS mRNA also showed daily and circadian rhythmicities, but the peaks and nadirs were not closely associated with those of peptide content; peak SS mRNA levels occurred during the late night-early day, with lowest levels at dusk-early night. Although VIP levels showed diurnal variation, with higher content during the night than during the day, rhythmic content did not persist in DD. A similar pattern was observed with VIP mRNA. SCN content of GRP peptide and mRNA was elevated during the late day, and declined at night in an LD cycle; and as with VIP, the rhythm of GRP peptide did not persist in DD. These data suggest that the rhythms of AVP and SS content in SCN are endogenously generated, but those of VIP and GRP are driven by the LD cycle. The work on VIP and GRP also suggest that these two neuropeptides are differentially regulated by light. This information, coupled with

Table 1
Rhythmic properties of neuropeptides in the SCN

Neuropeptide[1]	*Location of cell bodies*	*Rhythmic mRNA/peptide*	*Rhythmic secretion*	*Effects on circadian system*
AVP	dorsomedial SCN	yes/yes[2]	yes	no
SS	dorsomedial SCN	yes/yes[2]	yes	?
VIP	ventrolateral SCN	yes/yes[3]	?	yes
GRP	ventrolateral SCN	yes/yes[3]	?	yes

[1] Arginine vasopressin, AVP; somatostatin, SS; vasoactive intestinal peptide, VIP; gastrin releasing peptide, GRP; peptide histidine isoleucine, PHI.

[2] Rhythmic in LD cycle and DD.

[3] Rhythmic in LD cycle, but not DD.

morphological data, suggest functional differences between these sets of SCN-containing neurons.

Both VIP and GRP are located in the ventrolateral SCN, an area that contains input from the retina via the RHT. Could VIP- and GRP-containing neurons in the SCN be involved in photic entrainment of the circadian system? Support for this hypothesis comes from studies in which VIP or GRP were microinjected into the SCN region of Syrian hamsters free-running in constant conditions. Both neuropeptides caused phase delays of the locomotor rhythm when injected during the early subjective night, and phase advances when injected during the late subjective night; no effect was seen in response to injections given during the subjective day. Notably, the phase-shifting response to VIP and GRP is similar to that of light. Furthermore, light induces immediate-early gene expression in GRP and VIP-containing neurons in the SCN at the same phases of the circadian cycle at which it causes phase shifts in the circadian pacemaker. (Importantly, changes in cell content of a neuropeptide and changes in its release are not necessarily tightly coupled events. GRP levels in SCN tissue are highest during the day, but phase shifting effects of GRP occur during the night. Without information regarding the pattern of GRP secretion, interpretation of this type of result is difficult.) Based on the above information, the following working hypothesis has been postulated: The effects of light on the retina are conveyed to GRP and VIP-containing neurons in the SCN, and release of these neuropeptides have autocrine/paracrine effects modulating photic entrainment of the circadian pacemaker.

What about a role for AVP and SS in the circadian system? Although there has been clear demonstration of a circadian rhythm of AVP secretion from SCN tissue, there is no evidence that this neuropeptide plays an important role in regulating circadian rhythmicity. Brattelboro rats have no vasopressin, and yet their circadian system seems not to be affected by this omission. Furthermore, administration of AVP to hamsters had no significant effect on phase of the locomotor rhythm. Presumably, AVP could play a role in the output pathway of the SCN, but evidence for this is lacking. Likewise, a functional role for SS in regulating circadian rhythmicity has not yet been demonstrated.

4.2. Humoral Regulation of Circadian Rhythms in Behavior

How does the SCN transmit signals to the rest of the brain to regulate circadian rhythms in physiology and behavior? One possibility is that SCN neurons send neural projections to other brain structures, and regulate their circadian activity via synaptic transmission. Another possibility is that SCN neurons secrete a substance(s) into the circulation (blood and/or cerebral spinal fluid), and this rhythmic secretion regulates circadian activity in other parts of the body. There is now evidence that both neural and humoral communication provide efferent signaling to control circadian rhythms. This section will focus on recent studies supporting the latter. Animals that have had their SCN lesioned become arrhythmic. Transplant of fetal SCN tissue into the third ventricle of a host animal that had its SCN ablated restores circadian rhythmicity (although these animals' rhythms are no longer entrained by light). The period of the recovered circadian rhythm is that of the donor animal (*see* Fig. 9). For example, a wild-type hamster has a circadian period (τ) of about 24 h, and a homozygous tau-mutant hamster has a τ of about 20 h. If SCN tissue from a wild-type donor (τ = 24 h) is implanted into a mutant host (τ = 20 h), the period of the recovered rhythm will be about 24 h. Studies in which donor SCN tissue was encapsulated in a semipermeable polymeric capsule before transplantation prevented neural outgrowth from the donor tissue into the host

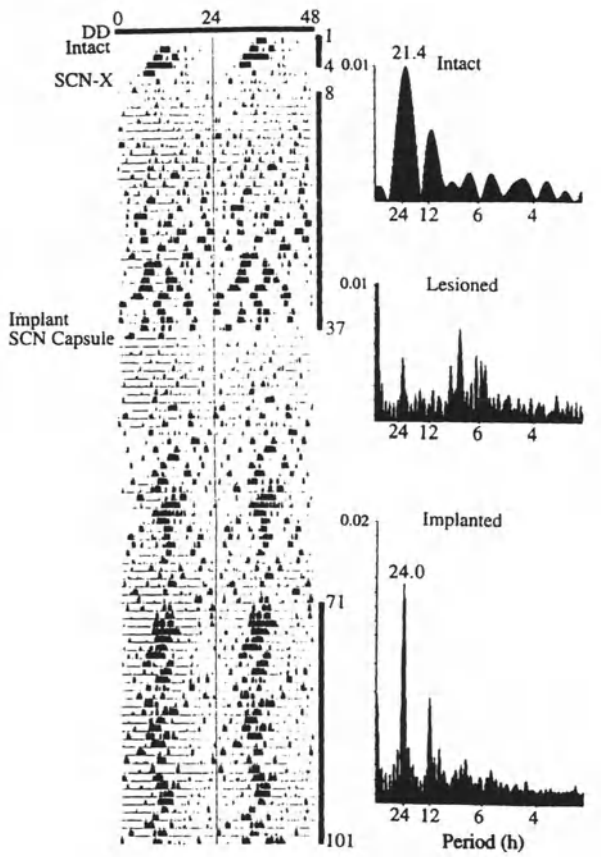

Fig. 9. Implantation of an encapsulated SCN restores circadian rhythmicity in an SCN-lesioned hamster. Here, the rhythm of wheel-running activity is used as an indicator of circadian-pacemaker function. *Left:* The actogram is double plotted (two 24-h periods of data plotted side-by-side) to facilitate interpretation of the data. Each dark spot represents a bout of wheel-running activity. The vertical bars on the right of the actogram indicate the days on which spectral analyses were performed. *Right:* Spectral analyses of wheel-running activity under the three conditions of the experiment: SCN-intact, SCN-lesioned, postimplantation. The endogenous rhythm of the intact tau-mutant host is 21.4 h in constant darkness (DD). Lesioning the SCN (SCN-X) produced arhythmicity. Several weeks after the lesion, the mutant hamster was implanted with an ecapsulated SCN from a wild-type donor hamster. Recovery of circadian rhythmicity emerged over the next several weeks, with a restored rhythm of the donor wild-type SCN (period = 24 h). Reprinted from *Nature, Vol 382*, Silver R, LeSauter J, Tresco PA, Lehman MN. A diffusible coupling signal from the transplanted suprachiasmatic nucleus controlling circadian locomotor rhythms, 1996: 810–813. With permission from Macmillan Magazines Ltd.

brain, but allowed chemical substances to diffuse into surrounding tissue. Encapsulated donor SCN tissue from wild-type hamsters were implanted into heterozygote tau-mutant host brain in which the SCN was lesioned. Prior to SCN ablation the period of locomotor activity was about 21–22 h; after SCN ablation activity became arrhythmic. Within 1–2 wk after transplant of the encapsulated wild-type SCN, a circadian rhythm of locomotor activity emerged with a period of about 24 h (*see* Fig. 9). Histological analysis confirmed no neural outgrowth from the capsule into host brain. These findings support the hypothesis that a diffusible substance from SCN tissue plays an important role in regulating circadian rhythmicity. The identity of this diffusible substance has yet to be determined.

5. CONCLUSION

The focus of this chapter has been considering evidence supporting a model of neuroendocrine feedback to the circadian system. There are several criteria that must be met in order to favor such a model. Does the SCN regulate rhythmic secretion of the neurohormone? Does removal of the neurohormone alter circadian or SCN physiology? Does replacement of the neurohormone restore appropriate circadian function? A review of the literature indicates that none of the neurohormones investigated so far meet all of these criteria in the adult mammal. Although application of melatonin and other neurohormones have been shown to phase shift the circadian pacemaker, that does not indicate a physiologically relevant role in a feedback mechanism. A case for physiological relevance of pineal melatonin in feedback to the SCN in the adult is weakened by the fact that pinealectomy has little to no significant effect on circadian rhythmicity. Perhaps the strongest support for a feedback model is from work done in fetal and neonatal animals in which the physiological relevance of a maternal melatonin signal is more convincing. SCN lesions of a pregnant female leads to desynchronization of circadian rhythmicity within her litter; entrainment of the litter is restored if the lesioned mother is injected with melatonin. Nevertheless, recent evidence of a diffusible substance from SCN tissue that can restore circadian rhythmicity in SCN-lesioned animals supports the existence of a neurohumoral feedback mechanism in the adult mammalian circadian system.

SELECTED READINGS

Albers HE, Liou S-Y, Ferris CF, Stopa EG, Zoeller RT. Neurochemistry of circadian timing. In: Klein DC, Moore RY, Reppert SM, eds. Suprachiasmatic Nucleus: The Mind's Clock. New York: Oxford University Press, 1991; 263.

Cassone VM. Melatonin and suprachiasmatic nucleus function. In: Klein DC, Moore RY, Reppert SM, eds. Suprachiasmatic Nucleus: The Mind's Clock. New York: Oxford University Press, 1991; 309.

Czeisler CA, Turek FW, eds. Special issue: Melatonin, sleep, and circadian rhythms: current progress and controversies. J Biol Rhythms vol 12(6). Thousand Oaks: SAGE Publications, 1997; 489.

Gillette MU. SCN electrophysiology *in vitro*: rhythmic activity and endogenous clock properties. In Klein DC, Moore RY, Reppert SM, eds. Suprachiasmatic Nucleus: The Mind's Clock. New York: Oxford University Press, 1991; 125.

Gillette MU, McArthur AJ. Circadian actions of melatonin at the suprachiasmatic nucleus. Behav Brain Res 1996; 73:135.

Inouye S-IT. Circadian rhythms of neuropeptides in the suprachiasmatic nucleus. In: Buijs RM, Kalsbeck A, Romijn HJ, Pennartz CMA, Mirmiran M, eds. Progress in Brain Research, vol 111. New York: Elsevier Science, 1996; 75.

Liu C, Weaver DR, Jin X, Shearman LP, Pieschl RL, Gribkoff VK, Reppert SM. Molecular dissection of two distinct actions of melatonin on the suprachiasmatic circadian clock. Neuron 1997; 19:91.

McArthur AJ, Hunt AE, Gillette MU. Melatonin action and signal transduction in the rat suprachiasmatic circadian clock: activation of protein kinase C at dusk and dawn. Endocrinology 1997; 138:627.

Piggins HD, Antle MC, Rusak B. Neuropeptides phase shift the mammalian circadian pacemaker. J Neurosci 1995; 15:5612.

Reppert SM. Interaction between the circadian clocks of mother and fetus. In: Chadwick DJ, Ackrill K, eds. Circadian Clocks and Their Adjustment. Chichester; New York: Wiley, (Ciba Foundation Symp 183), 1995; 198.

Silver R, LeSauter J, Tresco PA, Lehman MN. A diffusible coupling signal from the transplanted suprachiasmatic nucleus controlling circadian locomotor rhythms. Nature 1996; 382:810.

Tosini G, Menaker M. Circadian rhythms in cultured mammalian retina. Science 1996; 272:419.

Viswanathan N, Davis FC. Single prenatal injections of melatonin or the D1-dopamine receptor agonist SKF 38393 to pregnant hamsters sets the offsprings' circadian rhythms to phases 180 degrees apart. J Comp Physiol A 1997; 180:339.

Weaver DR, Rivkees SA, Carlson LL, Reppert SM. Localization of melatonin receptors in mammalian brain. In: Klein DC, Moore RY, Reppert SM, eds. Suprachiasmatic Nucleus: The Mind's Clock. New York: Oxford University Press, 1991; 289.

Weaver DR, Liu C, Reppert SM. Nature's knockout: the Mel_{1b} receptor is not necessary for reproductive and circadian responses to melatonin in Siberian hamsters. Mol Endocrinol 1996; 10:1478.

25 The Neuroendocrine Control of Seasonal Rhythms

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CONTENTS

1. INTRODUCTION

In many parts of the world, animals are exposed to large seasonal fluctuations in environmental conditions, particularly temperature and food availability. To cope with these changes, most long-lived species that spend at least part of the year in regions where annual differences in environmental conditions are very pronounced, exhibit seasonal cycles of physiological functions and morphological changes. The main adaptive mechanism widely observed in the wild is the ability to restrict breeding activity to the time of the year that coincides with the most propitious conditions for the survival of the neonate. Gametogenesis, sexual behavior, and pregnancy are timed accordingly. Under cold and temperate climates, these conditions are met in the spring and early summer. In tropical and arid areas, the rainy season is often the limiting factor. The adaptive importance of this trait was illustrated in a program of reintroduction of European ibex into Czechoslovakia. Native ibex were crossbred with the Turkish and Nubian subspecies, but because these subspecies had different breeding seasons, the offspring bred too early and their progeny were born in winter when food was scarce and the climate harsh and, consequently, died. Other physiological and behavioral changes take place to adapt to the harsh conditions of winter. These may include, depending on the species, migration, pelage changes, metabolic rate, and growth changes. This chapter will provide a brief overview of the neuroendocrine control of annual rhythms and a significant part will be devoted to the most widely studied annual rhythm: the photoperiodic control of the annual rhythm of reproduction.

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2. SEASONAL RHYTHMS AND ENVIRONMENTAL FACTORS

2.1. Description of Annual Rhythmicity

2.1.1. SEASONAL RHYTHM OF REPRODUCTION

The characteristics of the seasonal rhythm of reproduction vary greatly among species and among latitudes. As pointed out by Bronson, from an analysis of three groups of animals in different locations of the Northern Hemisphere (Peromyscus, lagomorphs, deer), the annual pattern of reproduction has three main characteristics. All species show a short, well-defined breeding season in the northern part of their ranges and all become year-around breeders in the southern part. Second, the latitude at which the shift

from restricted to continuous breeding occurs varies with life-span; the shift occurs at higher latitudes as life-span decreases. Third, the degree of year-to-year variation as well as locale-to-locale variation decreases as the life-span of the animal increases.

It is also important to point out that the mode of expression of seasonality is species-dependent. First, some species become reproductively quiescent during some seasons whereas others only show a reduction in fertility or no change at all. Second, the time of the year when animals are reproductively active varies from species to species and depends mainly on the length of gestation. The period of mating is observed either in spring and early summer in animals with either a very short gestational period (e.g., the ferret) or a gestational period of about a year (e.g., the horse). In contrast, mating occurs in autumn in animals with a gestational period of around 6 mo (e.g., the sheep). Third, in some mammals, in addition to timing fertilization, environmental cues also time the implantation of the embryo after an obligatory diapause of the blastocysts (e.g. European badger, mink, roe-deer). For example, in roe-deer, ovulation and fertilization take place in July and, after 5 mo of embryonic diapause, implantation occurs in December and birth in May.

The question of whether humans show seasonal variations in fertility has been raised many times. Pooling climatic and photoperiodic data from 380 geographical localizations, it appears that photoperiod and temperature may influence the physiology of human reproduction. At higher latitude, where changes in daylength are pronounced, a steep increase in human conceptions coincides with the spring equinox. Extreme temperatures (above 20°C and below 5°C) decrease the probability of conception. Interestingly, it appears that the impact of photoperiod was dominant before 1930, but temperature has become more important subsequently. More generally, the influence of environmental factors is decreasing in recent decades as people become increasingly shielded from photoperiod (by indoor work) and temperature (by heating and air-conditioning), which may explain a desensitization of human conception to seasonal rhythms.

2.1.2. Nonreproductive Seasonal Rhythms

In addition to the annual rhythm of reproduction, animals adapt their physiology to the variable environmental conditions in many ways. The annual rhythm in pelage is well known, as mammals in temperate areas frequently develop a winter coat that has greater insulating capacity as compared to the summer pelage. Mink have two molts: one in spring and one in autumn. The summer coat is characterized by a low number of hair follicles and the winter coat by a high number. The spring molt begins when the testes have already regressed and testis recrudescence in November occurs after the completion of the autumn molt. A relationship between the degree of expression of coat growth cycle and reproduction may also be observed. Primitive sheep breeds such as Soay or Moufflon have a short breeding season and a pronounced seasonal cycle of wool follicle activity and shedding. In contrast, the Merino breed has a long breeding season with little or no seasonal variation in wool growth.

Other important seasonal variations concern the body metabolic rate of the animals that takes different forms among species: variations in body weight, food intake, growth rate, milk production, fat storage, and mobilization. Deer show a rhythmic pattern of growth characterized by a rapid weight gain in spring and summer and weight loss during autumn and winter when they are sexually active. These changes in body weight are linked to changes in voluntary food intake, which is reduced during winter. In contrast, in the Syrian hamster, body weight increases in autumn and decreases in the spring.

In humans, the influence of season is also present. Seasonal affective disorder is a pattern of major depressive episodes that occur and remit with seasons. The most recognized form of seasonal affective disorder, "winter depression," is characterized by recurrent episodes of depression, hypersomnia, augmented appetite with carbohydrate craving, and weight gain that begin in the autumn and continue throughout the winter months. Although the mechanisms involved in this seasonal disorder are poorly understood, they appear to be related to a disturbance of the circadian clock, possibly because of a weak zeitgeiber during the winter months. Light therapy, which consists of exposing the patients to artificial light for a few hours every day during autumn and winter has proved useful to reduce the symptoms.

2.2. Environmental Factors Cueing for Seasonal Rhythms

2.2.1. Ultimate vs Proximate Factors

Environmental factors that act upon annual rhythms operate at two levels, the ultimate and the proximate. Ultimate factors are variables that, in the course of evolution, exert selection pressure to restrict

an activity to a particular time of the year when it is most likely to be successful. Reproductive timing mechanisms are under high selection pressures because temporal errors can result in wasted effort and can jeopardize survival. The main ultimate factors include dietary and climatic factors, particularly food availability, rainfall, and temperature. Some of the other ultimate cues of seasonal breeding are predator pressure and competition between species.

Proximate factors provide immediate cueing for the control of annual rhythms by regulating physiological processes. Many seasonal functions including reproduction, migration, and hibernation require long periods of development and preparation. This preparation must often be initiated when the conditions are far from optimal and the fitness of an animal is improved if it is capable of obtaining predictive information concerning future environmental conditions. Thus, mechanisms have evolved for the use of reliable, predictive information so that the individual is prepared for the times at which the ultimate factors are optimal. For a proximate factor to be a reliable predictor of seasonal events, it must be consistent and stable year after year. The annual cycle of photoperiod, the light fraction of the 24-h day, is the most noise-free environmental variable, and consequently is the major source of environmental information in the control of a variety of seasonal activities. It should be noted, however, that some animals do not differentiate between proximate and ultimate factors and breed when critical ultimate factors provide positive signals; these animals are referred to as opportunistic breeders and do not reproduce on a seasonal basis, but do so whenever a favorable opportunity arises. Mammals that are opportunistic breeders are generally small rodents that have the ability to become reproductively competent rapidly.

2.2.2. Photoperiod as the Main Proximate Factor

Although the Dutch manipulated light to get birds to sing out of season as early as the seventeenth century, the use of photoperiod as a predictive cue was first demonstrated in a mammal by Baker and Ransom. They observed that, in a colony of field voles held on 15 h of light, reproduction was normal. However, when exposed to 9 h of light/d, reproduction was blocked. Since then, photoperiodism has been shown to influence reproduction in most mammalian orders, but has been most thoroughly studied in hamsters and sheep. In Djungurian hamsters, exposure to short days induces reproductive inhibition, winter molt, onset of hibernation, increase in body mass, whereas maintenance of animals in long days prevents these changes. In sheep, the reversal of the photoperiodic cycle, without any modification of other environmental factors, causes the breeding season to phase shift by 6 mo; the reduction of its period to 6 mo induces the appearance of two periods of activity every year.

A clear feature of photoperiodic responses that is observed in all these treatments is that the majority of the photoperiod-induced changes are expressed after a latency of several weeks. Stimulation of ovulation after short-day exposure takes about 6 wk in sheep, induction of testicular regression by short days in the hamster about 8 wk, and stimulation of food intake by long days takes 6 to 8 wk in deer. This feature of the response is consistent with the predictive nature of photoperiodic information.

The response to photoperiod in mammals is not based solely on absolute daylength. Animals respond differently to a given daylength if it increases or decreases. In the ewe, 13 h of light/d cause a stimulation of LH secretion if animals have been exposed previously to 16 h of light. In contrast, 13 h of light/d inhibits LH secretion if animals had been exposed previously to 10 h of light. Exposure to the same photoperiod produces opposite effects depending on the photoperiodic history of the animals. Similar results have been obtained in several other species and the importance of photoperiodic history appears to be a general characteristic of photoperiodic responses. Progressively changing photoperiods throughout the year is also important to determine the length of the breeding season. Ewes submitted to a single-step decrease in photoperiod from 16L:8D (16 h of light: 8 h of darkness) to either 8L:16D or 12L:12D show a stimulation of LH secretion that lasts for only 50 to 60 d. In contrast, if daylength is decreased first from 16L:8D to 12L:12D, and then, at the time of LH increase, to 8L:16D, the period of elevated LH levels lasts almost twice as long. It should be noted that photoperiodic history is also important prenatally. Postnatal testicular development of Djungurian hamsters in 14 h of light is rapid if mothers were exposed during gestation to a photoperiod shorter than 14 h of light. In contrast, if the photoperiod of gestation is longer than 14L:10D, postnatal testicular development occurs slowly. *Because each daylength occurs twice a year, once in summer/autumn, once in winter/spring, it is the ability of integrating photoperiodic history and the change in photoperiod that allows animals to determine precisely the time of*

year. However, it should be noted that experiments in the Djungurian hamster using progressive increases and decreases in photoperiod have shown that not only is the pattern of gradual change in daylength important, but the absolute length of the day is also communicating photoperiodic information.

2.2.3. Circannual vs Noncircannual Species

Photoperiodic species are divided into two main categories depending on how photoperiod controls the annual rhythms and on the endogenous nature of the annual changes. Circannual species display endogenously generated rhythms and photoperiod synchronizes these internal rhythms. However, some species fail to show circannual rhythms and photoperiod drives the seasonal changes in these species directly.

2.2.3.1. Circannual Species. A circannual rhythm is composed of an endogenously generated sequence of events that takes approximately 1 yr to complete. Three conditions must be met for an annual rhythm to be considered as circannual. First, the cycle must persist for at least 2 yr in experimental conditions that provide no external information about its period (e.g., constant photoperiod, temperature). Second, its period in such conditions must differ from 365 d demonstrating that the cycle is not synchronized by an annually recurring environmental cue. Third, desynchrony must be observed among individuals in such conditions, also demonstrating that the cycle is not being driven by an environmental cue.

A good example of a circannual rhythm has been described in the Pekin duck, which continues to display fluctuations in testis size for up to 5 yr in constant darkness with a free-running period averaging 319 d. Several studies have described circannual cycles in animals exposed to a constant photoperiod, instead of constant darkness, which is sufficient to prevent the animal from receiving any information about time of the year. Circannual rhythms are expressed by many mammals including bats, ferrets, squirrels, marmots, sheep, deer, and primates. One of the best-studied examples is the change in body weight, reproduction, and hibernation in the ground squirrel. During the summer, body weight increases as a result of fat storage and reaches a plateau in the autumn. At this time, animals enter their underground burrows where they spend the winter. During the winter, they enter deep torpor with occasional arousal every 1 to 14 d. The final arousal in the spring leads to the resumption of activity above the ground. Upon emergence, ground squirrels are in reproductive condition and soon mate; females bear a single litter each year. The cycle begins again with the completion of the reproductive phase. All these annual changes in physiology and behavior persist under constant conditions of temperature, photoperiod and food availability.

How photoperiod entrains a circannual rhythm has been well studied in the case of reproductive activity in the ewe. This animal normally enters the breeding season in the autumn and, in the absence of pregnancy, has regular ovulatory cycles until midwinter. Ovulations then cease and the ewe remains anovulatory throughout the summer. When these animals are deprived of information about time of year, either by being kept in constant photoperiod, or by blinding, they continue in most cases to show changes in ovulatory activity or in LH secretion. If ewes are kept for 5 yr in constant short photoperiod (8L:16D), a cycle of LH secretion is observed with a period shorter than a year and cycles are desynchronous among individuals and out of phase with respect to the cycles of sheep maintained outdoors. Interestingly, it is possible to render ewes incapable of perceiving photoperiod by pinealectomy and then to infuse specific circadian patterns to restore "photoperiodic information" (Section 3.1.1.). Using this model the importance of a specific portion of the annual photoperiodic cycle can be tested by replacing melatonin in pinalectomized ewes only at this portion, for the entrainment of the circannual rhythm of LH secretion. Results of these studies indicate that the entire annual photoperiodic cycle is not required for the synchronization of the rhythm and, therefore, synchronization of annual cycles do not require continuous input from the environment (Fig. 1). Not all cues are equally effective at synchronization. In this animal, the increasing daylength of winter and spring are critically important to impose a 365-d period to the rhythm.

The adaptive advantage of circannual rhythms is relatively obvious in hibernating animals. The endogenous cyclicity allows them to measure the passage of time and to prepare for future events to come while they are deprived of external information about time of year. However, why are other animals equipped with circannual rhythms when external cues are present all the time? The seasonal variations in physiological functions could be directly driven by seasonal cues. One possible answer is that circannual rhythms like other self-sustained rhythms show a certain amount of inertia towards zeitgeber stimuli. The endogenous rhythms do not react immediately to changes in environmental cues and, therefore, are relatively nonsusceptible to irregular fluctuations in

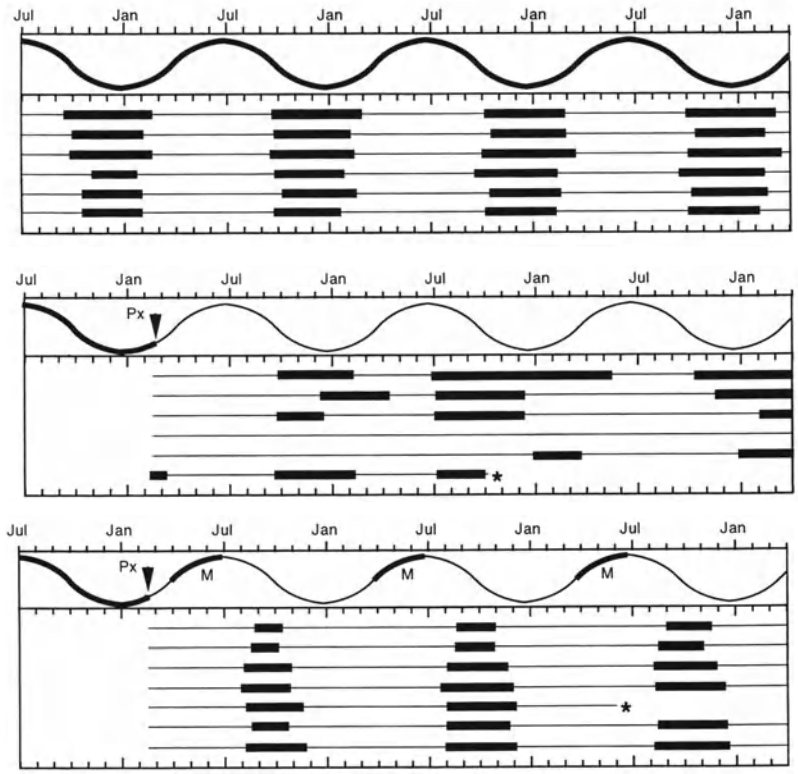

Fig. 1. Synchronization of the annual cycle of reproduction in the ewe. Top: annual cycle of LH secretion in pineal-intact ewes (black bars represent the periods of elevated LH secretion indicative of high reproductive neuroendocrine activity in the model of ovariectomized ewes treated with estradiol used in this experiment). Middle: circannual changes in LH secretion in pinealectomized ewes deprived of photoperiodic information (changes are not synchronous among ewes and are desynchronized from the natural changes represented in top panel). Bottom: entrained changes in LH secretion in pinealectomized, which receive "photoperiodic information" by infusion of melatonin during the spring only (heavy photoperiodic curve). This 90-d period of information is sufficient to synchronize the rhythms of the ewes. Arrow represents the time of pinealectomy and stars the time of death of 2 animals. (Adapted from Woodfill CJ, Wayne NL, Karsch FJ. *Biol Reprod* 1994; 50:965.)

environmental conditions. This characteristic is probably particularly important in migrating animals by allowing them to keep an internal timing of the year when they are exposed to conflicting or disturbing information about time of year as they travel across latitudes and environmental influences.

2.2.3.2. Non Circannual Species. Many mammalian species fail to show endogenous cycles of seasonal functions when housed under a fixed photoperiod. The best examples of this lack of circannual rhythm are many short-lived rodents that remain reproductively active as long as they are maintained in long photoperiod. The Syrian hamster requires short days to induce gonadal regression at the end of the breeding season. Subsequent gonadal recrudescence, which occurs in midwinter does not require an active photoperiodic drive; the transition into breeding season is generated by the development of refractoriness to the short days of winter, which allows this process to occur when days are still short. In order for the appropriate response to the decreasing daylengths of the following autumn to occur, the state of photoresponsiveness must be restored. This is accomplished only if the individual experiences several weeks of exposure to long days, as occurs each summer in the wild. Although the development of refractoriness is a form of endogenous timekeeping, the requirement for short days to inhibit reproductive activity and that for long days to restore photoresponsiveness, make hamsters unable to exhibit circannual cycle of reproductive activity whether they are exposed to short or long days. This classical model of seasonality is exemplified on Fig. 2A. However, this model was developed on the basis of laboratory experiments using only constant short and long days and it was assumed that the critical threshold for the two effects of short days, induction of gonadal regression and initiation of the timekeeping process leading to the development of refractoriness, are identical. More recent data based on the analysis of the importance of the gradually changing photoperiod throughout the year lead to a more complex model in which the decreasing photoperiod of summer crosses different thresholds (at different times) for the different actions of short days to be initiated (gonadal regression, winter molt, triggering of internal processes; Fig. 2B).

2.2.4. Other Environmental Factors

2.2.4.1. Nutrition. Nutrition can influence seasonal rhythms through the levels of nutrients (food availability). Food deprivation can affect the timing of hibernation in the ground squirrel; however, it does not seem to synchronize circannual rhythms as hibernation occurs at the normal time the following year. Similarly, food deprivation can drastically reduce

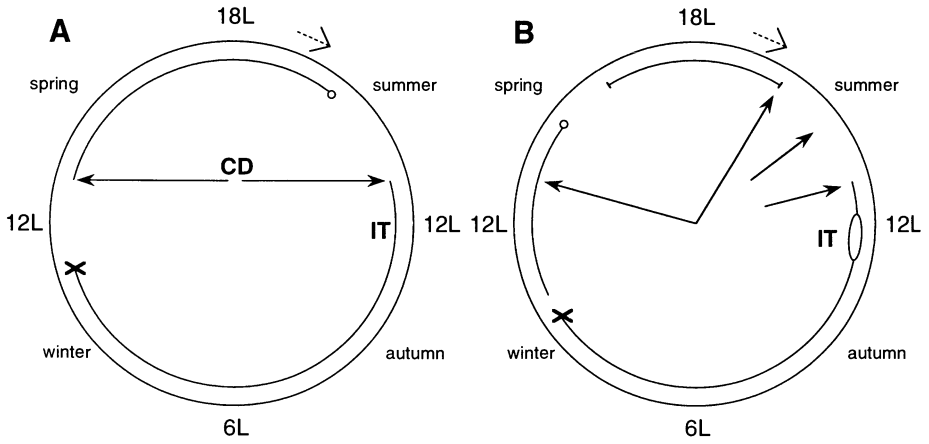

Fig. 2. Conceptual models of photoperiodic regulation of seasonality in hamsters. Large unbroken outer circle represents cyclical progression of daylength (summer and winter solstices at top and bottom; vernal and autumnal equinoxes at left and right, respectively). **(A)** The crossing of a single critical daylength (CD) in late summer (arrow) initiates gonadal regression and triggers an interval timer (IT) which programs timing of gonadal recrudescence in late winter (x). Crossing of the same critical daylength in spring (arrow) begins the process of breaking refractoriness (the completion of which is designated by circle). **(B)** In late summer, multiple critical daylengths initiate gonadal regression, winter molt, and triggering of interval timer(s), respectively (various arrows). The interval timer is affected by the length of various short daylengths (represented by oval). Increasing winter/spring daylengths that are still shorter than those that initiate gonadal regression in autumn can break refractoriness (circle) or stimulate gonadal growth directly (arrow). Very long daylengths later in spring/summer induce nonresponsiveness to future daylengths (bracketed arc). (From Gorman MR, Zucker I. In: Touitou Y, ed. *Biological Clocks. Mechanisms and Applications.* Amsterdam: Elsevier Science, 1998: 195.)

reproductive ability, but it does not have a profound effect on the timing of the subsequent breeding season. In arid climates, food availability can be the major factor in timing reproduction. In Microtus californicus, reproduction is observed in spring and autumn when grass is abundant and gonadal activity cannot be controlled by photoperiodic treatments suggesting that food availability is the primary environmental factor.

Nutrition can also influence seasonal rhythms through specific plant compounds being present at specific time of the year. The plant compound, 6-methoxy-2-benzoxazolinone (6-MBOA), can induce breeding in winter in natural populations of microtine rodents. In Microtus montanus, increase in testicular size and rate of conception are observed after addition of 6-MBOA in the food. The signals given by this compound appear to override the inhibitory photoperiodic effect.

2.2.4.2. Temperature. Ambient temperature is one of the most important environmental factors in many ectotherm vertebrates (e.g., amphibians and reptiles) but there is little evidence for the importance of temperature to time-seasonal changes in mammals. However, temperature can modulate the response to photoperiod. In sheep, cold temperatures in late summer hastens the onset of the breeding season, but if sheep are kept in constant photoperiod, temperature is unable to entrain the reproductive rhythm. In the ground squirrel, low temperature in the spring can delay the circannual cycle of hibernation. Temperature plays a more important role in the regulation of prolactin secretion with an inhibitory effect of low temperature and a stimulatory effect of high temperature. High prolactin concentrations in summer result from the combined stimulatory effects of long photoperiod and high temperature.

2.2.4.3. Hierarchy and Interactions Among Factors. In many cases, there is not a single proximate factor that acts upon annual rhythms. The action of a main proximate factor (photoperiod in the vast majority of cases) is enhanced or canceled by that of secondary factors, which inhibit or stimulate a function. In this respect, the annual reproductive cycle in sheep is under the primary control of photoperiod, but temperature, nutrition, and social factors can act as secondary factors. A poor nutrient supply will reduce the reproductive ability during the breeding season; low temperature in summer can advance the breeding season, which normally occurs in the autumn; introduction of a strange male in a flock of females that have been separated from males for several weeks induces ovulation. In this animal, social cues can also act on a longer term basis to entrain

the circannual rhythm of reproduction if the primary cue (photoperiod) is removed. Indeed, ewes, that are pinealectomized and, therefore, deprived of photoperiodic information (Section 3.1.1.), display circannual cycles of LH secretion. If these ewes are isolated from their congeners, they display cycles that are not synchronized among animals, but, if kept with a flock comprised of intact ewes and rams, their rhythms become synchronized with the intact animals. This suggests that they received cues, possibly pheromonal, from the other animals, which replaced photoperiod to inform them about time of the year.

The laboratory rat presents a contrasted and interesting example of hierarchy among factors. Although these animals are considered as nonphotoperiodic, removal of the olfactory bulbs induces males to go through puberty earlier if they are kept in short days compared to others kept in long days. Removal of the olfactory bulbs and, therefore, of pheromonal cues, reveals an underlying responsiveness to photoperiod suggesting that reproduction is controlled primarily by social factors and that photoperiod is secondary to them. Similar results have been obtained in the domestic sow.

A good example of interaction between photoperiod and food availability is obtained in the field vole. In this animal, when kept in long days, testicular weight and ovulation rate are higher if they are fed with alfalfa harvested in early spring during the growing phase rather than in summer or autumn; when voles are kept in short days, testicular regression is more complete when they are fed with autumn than spring alfalfa.

3. NEUROENDOCRINE MECHANISMS

Photoperiod is the most reliable and important environmental cue for the synchronization of annual rhythms. Furthermore, it is the most-studied seasonal regulation at a neuroendocrine level. This Section will therefore be based to a large extent on the photoperiodic regulation of seasonal rhythms.

3.1. Photoperiodic Regulation of Seasonal Rhythms

Previously in this chapter, we have mentioned that photoperiod may act on reproductive activity by driving a response or by synchronizing a circannual rhythm of reproduction. Although the mechanisms involved in these two types of effect may be different, the study of the mechanisms was, in most cases, performed using simplified experimental models in which animals are exposed to cycles of long days and short days with periods much shorter than 1 yr and in which the entrainment of circannual rhythm was not taken into consideration. Regardless of whether it is a driving action of photoperiod or an entrainment of the circannual rhythm of reproduction, the interpretation of daylength involves two major components. First, the implication of the circadian system that transforms the light/dark information into an endocrine signal, namely the circadian secretion of melatonin (*see* Chapter 24). The transduction of photoperiodic information into a rhythmic secretion of melatonin is common to the photoperiodic regulation of all seasonal functions. Second, the rhythm of secretion of melatonin will be decoded to control different physiological functions. In this case, the mechanisms involved in the action of melatonin are at least in part different among the different physiological functions and will be considered separately below.

3.1.1. Transduction of Photoperiodic Information into the Secretory Rhythm of Melatonin

The pineal hormone, melatonin, transduces photoperiodic information (*see* Chapter 24). The importance of the pineal gland was demonstrated by numerous experiments showing that the effect of photoperiod on seasonal functions is profoundly altered in pinealectomized animals. In Syrian hamsters, pinealectomy prevents the seasonal reduction in gonadotropin secretion and the gonadal regression normally brought about by experimental or natural short photoperiod. Injection of melatonin reverses the effect of pinealectomy on gonadotropin secretion and causes gonadal regression. This early work, mainly in hamsters, led to the concept of the antigonadal action of melatonin. However, more recent data, particularly those obtained in short-day breeders, lead to a more generalized role of melatonin in the control of seasonal rhythms. Specifically, pinealectomy suppresses responses to both short and long photoperiod and melatonin, depending on its specific pattern, reinstates both these responses. Thus, the *role of melatonin is to provide an endocrine code for daylength.* This role was well illustrated by replacement studies in pinealectomized animals. Melatonin delivered into the peripheral circulation to mimic short-day or long-day profiles can reproduce the stimulatory effects of short days or the inhibitory ones of long days on LH secretion in the ewe. Similar conclusions for a role of melatonin were obtained concerning other physio-

logical seasonal functions such as molt and pelage changes, prolactin secretion, thermoregulation, hibernation, and body mass changes.

The characteristic of the melatonin secretory rhythm that conveys the photoperiodic information to affect seasonal rhythms has been a matter of controversy. Three main hypotheses have been considered: the amplitude (difference between nighttime and daytime levels), the phase (presence of melatonin at a given time of day to coincide with a period of sensitivity to melatonin), or the duration of secretion (length of time with elevated levels). Amplitude is highly variable among individuals, does not consistently differ between short and long days, and experimental manipulations of the amplitude of the nighttime rise does not alter the reproductive response to melatonin. The amplitude of the nocturnal elevation of melatonin above a minimum level that is necessary for determining that a rise has occurred, therefore, does not appear to be important in the discrimination in daylength. Evidence for the phase hypothesis was obtained from studies in which single injections of pharmacological doses of melatonin administered daily in late afternoon or morning to Djungurian or Syrian hamsters cause gonadal regression. Injections at other times of the day are ineffective. A severe limitation of these studies employing daily injections of melatonin was that it is virtually impossible to control either the duration or the amplitude of the elevation of melatonin in the blood. When physiological doses of melatonin are infused into pinealectomized Djungurian hamsters and sheep for fixed durations, but at different times relative to the light/dark cycle, the response varies only with the duration of the infusion, and not with time of the day. These results and others obtained in several species led to the concept that *the nocturnal duration of the melatonin elevation is the critical feature of the melatonin signal for its action on photoperiodic physiological functions.* This is also in keeping with the fact that the duration of melatonin secretion is always positively correlated to the length of the night.

The definition of duration is not absolute, but relative. The interpretation of the duration of a given melatonin pattern depends upon the photoperiodic and melatonin history of the animal. As discussed earlier, the change is more important than the absolute duration of the photoperiod to determine a physiological response (Section 2.2.2.). A change in daylength is always accompanied by a similar change in the duration of melatonin secretion. Furthermore, experiments in Djungurian hamsters in which melatonin was infused with gradual duration changes confirm the importance of the change in the duration of melatonin presence for the photoperiodic response. *Therefore, the response to a given melatonin duration depends on the previous pattern of change in duration and the ability to perceive gradual changes in photoperiod is the reflection of mechanisms allowing the discrimination of similar changes in the duration of melatonin secretion.* An exception to this rule concerns the regulation of the secretion of prolactin which depends primarily upon the absolute photoperiod and duration of the melatonin presence, and not upon their changes.

3.1.2. Sites of Action of Melatonin

The identification of the sites of action of melatonin is difficult because melatonin influences many physiological functions. Melatonin could either act at a single site in the brain or pituitary, which would then be implicated in the regulation of many seasonal functions, or melatonin could act at multiple sites, each involved in regulating one seasonal function. This difficulty is expanded by the localization of high-affinity melatonin receptors in a wide variety of tissues in the body (*see* Chapter 23), although whether melatonin acting at these sites could affect seasonal functions remains debatable.

The first demonstration that melatonin acted in the brain was provided by studies in the white-footed mouse. Small beeswax pellets impregnated with melatonin and implanted in the anterior hypothalamus and medial preoptic area cause gonadal regression similar to that induced by short days. Pellets placed in other areas of the brain and subcutaneously have no effect on the gonads. Interestingly, similar results were obtained with a rhythmic pattern of intracerebral melatonin delivery. Melatonin delivered for 10 h, but not 5 h, in the anterior hypothalamus or medial preoptic area causes gonadal regression of pinealectomized white-footed mice. Similar results were obtained in Djungurian hamsters and gerbils. However, these studies could not define a precise target within the brain.

A major breakthrough was made with the development of the melatonin probe, ^{125}I-melatonin, which allowed us to identify putative target sites of melatonin within the hypothalamo-hypophyseal system whose importance was then tested by functional studies, mainly in hamsters and sheep. Although binding was found in several areas within the brain and the pituitary (*see* Chapter 23), it was the pars tuberalis that drew most attention because the density of bind-

ing was much higher that in any other hypothalamic or pituitary site and because it is a consistent feature among species investigated to date. Several studies have also found a seasonal variation in the melatonin binding site density and in the cytology of the pars tuberalis. Finally, the pars tuberalis is in a prime anatomical position for interacting with both the median eminence and the pars distalis of the pituitary and could, therefore, influence hypothalamo-hypophyseal function.

Surprisingly, studies in sheep and hamsters have led to the conclusion that the pars tuberalis does not mediate the action of melatonin on the neuroendocrine reproductive axis. Indeed, in the ewe, melatonin implants, directly apposed to the pars tuberalis or inserted within it, have no effect on plasma LH secretion. In this species, melatonin binding was found in the premammillary hypothalamic area and effectiveness of melatonin microimplants to stimulate LH secretion was linked to how close they were located from the area of binding (Fig. 3). In hamsters, specific binding was found in the medial basal hypothalamus and lesioning of this area blocks the ability of melatonin, infused as a short-day profile, to induce gonadal atrophy and to decrease blood concentrations of LH. Although it remains to be established whether the areas identified in hamsters and sheep are functionally related, these data suggest a discrete target for melatonin in the hypothalamus.

The pars tuberalis mediates, at least in part, the action of melatonin on prolactin secretion. Indeed, in the ewe, melatonin implants, directly apposed to the pars tuberalis or inserted within it, have an inhibitory effect on prolactin release. Also, in rams with a surgical hypothalamo-pituitary disconnection in which the central control of prolactin secretion is abolished, the ability of melatonin to inhibit the secretion of prolactin is maintained.

The dual site of action of melatonin for controlling reproduction and prolactin is interesting in relation to the photoperiodic regulation of these two functions. Indeed, the action of photoperiod and melatonin on reproductive activity is characterized by long latency and the importance of photoperiodic history, which is consistent with an action at the level of the brain for the storage of information. In contrast, photoperiodic history is not critical to the regulation of prolactin secretion and the action of melatonin is relatively rapid (only a few days) which is consistent with a more "classical" endocrine regulation at the level of the pituitary.

In mammals, two subtypes of high affinity G-protein coupled melatonin receptors have been cloned (Mel-1A and Mel-1B, *see* Chapter 23). The high level of expression of these receptors in the pars tuberalis suggests that they mediate the action of melatonin on prolactin secretion. In contrast, the photoresponsiveness of hamsters in which the Mel-1B receptor gene cannot encode a functional receptor and the failure to obtain a strong expression of these receptors in the hypothalamus outside of the suprachiasmatic nucleus leaves the possibility that the action of melatonin on reproduction is mediated by receptors which remain to be identified.

3.1.3. Melatonin Control of Reproduction

3.1.3.1. Steroid Dependent and Independent Effects of Photoperiod on Gonadotropin Secretion. Photoperiod induces changes in reproductive activity through modifications in the secretion of LH and FSH. Photoperiod regulates the secretion of gonadotropins by means of two complimentary mechanisms: one independent of, the other dependent on gonadal steroids. Melatonin mediates both the steroid dependent and independent effects of photoperiod on gonadotropin secretion.

The steroid-independent effects of photoperiod are observed in gonadectomized animals. In castrated rams and ovariectomized ewes, the secretion of LH is lower during inhibitory long days or the anestrus season than during short days or the breeding season, being 1 pulse/h or every 30 min, respectively, in ovariectomized ewes, i.e., a twofold difference. This difference between LH secretion during long d and short days is greatly increased in the presence of estradiol or testosterone, which demonstrates that there exists also a steroid dependent effect of photoperiod. An LH pulse is observed either every 12 or 24 h or every 30 min depending on whether the ovariectomized ewes treated with an estradiol implant mimicking midfollicular phase levels are experiencing long or short days, i.e., at least a 20-fold difference. A similar dual action of photoperiod on gonadotropin secretion is demonstrated in other species. However, the relative importance of the two mechanisms varies among species; the steroid-dependent effect is predominant in sheep, the steroid-independent one in the female Syrian hamster. The steroid-independent effect of photoperiod on gonadotropin secretion is not dependent upon adrenal steroids as the difference between short d and long days persist after both ovariectomy and adrenalectomy.

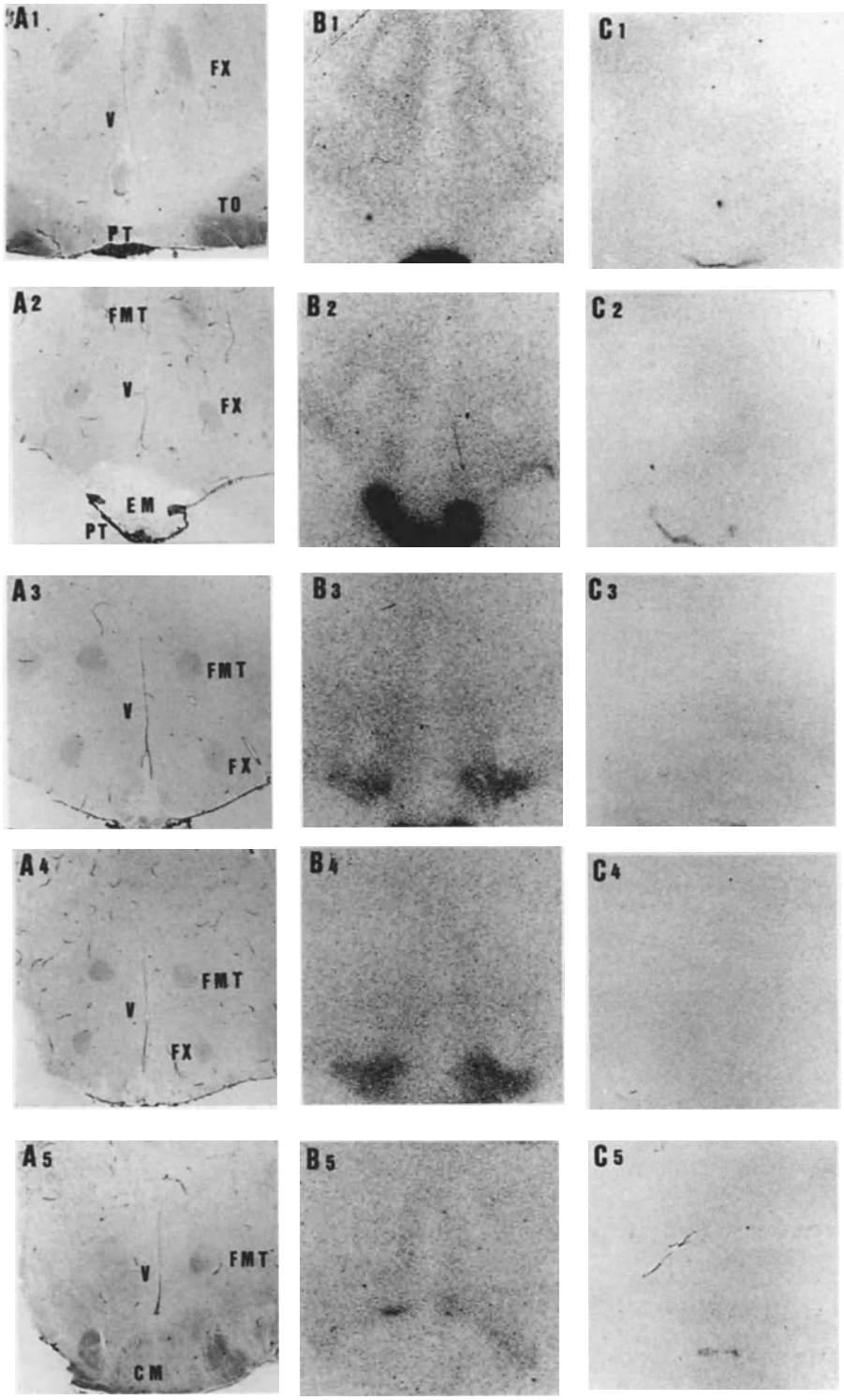

Fig. 3. Coronal sections of sheep hypothalamus illustrating distribution of melatonin binding sites. Top to bottom reflects rostral to caudal sequence (1 to 5). Left column (**A**): cresyl violet stained sections; middle column (**B**): autoradiographic image of total iodomelatonin binding; right column (**C**): nonspecific binding, estimated by incubating adjacent section with a 200-fold excess of cold melatonin. Note the specific binding of melatonin in the pars tuberalis (B1 and B2) and in the premammillary hypothalamic area (B3 and B4). Nomenclature: V: third ventricle; FMT: Fasciculus mamillothalamicus; Fx: fornix; TO: tractus opticus; CM: corpus mamillare; EM: Median eminence; PT: pars tuberalis. (From Malpaux B, Daveau A, Maurice-Mandon F, Duarte G, Chemineau P. *Endocrinology* 1998; 139:1508.)

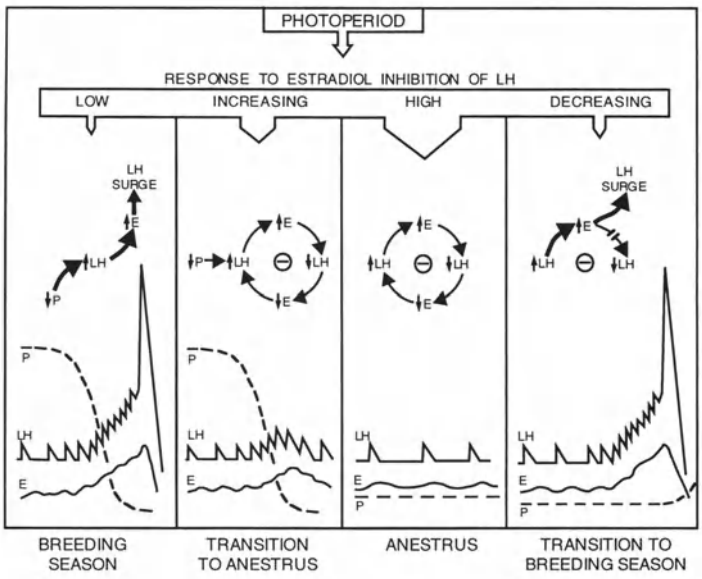

Fig. 4. Model for control of seasonal breeding in the ewe. Left to right: Endocrine events: during the follicular phase of the breeding season; at luteolysis during the transition to anestrus; throughout anestrus; during the first follicular phase at the transition to the breeding season. (From Karsch FJ, Goodman RL, Legan SJ. *J Reprod Fertil* 1980; 58:521.)

How seasonal variations in the mechanisms controlling pulsatile LH secretion account for the seasonal variations in ovulatory activity has been particularly well studied in the ewe. The endocrine events taking place during the estrous cycle constitute a chain of causally related events. During the luteal phase of the breeding season, progesterone exerts a strong inhibition of GnRH and LH pulsatile release. After luteolysis, GnRH and LH pulse frequency increase, which stimulates ovarian estradiol secretion. The estradiol rise then triggers the LH surge and ovulation occurs. At the transition into anestrus, estradiol has become powerfully inhibitory and a slight increase in estradiol levels is followed by an immediate inhibition of LH secretion (Fig. 4). As a consequence, luteolysis is not followed by a large increase in GnRH and LH pulsatile secretion and estradiol concentrations cannot reach the threshold value critical to trigger the positive-feedback mechanism leading to ovulation and estrous behavior. Ewes remain in anestrus as long as the inhibitory mechanisms of LH secretion remain active and start cycling again in the autumn when they decline. The critical step in the events occurring during the estrous cycle, which explains the cessation of ovulation during anestrus, is, therefore, the follicular phase increase in LH pulse frequency that drives the preovulatory estradiol rise. Anestrus does not result from changes in other steps of the sequence of events of the estrous cycle. During anestrus, GnRH can stimulate LH secretion, LH can stimulate estradiol secretion, and estradiol, if given to produce late follicular phase levels, can induce the positive-feedback mechanism and cause ovulation.

The changes in steroid negative feedback take place mainly at the level of the hypothalamus, not the pituitary. Indeed, seasonal changes in pulsatile LH secretion in ovariectomized and estradiol treated ewes is a close reflection of the changes in GnRH pulsatile secretion. Furthermore, the inhibitory effect of peripheral estradiol or testosterone during the anestrous season can be mimicked by the local delivery (insertion of microimplants) of the steroids in the hypothalamus. Many studies show that dopamine and noradrenaline are involved in transducing the negative feedback of estradiol on GnRH secretion. In ewes, systemic injection of a dopamine antagonist (pimozide) during anestrus (strong negative feedback of estradiol) induces a temporary increase in LH secretion. In sheep, the A15 hypothalamic cell group is a dopaminergic structure and is involved in mediating the inhibitory effects of estradiol. Lesioning of the A15 nucleus during anestrus causes an increase in LH secretion. In addtion, estradiol increases tyrosine hydroxylase (TH, rate-limiting step enzyme of catecholamine synthesis) activity in inhibitory long-day treated ewes and induces *c-fos* gene expression in TH-immunoreactive cells of this structure in a season-dependent manner. A similar role has been proposed for the A14 dopaminergic cell group. Studies using local delivery of specific pharmacological blockers reveal that alpha-adrenergic and dopaminergic neurons would be organized in series in this regulation: alpha-adrenergic neurons would stimulate a dopaminergic neuronal system which, in turn, would inhibit GnRH neurons directly or indirectly. This implication of dopamine and noradrenaline would be a part of

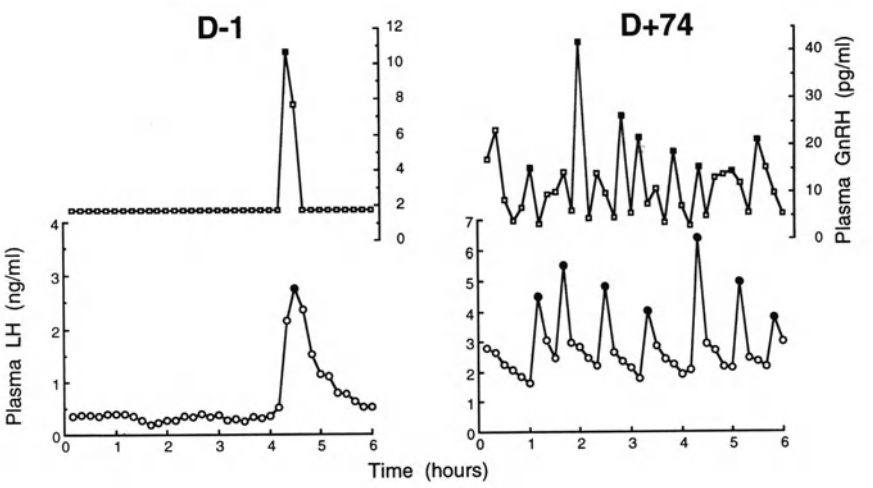

Fig. 5. Effect of melatonin on GnRH and LH pulsatile secretion. Examples of individual profiles of GnRH (top profiles) and simultaneous LH (bottom profiles) secretions before (D-1, left panels) or 74 d after (D+74, right panels) the insertion of a melatonin implant. Ewes were ovariectomized and treated with a subcutaneous estradiol implant and were exposed to long days (16L:8D). Blood samples were obtained every 10 min for 6 h on each occasion. The closed symbols depict detected pulses (adapted from Viguié C, Caraty A, Locatelli A, Malpaux B. *Biol Reprod* 1995; 52:1114).

the seasonal regulation of gonadotropin secretion by mediating estradiol negative feedback.

3.1.3.2. GnRH Neurons as the Final Nervous Pathway of Melatonin Action. The final step of the action of melatonin at the level of the central nervous system is a modulation of GnRH secretion, partly as a consequence of the modification of steroid feedback. Melatonin treatment causes an increase in the immunoreactivity of GnRH in cell bodies located in the hypothalamus and increased density of GnRH-containing elements in the median eminence, in addition to its suppressive effects on gonadal function in white-footed mice. In Djungurian hamsters, a rapid and transient increase in the distribution of detectable GnRH mRNA-containing cells is observed 2 d after transfer to stimulatory long days. This increase may be an early step in the stimulation of FSH secretion and gonadal growth, which occur days later. In sheep, it is possible to measure directly and reliably the release of GnRH in the hypophyseal portal system and melatonin, given as a short-day profile, causes an increase in the frequency of pulsatile GnRH release (Fig. 5). This increase in GnRH secretion is observed after 40 to 60 d when LH secretion is stimulated. Despite this effect of melatonin on the GnRH system, it is thought that melatonin does not act directly on the GnRH neurons; rather it acts indirectly through some interneuronal route that synapses on the GnRH neurons. The initial step of this route has not been isolated as the nature of the target cells of melatonin in the medial basal hypothalamus of hamster or the premammillary hypothalamus of sheep is not known. Nevertheless, some of the neurotransmitters playing a role of relay between the target of melatonin and the GnRH neurons are established.

3.1.3.3. Interneurons Between Melatonin Target Neurons and GnRH Neurons. A large number of neurotransmitters are involved in the regulation of the GnRH neurons and constitute potential candidates to mediate the effects of melatonin on GnRH release. Indeed, experimental evidence implicating several neuronal systems has been obtained.

Dopamine

In the ewe, exposure to stimulatory short days results in decreased dopaminergic activity in the median eminence as assessed by both a reduction in dopamine content and in TH activity. No effect of short-day exposure on noradrenaline content in this structure is found. This effect of photoperiod on TH activity is mediated by melatonin. The inhibition of median eminence TH activity by short days or by treatment with a melatonin implant is expressed at a time when the inhibition of prolactin secretion is already maximal indicating that these photoperiod-induced changes in TH activity are independent of the regulation of prolactin secretion (Fig. 6). Rather, they are related to the photoperiodic regulation of LH secretion. Indeed, the pharmacological blockade of TH locally in the median eminence of long-day photoinhibited ewes leads to an increase in LH secretion. A reduction in TH activity in the median eminence is, therefore, an important component of the stimulatory effect of melatonin on GnRH output. However, such modulation of TH activity is estradiol-independent. Thus, in contrast to the A15 dopaminergic nucleus which is involved in the modulation of the estradiol negative feedback, the dopaminergic neurons projecting into the median eminence appear to be involved upstream relative to the integration of the

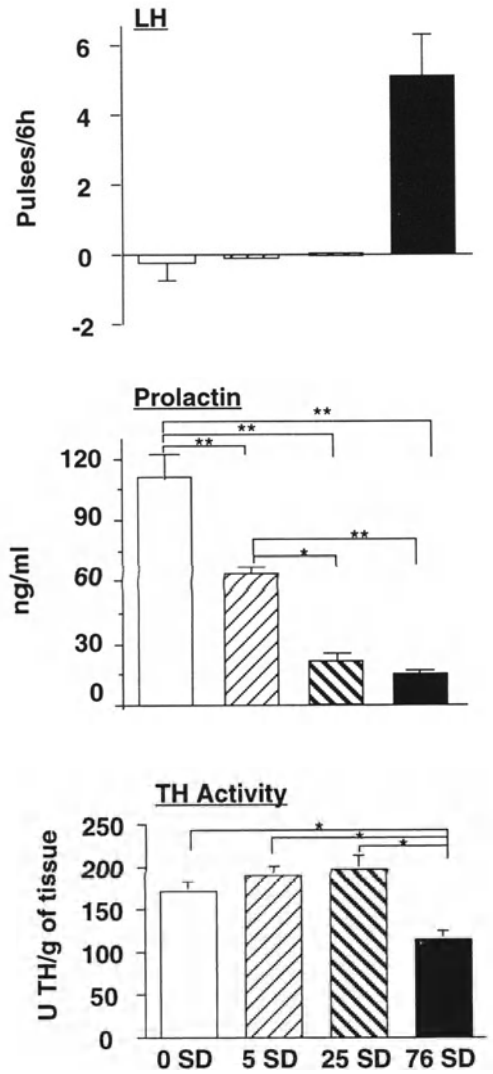

Fig. 6. Neuroendocrine changes in ewes treated with 0, 5, 25, or 76 short days. Mean (±SEM) difference in the number of LH pulses/6h between the control long-day period and the end of the experiment(top), mean (±SEM) plasma prolactin concentrations at the end of the experiment (middle) and mean (±SEM) TH activity in the median eminence of ewes. All ewes were ovariectomized and treated with a subcutaneous implant of oetradiol. They were pretreated with long days (16L:8D) and then allocated to one of 4 groups treated with 0, 5, 25, or 76 short days. LH secretory profiles were determined from LH concentration measured in blood samples obtained every 10 min for 6 h at two different periods of the photoperiodic treatment: 1) 7 d before the end of the last long-day period, and 2) 3 to 4 d before the end of the experiment (i.e., in long-day or on average after 3, 23, or 74 short days). Prolactin concentrations were determined in every third sample during the last 3 h of the second sampling period. TH activity was measured individually in the stalk-median eminence of the same ovariectomized ewes ($n = 7$ per group) *: $p < 0.05$; **: $p < 0.01$ (adapted from Viguié C, Thibault J, Thiéry JC, Tillet Y, Malpaux B. *Endocrinology* 1997; 138:499).

estradiol signal. The median eminence in sheep is a structure rich in dopaminergic terminals, but contains no TH-immunoreactive perikarya. The localization of the cell bodies projecting their axons to the median eminence, and more generally the anatomical and functional relationship between the A14 and A15 nuclei and the median eminence, have yet to be determined. In the median eminence, dopamine probably acts directly on GnRH neurons as dopaminergic synapses on GnRH terminals have been observed, and this action is mediated through D-2 type receptors.

In male Syrian hamsters, exposure to inhibitory short days reduces dopamine and noradrenaline turnover in the median eminence and this effect is mediated by melatonin. This change is associated with a decrease in L-aromatic amino acid decarboxylase positive cells in the arcuate nucleus without changes in TH-immunoreactive cell number or TH activity; furthermore, it is independent of gonadal steroids. Suppression of LH and FSH release in this species is presumably related to reduced noradrenaline activity, whereas reduced dopamine turnover may represent a consequence of suppression of prolactin levels by short days.

Serotonin

Evidence for the implication of serotonin was also obtained in sheep. Serotonin inhibits LH pulsatile secretion during seasonal anestrus, and not during the breeding season. This inhibitory effect of serotonin is mediated by 5-HT2 receptors and interestingly a photoperiod-induced change in the density of 5-HT2 receptors in the ventrolateral posterior hypothalamus was described.

Excitatory aminoacids

The implication of excitatory aminoacids in the photoperiodic regulation of LH secretion was suggested by experiments involving administration of N-methyl-D,L-aspartic acid (NMDA), an agonist of neuroexcitatory aminoacids. Such an administration acutely stimulates GnRH and LH secretion in seasonal species such as sheep and hamsters. However, this stimulatory effect is larger during periods of photoinhibited LH secretion than during photostimulated periods. Also, in male hamsters, the inhibitory effect of short days on testicular activity can be blocked by chronic treatment with NMDA. Although glutamate is involved in the photic regulation of circadian rhythm at the level of the retinohypothalamic tract, this action of NMDA cannot be explained by an effect on the melatonin

secretory rhythm. Indeed, the differential effect of NMDA in photoinhibited and photostimulated ewes is maintained if stimulation is induced by melatonin administration instead of short-day exposure. Furthermore, in Djungurian hamsters, the chronic effect of NMDA is observed on the photoperiod-induced changes in reproduction, but not on other functions (pelage color, body weight). *These experiments suggest that a change in excitatory amino acid input may be part of the mechanism mediating the action of melatonin on GnRH secretion.* Consistent with this hypothesis, photoperiod modifies the density of NMDA receptors in the preoptic area of hamsters.

3.1.3.4. Neuronal Network Linking Melatonin Targets and GnRH Neurons. Besides the neurotransmitters or receptors mentioned above, future research will probably reveal the involvement of other neuronal systems in mediating the action of melatonin on GnRH neurons. Regardless of the nature of these neurons, the action of melatonin on GnRH neurons most likely involves a complex network of interacting neurons. These neuronal networks may differ greatly between species. For instance, in two species that present similarities in the reproductive response to melatonin, the Syrian and Djungurian hamsters, the suprachiamatic nucleus does not appear to play the same role. In Djungurian, but not Syrian hamsters, lesion of the suprachiasmatic nucleus blocks short-day induced testicular regression.

Part of the mode of action of photoperiod could be to cause morphological changes in this neuronal network. Evidence for such an effect was obtained at the level of the GnRH neurons of the ewe. At the light microscopic level, immunostained GnRH neurons in the preoptic area have longer and more numerous dendrites during anestrus than during the breeding season. However, these changes could reflect alterations in the content or localization of immunodetectable GnRH, rather than actual morphological changes. At the electron microscopic level, GnRH neurons in the preoptic area receive more than twice the mean number of synaptic inputs per unit of plasma membrane during the breeding season as during anestrus. Interestingly, these changes are not dependent on changes in ovarian steroid levels, which differs markedly with the examples of seasonal plasticity described in the literature because of changing levels of steroids. The changes observed in the preoptic area of the ewe could, therefore, reflect the action of photoperiod or the expression of the endogenous rhythm of reproduction.

In relation to possible phenomena of neuronal plasticity, it is worth noting that thyroid hormones have been strongly implicated in allowing seasonal changes in reproductive activity. Reproductive transition from the breeding season to anestrus is dependent upon the presence of thyroid hormones. Thyroidectomized ewes do not exhibit a seasonal decline in episodic secretion of GnRH in hypophyseal portal blood and thus fail to enter anestrus. Thyroxine replacement reverses the effect of thyroidectomy and the seasonal changes in thyroid hormone concentrations are not necessary for this reversal. Because thyroid hormones are essential for the normal morphological maturation of the central nervous system, the permissive role of thyroid hormones for seasonal changes to occur may be to cause the prerequired morphological rearrangements for the changes in GnRH secretion.

3.1.4. Control of Seasonal Pelage Cycles

There are three lines of evidence suggesting that the *influence of melatonin on hair follicles is indirect and is mediated mainly via changes in the secretion of prolactin.* There is a temporal relationship between the seasonal cycle of prolactin secretion and the pelage cycle. In winter, the Moufflon has a coarse outer coat of guard hairs and a dense undercoat. Growth of this coat is complete by December and there is no fiber growth from January to March when prolactin concentrations are low. As prolactin concentrations increase in April, growth of guard hair resumes and a molt occurs, resulting in a short summer coat. As prolactin levels decline in autumn, both guard hairs and down grow to produce the winter coat. Second, treatment with prolactin has a profound effect on pelage, as demonstrated in several species (Djungurian hamster, mink, blue fox, red deer, cashmere goat). For example, administration of prolactin to minks at the end of winter advances the spring molt. Third, the effect of lengthening spring photoperiod on pelage can be overridden by injection of a dopaminergic agonist, which prevents the seasonal increase in prolactin levels. Similarly, dopaminergic antagonists can prevent the changes occurring during shortening days. In the Djungurian hamster, treatment with bromocriptine, a dopamine agonist, prevents the spring molt and concomitant administration of prolactin allowed the spring molt to occur. Prolactin has therefore a causal role in the seasonal control of hair follicle, but it is not clear whether it acts directly at the hair follicle

although the presence of prolactin binding sites in hair follicles of sheep and the stimulation of growth of hair follicle of goats in vitro favor a direct-action hypothesis. Other factors that vary seasonally (IGF-1, thyroid hormones) have also been implicated in the control of pelage cycles.

3.1.5. Melatonin Control of Growth and Metabolism

The mechanisms by which melatonin controls cycles of body weight, food intake, and fat storage/mobilization are poorly understood and only sparse data are available. Part of the changes in body weight are secondary to changes in reproductive activity. In male Djungurian hamsters, the loss of body weight following exposure to short days is partly related to a decrease in testosterone secretion; however, an additional component is independent of androgen action because a short-day induced loss of body weight can be observed in castrated animals.

Photoperiod influences the secretion of growth hormone (GH). In Syrian hamsters, mean blood concentrations of GH are higher in short than in long days, but in sheep and reindeer, GH is stimulated by increasing daylength. As a result, in these species, a good temporal relationship between increases in GH secretion and improved growth exists. A possible role of GH would be to stimulate the production of IGF-1 whose blood levels vary seasonally. In the reindeer, exposure to long days causes an increase in IGF-1 blood levels and, in male red deer, melatonin treatment alters the seasonal cycle of IGF-1. In the male Syrian hamster, melatonin causes a concomitant increase in body weight and IGF-1 blood levels.

In sheep and cow, exposure to long days increases the milk yield and this effect is mediated by melatonin. The stimulatory effect of long days on prolactin and, to a lesser extent, on GH blood concentrations could explain the effect of long days on milk production. In the lactating cow, an increase in IGF-1 blood levels is also observed during long-day exposure and could be a mechanism involved in the stimulatory effect of long days.

Neuropeptide Y (NPY) is involved in the control of food intake and there are photoperiod-induced changes in NPY immunostaining, particularly in the median eminence. However, in Djungurian hamsters, although NPY stimulates food intake, this effect is not photoperiod dependent. Therefore, changes in NPY do not appear to be part of the seasonal regulation of food intake in this species.

In Djungurian hamsters, short photoperiod reduces leptin gene expression in white and brown adipose tissue. Because leptin is a powerful regulator of food intake and energy expenditure, it may mediate the effect of photoperiod on some seasonal adaptations.

3.1.6. Melatonin Control of Prolactin Secretion

The seasonal cycle of prolactin secretion, which is well-conserved among species with a peak of secretion in spring or summer, in most species plays an important role in mediating the effect of photoperiod and melatonin on different functions (see above). However, the pathways involved in the control of prolactin secretion by melatonin are poorly known. There is no evidence that photoperiod interacts with the release of hypothalamic factors modulating prolactin secretion, which reinforces the hypothesis of an action of melatonin mainly at the level of the pars tuberalis to control prolactin secretion (Section 3.1.2.). It is currently hypothesized that, under the control of melatonin, the pars tuberalis produces a secretory product that can modulate prolactin secretion at the levels of the lactotrophs. In support of this hypothesis, pars tuberalis cells in primary culture release a factor that activates *c-fos* gene expression in pituitary lactotrophs and stimulates the secretion of prolactin by primary culture of pars distalis cells. This factor has yet to be isolated.

3.2. Generation and Expression of Circannual Rhythms

The physiological mechanisms underlying the generation and synchronization of circannual rhythms are poorly understood. The poor understanding is mainly related to the long-term nature of the rhythm, which requires long and expensive experimentation and also to the fact that it can be studied only on long life-span species that can express a sufficient number of cycles, but are usually not the most widely used laboratory species. This paragraph will review the hypothetical models proposed for the generation of circannual rhythms and the structures for which involvment in the generation or expression of these rhythms has been studied.

3.2.1. Hypothetical Models for the Generation of Circannual Rhythms

Three hypothetical models have been proposed to explain the generation of circannual rhythm: the fre-

quency demultiplication model, the oscillator model, and the sequence of stages model.

3.2.1.1. Frequency Demultiplication of Circadian Rhythms. This hypothesis proposes that circannual rhythms might be generated by a rhythm with higher frequency, for instance circadian rhythms, in a process called frequency demultiplication. An analogy often used is that of the electrical clock that transforms the frequency of 50 to 60 cycles/s of the commercial electrical current into 1 cycle/d. This hypothesis was tested in two ways. The first way consisted in determining whether there is a relationship between the period of the circadian clock and that of circannual rhythms, either by entraining the animals to light–dark cycles of different periods or by letting the circadian system free-run in constant conditions. For instance, in female ground squirrels, entrainment of circadian rhythms to periods of 23, 24, or 25 h did not cause a change in the period of the circannual cycles of body mass or estrous cyclicity. The second way consisted in disrupting the circadian system, for instance by lesioning the suprachiasmatic nucleus in mammals, and analyzing the consequences on the expression of circannual rhythms. Both of these approaches used in many experiments in many species did not support the hypothesis as no obvious relationship was found between period of circadian and circannual rhythm and disruption of the circadian system did not prevent the expression of circannual rhythms. The lack of support for this model is also consistent with the fact that it is possible to entrain circannual rhythm with a period much shorter than 1 yr, by manipulating daylength. Because the period of the light–dark cycle, and therefore, that of the circadian system, remains 24 h, the number of circadian cycles within such an entrained circannual rhythm is reduced which is not consistent with the frequency demultiplication hypothesis.

3.2.1.2. The Oscillator Model. This hypothesis proposes that, in analogy with the circadian rhythms (*see* Chapter 24), circannual rhythms are generated by an oscillator with a periodicity of about 365 d. The types of experiments that have been performed to characterize the formal properties of circadian oscillators (*see* Chapter 22) would be impossible for studying the formal properties of a putative circannual oscillator because of the long timespans involved. In contrast to circadian rhythms whose period is stable over time, circannual rhythms appear more unstable, which may appear in conflict with the theory of the oscillator in which periodicity should be stable and protected from environmental perturbations. The failure to identify one structure, responsible for the generation of the rhythm, also considerably weakens this hypothesis (Section 3.2.2.).

3.2.1.3. Sequence of Stages Model. This hypothesis proposes that circannual rhythms are composed of a series of stage, the termination of each stage triggering the onset of the next. Each stage of the series is linked to the previous one and the first one is linked to the last one so that the cycle can persist in constant conditions. The analogy often used in this case is that of the estrous or menstrual cycle. The period of such a cycle is the sum of the duration of each individual stage and a change in the period of the cycle can be obtained by lengthening or shortening (temporary block or acceleration) an individual stage. Synchronization of the rhythm by the environment could be accomplished by specific cues acting at one or more critical stages of the annual cycle to advance or delay the onset of the next stage and thus influence the timing of the subsequent cycle events and cycle periodicity. This model allows the possibility of modifying the periodicity and waveform of the rhythm by disturbing various parts of the rhythm. In this sense, it may better reflect the lack of stability observed in circannual rhythms. The requirement for thyroid hormones at a particular phase of the reproductive cycle for it to be expressed is consistent with this model; the presence of thyroid hormones may be necessary for the cycle to proceed from one particular stage to the following one (Section 3.1.3.4.).

3.2.2. Neural Substrates for the Circannual Rhythms

In an attempt to identify a putative circannual oscillator (or set of oscillators), investigations have focused on identifying structures necessary for circannual rhythms to be expressed. Studies have been focused on the suprachiasmatic nucleus, the pineal gland, and various hypothalamic areas. None of these structures has been found to be essential for the generation of the circannual rhythms because circannual cycles of LH secretion persist after pinealectomy in ewes and lesions of the suprachiasmatic nucleus in ground squirrels did not suppress the circannual body weight and reproductive rhythms. Similarly, circannual body weight rhythms persist after lesion of the ventromedial hypothalamus in ground squirrels. These structures are not critical for the generation of circannual rhythms; however, they may be involved in their expression as their destruction modifies the characteristics of the rhythms. For instance, a dissoci-

ation of the circannual body weight and hibernation rhythms can be observed in ground squirrels after lesion of the suprachiasmatic nucleus; weight gain is often observed during hibernation, whereas weight loss is normally associated with hibernation. Also, lesions of the suprachiasmatic nucleus modifies the temporal organization of seasonal hibernation in these animals, particularly by lengthening the duration of hibernation season. In pinealectomized ground squirrels, pinealectomy reduces the period of the rhythm of body mass and of estrous activity. Also, most pinealectomized ewes show circannual rhythm of LH secretion, but some fail to do so and the cycles in others often become dampened with time. Similarly, in ewes kept in short days for 5 yr, a spontaneous change in the secretory rhythm of melatonin appears occasionally and may have contributed to the expression of the circannual rhythm of LH secretion. Melatonin is not a part of the mechanisms generating the circannual rhythm in LH secretion; however, it may be involved in modulating the amplitude and the length of the respective phases of the rhythm. *Therefore, the generation of circannual rhythm does not rely on a single neural structure as the suprachiasmatic nucleus generates circadian rhythms in mammals. Many structures are most likely involved in the expression of circannual rhythms and it is possible that different circannual rhythms are controlled by different critical structures.*

4. SUMMARY AND PERSPECTIVES

Seasonal rhythms are various and constitute a physiological basis for adaptation to annually changing environmental conditions. Among the different factors cueing for seasonal rhythms, photoperiod is the most widely used and it is also the best understood in terms of neuroendocrine mechanisms. The action of photoperiod on the different seasonal rhythms involves a first step of traduction of daylength information into an endocrine code, the melatonin secretion rhythm. This rhythm, characterized mainly by the duration of presence of melatonin, is then decoded by the central nervous system to control the timing of the different photoperiod-dependent annual rhythms. The localization of sites of action for melatonin and identification of its receptors constitute an intitial point of entry to unravel the neuronal network(s) of neurones by which the effects of this pineal indoleamine are mediated and modulated by the action of other environmental factors. Annual rhythms in many species are generated by endogenous rhythms but the mechanisms involved in this internal timing process are not known and remain purely hypothetical. The unraveling of these long-term time-keeping mechanisms is a challenge for future research.

SELECTED READINGS

Arendt J. Melatonin and the pineal gland: influence on mammalian seasonal and circadian physiology. Rev Reprod 1998; 3:13.

Bronson FH. Mammalian Reproductive Biology. Chicago: The University of Chicago Press, 1989.

Curlewis JD. Seasonal prolactin secretion and its role in seasonal reproduction: a review. Reprod Fertil Dev 1992; 4:1.

Goldman BD, Nelson RJ. Melatonin and seasonality in mammals. In: Yu HS, Reiter RJ, eds. Melatonin: Biosynthesis, Physiological Effects, and Clinical Applications. Boca Raton: CRC, 1993; 225.

Goodman RL. Neuroendocrine control of the ovine estrous cycle. In: Knobil E, Neill JD, eds. The Physiology of Reproduction, 2nd ed., New York: Raven, 1994; 659.

Goodman RL, Thiery JC, Malpaux B. The circannual rhythm controlling ovarian function in the ewe: possible neural elements. In: Touitou Y, ed. Biological Clocks. Mechanisms and Applications. Amsterdam: Elsevier Science B.V., 1998; 363.

Gwinner E. Circannual Rhythms. Berlin: Springer-Verlag, 1986.

Karsch FJ, Bittman EL, Foster DL, Goodman RL, Legan SJ, Robinson JE. Neuroendocrine basis of seasonal reproduction. Recent Prog Horm Res 1984; 40:185.

Lehman MN, Goodman RL, Karsch FJ, Jackson GL, Berriman SJ, Jansen HT. The GnRH system of seasonal breeders: anatomy and plasticity. Brain Res Bull 1997; 44:445.

Malpaux B, Chemineau P, Pelletier J. Melatonin and reproduction in sheep and goats. In: Yu HS, Reiter RJ, eds. Melatonin: Biosynthesis, Physiological Effects, and Clinical Applications. Boca Raton: CRC, 1993; 253.

Malpaux B, Viguié C, Skinner DC, Thiéry JC, Chemineau P. Control of the circannual rhythm of reproduction by melatonin in the ewe. Brain Res Bull 1997; 44:431.

Martinet L. Seasonal reproduction and photoperiodism. In: Thibault C, Levasseur MC, Hunter RHF, eds. Reproduction in Mammals and Man. Paris: Ellipses, 1993; 605.

Morin LP, Dark J. Hormones and biological rhythms. In: Becker JB, Breedlove SM, Crews D, eds. Behavioral Endocrinology. Cambridge: MIT Press, 1993; 473.

Roenneberg T, Aschoff J. Annual rhythm of human reproduction: II. Environmental correlations. J Biol Rhythms 1990; 5:217.

26 The Neuroendocrine Control of Ultradian Rhythms

Johannes D. Veldhuis, MD

CONTENTS

1. DEFINITION OF ULTRADIAN RHYTHMS

Endocrine glands typically communicate with their remote target tissues via *intermittent chemical* signaling. The release of such communicative signals, or blood-borne hormone molecules, tends to be *episodic* on various time scales, as illustrated in Fig. 1. For example, opening or closing of ion channels triggers rapid neuronal depolarization and abrupt neurotransmitter discharge over a time scale of milliseconds, such as might occur for hypothalamic *gonadotropin-releasing hormone (GnRH) release* by nerve terminals from GnRH neurons impinging on the portal microvasculature in the median eminence at the origin of the pituitary stalk. GnRH transportation via the hypothalamo-pituitary portal venous system to anterior-pituitary gland *gonadotrope cells* probably occurs over seconds to minutes. Gonadotrope cells respond to GnRH over minutes, after engagement of GnRH with its cognate receptor, thus marshaling immediate secretion of prestored (granule-encapsulated) luteinizing hormone (LH) [and follicle stimulating hormone (FSH)]. GnRH also initiates the less rapid gene expression, biosynthesis, and processing of *gonadotropin subunits* over many minutes (producing a secondary rise in LH secretion following GnRH stimulation). Secreted LH molecules enter the local and systemic circulation for delivery over seconds to minutes to target tissues (e.g., testis Leydig cells in the male, theca and granulosa-luteal cells in the female, brain LH/hCG receptors, etc.). The *target cells of gonadotropic hormone action* then typically release and produce sex-steroid hormones over several minutes (e.g., *testosterone* over 5–30 min) as well as selected glycoprotein signaling molecules (e.g., inhibin, activin, follistatin) for both local intraglandular (paracrine, autocrine) actions and *feedback* on the parent regulatory sites (GnRH neurons and gonadotrope cells) via direct or indirect actions. This recurrence of *hormone signaling* or *secretory pulses* on a time scale of minutes to hours (specifically, multiple times per day) is termed *ultradian*, as distinct from *circadian* (occurring approximately once per 24 h) or *infradian* (recurring less often than once daily).

All pituitary and most target-tissue hormones in

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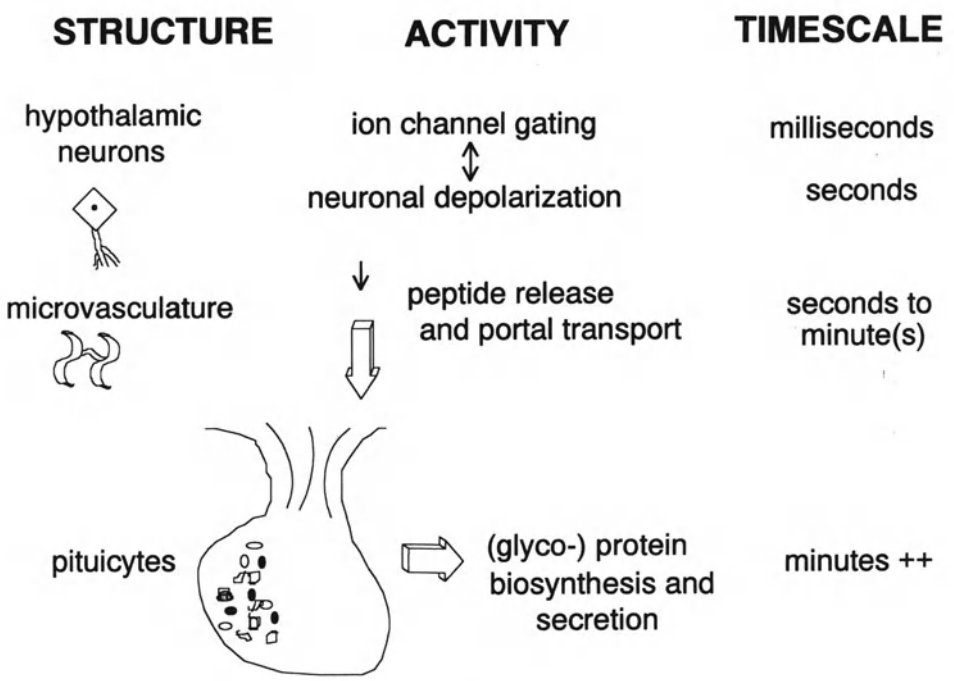

Fig. 1. Approximate time-scales of neuroendocrine signaling. Time scales of chemical communication vary across a spectrum extending from milliseconds (neuronal activation potentials) to seconds (neurosecretory granule release), a few minutes (secretagogue actions on responsive pituitary cells), or many minutes (pituitary-hormone stimulation of remote target tissues, and target-tissue feedback effects). Longer term (24 h, seasonal and annual) rhythms are not shown. [Adapted with permission from Veldhuis JD, (1987). Pathophysiological features of episodic gonadotropin secretion in man. *The American Journal of the Medical Sciences* 294:150–160.]

healthy (physiological) conditions exhibit an ultradian time scale of *intermittent secretory activity*. This is illustrated in Fig. 2A for cortisol, reflecting the adrenal cortex's secretory response to intermittent (pituitary) adrenocorticotropin hormone (ACTH) *pulsatile signaling*. Comparably, prominent ultradian pulsatility is evident for growth hormone (GH) (Fig. 2B) and prolactin (PRL) (Fig. 2C). For anterior pituitary hormone release, the (ultradian) *interpulse intervals* (average time between successive secretory events) typically range from 45 min to 4 h, with the most common interval approximately 60–90 min, e.g., for LH, FSH, PRL, thyroid stimulating hormone (TSH), or GH in young men and women. The most rapid ultradian *pulsatile frequency* is observed for ACTH (which fluctuates as often as 2–5 pulses per hour), and the least frequent for LH release in the midluteal phase of the normal human menstrual cycle (e.g., one pulse every 4–6 h). In addition, anterior pituitary hormones exhibit 24-h (nyctohemeral) rhythmicity (Table 1). Such 24-h variations in serum hormone concentrations arise from sleep-activity cycles, true circadian changes (see later), or both.

The *adaptive state*, *developmental age*, *physiological phase*, *gender*, *age*, and *species* of the organism all typically modulate intermittent or episodic pulse features, e.g., particularly their *frequency and/or amplitude*, but also their duration and possibly pulse shape. For example, infant boys, but not girls, in the first days and weeks of life manifest a temporary adult-like pattern of *circhoral* (nearly hourly) pulsatile LH release (Fig. 3A). GH, TSH, and PRL secretion in the human neonate are also markedly amplified, but prolactin release is virtually apulsatile, i.e., more nearly continuous, at this developmental stage (Fig. 3B). The neuroendocrine mechanisms underlying these physiological gender and developmentally based contrasts are not yet known.

Extrapituitary hormones, such as aldosterone, parathyroid hormone (PTH), and insulin, also maintain evident, and pathophysiologically regulated, pulsatile mode of secretion. The time scale of ultradian *rhythmicity* for some of these hormones can be on the order typically of several minutes, e.g., 8–12 min interpulse intervals for PTH and insulin release. If the interpulse interval is relatively constant, as is typically the case of insulin release in healthy individuals, the term *oscillatory* hormone release is appropriately applied, because a (regular) *sinusoidal periodicity* is approximated. In contrast, pituitary and pituitary-hormone

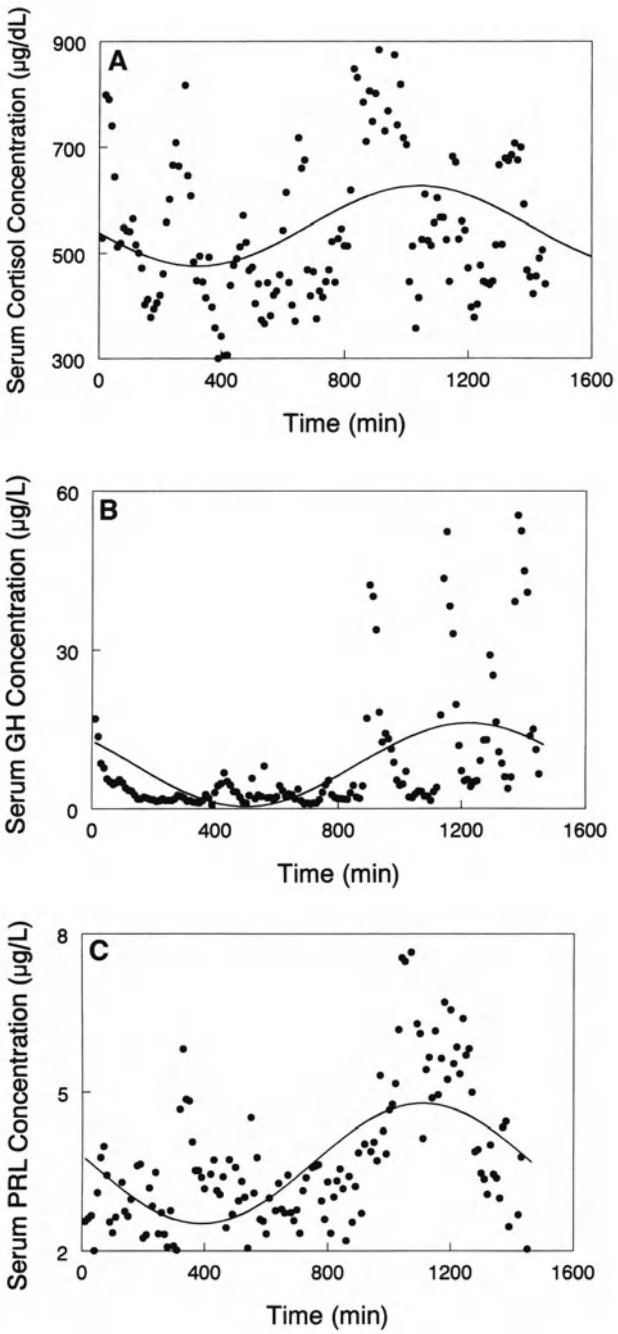

Fig. 2. Illustrative 24 h serum cortisol (*Panel A*), GH (*Panel B*), and prolactin (*Panel C*) concentration profiles fit to a single (cosine) circadian rhythmicity. Remarkable ultradian (short-term) pulsatility would be overlooked by this delimited formulation of hormone release.

driven (e.g., testosterone, progesterone, estrogen, and cortisol) secretory pulses are more often *episodic*, which denotes limited predictability of pulse times (or serial interpulse interval lengths). For example, in normal men, LH pulses occur episodically with a mean interpeak delay of approximately 90 min, but the *distribution of interpulse intervals* (so-called waiting times) may range from 30 min to 150 min within any given individual observed over 24 h. Moreover, successive interpulse intervals do not usually predict the next waiting time, indicating that GnRH-LH pulsatility conforms to a *renewal process,* i.e., there is no evident memory (or autocorrelation) in the (GnRH neuronal) pulse-generator system. A *Poisson distribution* approximates the expectation of such pulse numbers, which emphasizes that their counting error (variance) equals their mean (e.g., observing only 10 pulses in 12 h has a counting variance of a 10 and standard deviation of 3.2, or a 32% error). This concept highlights the need for adequately extended observation intervals in neuroendocrine research in pulsatile systems, e.g., GnRH-LH-testosterone, in order to minimize counting errors in pulse frequency estimates for ultradian rhythms.

Ultradian hormone pulsatility also imposes *clinical implications*, i.e., that repeated blood sampling is necessary to judge serum hormone concentrations reliably. For example, in conventional RIA's, single blood measurements of LH, as well as PRL, GH, or ACTH may vary by 2–10-fold in the same individual sampled moments apart (Fig. 4). More recent application of ultrahigh-sensitivity *immunofluorometric and chemiluminescence-based assays* in children and adults reveal that serum LH and GH concentrations may vary up to 10–30-fold and 30–100-fold, respectively, over 24 h in an individual. Indeed, in the GH axis, daytime serum GH concentrations may fall as low as 0.02–0.03 µg/L after meals and rise to 20–30 µg/dL at night during slow-wave sleep, thus displaying a 1000-fold variation in the young healthy adult. The normal ACTH-adrenal axis manifests analogous striking ultradian pulsatility and circadian variations (see Fig. 5A for illustrative ACTH profiles in men and women). Accordingly, accurate determination of mean serum hormone concentrations or secretion rates most often requires prolonged blood sampling, integrated estimates (e.g., isotopic clearance or 24-h urinary production measures), or indirect inference (e.g., measurement of plasma insulin growth factor-1 (IGF-I) as a marker of excessive or deficient long-term GH variations).

The ultradian release patterns of pituitary hormones are endowed principally by hypothalamically derived *neural signals* acting upon responsive pituitary cells, which are modulated in turn by *intrapituitary paracrine and autocrine factors*, and also by systemically delivered feedback hormones. Unexpectedly, primate pituitary glands studied in vitro

Table 1
Twenty-Four Hour Rhythms
in Plasma Concentrations of Anterior Pituitary Hormones in Men

	*Amplitude** *(concentration)*	*Acrophase*** *(clocktime)*	*Mesor**** *(concentration)*
Hormone (units)			
LH (IU/L)	0.60 ± 0.10	2400 (± 114 min)	7.6 ± 0.53
FSH (IU/L)	0.38 ± 0.08	2253 (± 170 min)	6.8 ± 0.08
Prolactin (μg/L)	1.4 ± 0.39	0151 (± 96 min)	4.7 ± 0.57
TSH (mU/L)	0.51 ± 0.17	0233 (± 51 min)	1.8 ± 0.38
GH (μg/L)	0.41 ± 0.16	0032 (± 64 min)	0.50 ± 0.18
ACTH (ng/L)	5.9 ± 1.1	0915 (± 86 min)	13 ± 1.8
Beta-endorphin (ng/L)	5.2 ± 0.81	0626 (± 116 min)	32 ± 3.9

* Amplitude: one-half the difference between the zenith and nadir of the 24-h rhythm.

** Acrophase: time when the maximal value of the 24-h rhythm occurs.

*** Mesor: mean value about which the 24-h rhythm oscillates.

Data are mean estimates (N = 6–8 normal, middle-aged men studied for each hormone).

Plasma hormone concentrations were determined by radioimmunoassay (LH, FSH) or radioimmunometric assay (others) in blood collected at 10-min intervals over 24 h.

Adapted with permission from Veldhuis JD, Johnson ML, Iramanesh A, Lizarralde G. Rhythmic and non-rhythmic modes of anterior pituitary hormone release in man. In: Touitou Y, Haus E, eds., *Biological Rhythms in Clinical and Laboratory Medicine.* New York: Springer-Verlag, 1992: pp. 277–291.

show variable high-frequency spikes of hormone release (e.g., every 3–20 min), when disconnected from in vivo hypothalamic neural signals (releasing and inhibiting factors) and systemic feedback effectors. This *episodic hormonal discharge* is of low amplitude, and likely independent of, or subordinate to, intact hypothalamo-pituitary pulsatility, because the latter is marked physiologically (but not pathologically) by well-organized, typically monophasic, high-amplitude, and largely reproducible secretory waveforms. On the other hand, pituitary tumors secrete hormones in an aberrant, low-amplitude, high-frequency, and more disorderly fashion with accompanying (markedly) elevated basal (nonpulsatile) hormone release, e.g., ACTH-GH-, and PRL-secreting pituitary tumors, thus evincing (profound) loss of physiological ultradian pulse regulation. Figure 5B illustrates a low-amplitude, high-frequency, and disorderly 24-h ACTH release pattern with elevated interpulse secretory rates in a patient with a pituitary ACTH-secreting tumor causing the clinical condition of Cushing's syndrome due to excessive (adrenal) cortisol production.

2. BIOLOGICAL RELEVANCE OF THE ULTRADIAN HORMONE PULSE SIGNAL

Several significant implications of ultradian hormone release are recognized. Foremost, the mechanistic origin of episodic secretion of pituitary hormone reflects primarily the intermittency of neural secretagogue and/or inhibitor input to pituitary cells via the hypothalamo-pituitary portal microcirculation. Thus, pituitary-hormone pulsatility mirrors hypothalamic signaling activity as transduced by responsive pituitary cells: *see* Fig. 6 for GnRH-LH pulsatile activities. This *neuroendocrine interface* provides therefore an instructive "window to the brain," except in some pathological states, e.g., hypothalamo-pituitary disconnection because disease interrupting the pituitary stalk.

The intermittent rather than continuous stimulation of a target gland also often preserves cellular responsiveness to the agonist, which otherwise, would result in *downregulation or desensitization* of the target tissue. Such downregulation is especially notable for GnRH's action on anterior pituitary gonadotrope cells (Table 2). Indeed, a time-invariant GnRH signal, whether imposed by continuous intravenous infusion of GnRH or by administering a long-acting GnRH agonist peptide (GnRH superagonist analog), stimulates LH (and FSH) secretion acutely over hours to 1–2 d, but then downregulates LH (and FSH) secretion profoundly after 3–7 d (Fig. 7). Continuous, rather than pulsatile, GnRH stimulation evokes profound abrogation of gonadotrope-cell expression of LH (and FSH)-beta subunit genes in vitro also, whereas free alpha subunit production continues unabated. This *ultradian signal-dependence of GnRH*

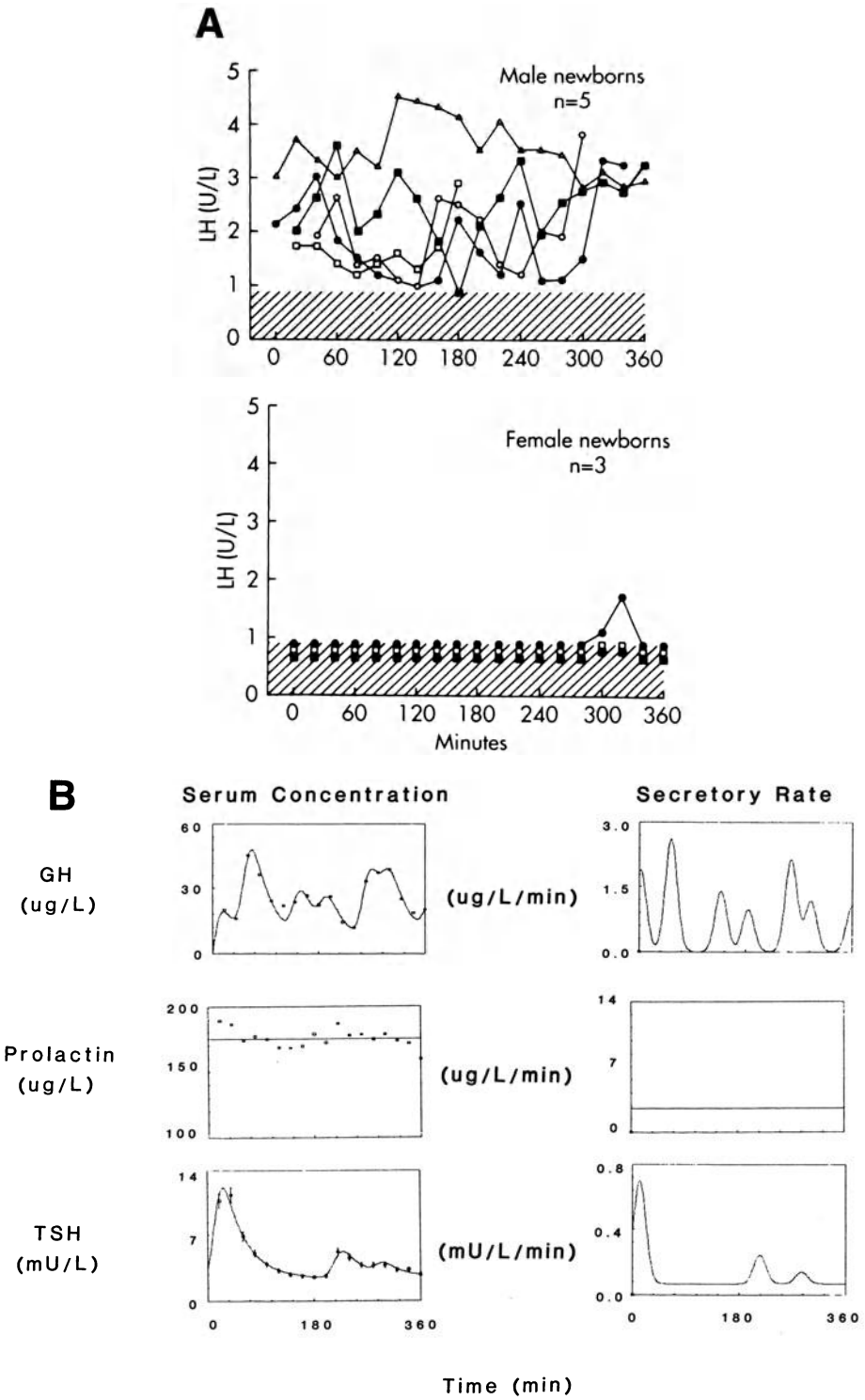

Fig. 3. Ultradian release of LH in the human male (but not female) neonate (*Panel A*), and amplified pulsatile secretion of GH and TSH compared to nearly continuous outpouring of PRL (*Panel B*) on the first day of life. Features of ultradian rhythmicity of pituitary hormone secretion are gender, developmental stage, and hormone-specific, as well as susceptible to disruption in pathophysiology (see text). [Adapted with permission from de Zegher F, Devlieger H, and Veldhuis JD, (1992). Pulsatile and sexually dimorphic secretion of luteinizing hormone in the human infant on the day of birth. *Ped Res* 32:605–607 and de Zegher F, Van den Berghe G, Devlieger H, Eggermont E, and Veldhuis JD, (1993). Dopamine inhibits growth hormone and prolactin secretion in the human newborn. *Ped Res* 34:642–645.]

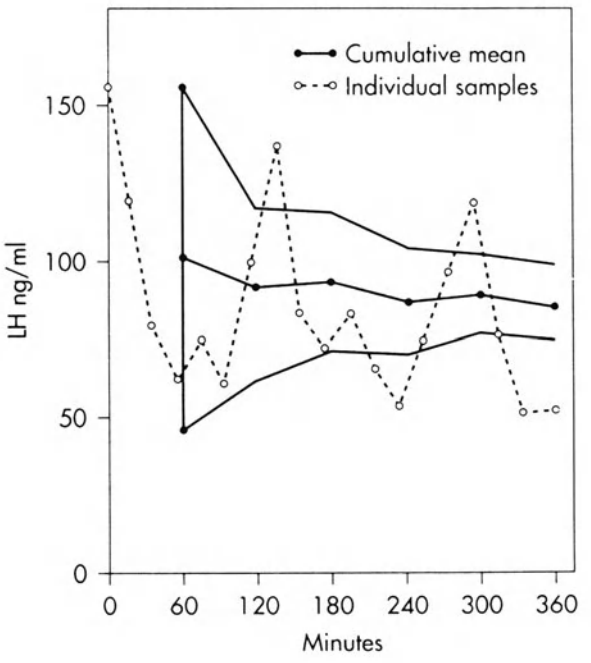

Fig. 4. Clinical sampling implications of the pulsatile (ultradian) release of LH (open circles and interrupted line), illustrating the progressive improvement in estimation accuracy of serum LH concentrations (solid circles and continuous line, cumulative mean ±95% confidence interval) with more prolonged blood withdrawal (here, at 20 min intervals). [Adapted with permission from Veldhuis, J. D. (1998) Male hypothalamic-pituitary-gonadal axis, in *Reproductive Endocrinology* (Yen SSC, Jaffe RB and Barbieri RL, ed.), W.B. Saunders Co., Philadelphia, PA, in press.]

action on gonadotrope cells is exploited clinically via the use of GnRH analogs with 300–1000-fold greater potency than native GnRH and formulated with markedly prolonged absorption and in vivo residence times (e.g., allowing monthly injections) to downregulate the gonadotrope-LH-Leydig cell-testosterone axis. This therapeutic strategy can achieve selective "medical castration" in patients with androgen-dependent prostate cancer, or downregulate the LH/FSH-ovarian axis in women with (estrogen-dependent) endometriosis or breast cancer.

Another plausible (but not proven) biological implication of neuroendocrine pulsatility is *ergonomic*. Specifically, intracellular synthesis, processing, transport, and storage of complex hormone macromolecules, such as the stress-responsive protein hormone, ACTH, by the metabolically replete and unstressed cell during the interpulse interval of secretory quiescence provides readily releasable hormone pools for later rapid and massive release when triggered by a relevant acute stimulus. In contrast, less overtly pulsatile hormones, such as testosterone, are not stored in available secretory granules and respond more slowly to secretagogue activation (e.g., an incoming LH pulse).

At the level of the target tissue, *receptor recycling* can occur within the interval between successive hormone pulses. This biological feature of hepatic GH receptor turnover allows renewal of cell membrane receptors every 60–90 min within the 3.0–3.3 h interpulse intervals of GH secretion in the (male) rat.

In the rat, strong *gender differences* exist in ultradian GH release patterns, with prominent three hourly pulses in the male but more nearly continuous GH release in the female animal (Fig. 8). Furthermore, in this species, vivid contrasts emerge in the effects of pulsatile versus continuous GH stimulation of target-tissue receptors. For example, at a biochemical level, *pulses of GH* (male pattern) effectively activate the specific STAT 5b intracellular signaling pathway in liver, whereas *continuous GH drive* (female pattern) downregulates this messenger pathway. At a molecular level, pulsatile GH stimulates muscle IGF-I gene expression more effectively than continuous GH delivery. And, in the whole animal, pulses of GH promote body growth more effectively compared with constant GH infusions (Table 3). Thus, both the regulation and the impact of ultradian GH release are significantly modulated by sex steroids and developmental stage in the rodent. Analogous, albeit less striking, gender distinctions operate in the human GH axis.

3. NEUROENDOCRINE CONTROL OF ULTRADIAN RHYTHMICITY

The primary sites of regulation of ultradian pituitary secretory activity are the central nervous system, pituitary gland, feedback by target-tissue hormones, and network coordination within the entire individual neuroendocrine axis, e.g., GnRH-LH-testosterone. Moreover, between-axis interactions are also evident, e.g., the somato- and gonadotropic axes react in a concerted manner in (human) puberty. The notion of multisite (feedback network) control of ultradian pulsatility is discussed below, and illustrated in Fig. 9 for the pulsatile ACTH-adrenal axis.

2.1. CNS

A cardinal locus of control of ultradian pituitary secretory activity resides in the CNS. Neurosurgical ablation and deafferentation experiments in animals, and brainstem diseases in patients, were among the first observations to define a critical role for suprapitu-

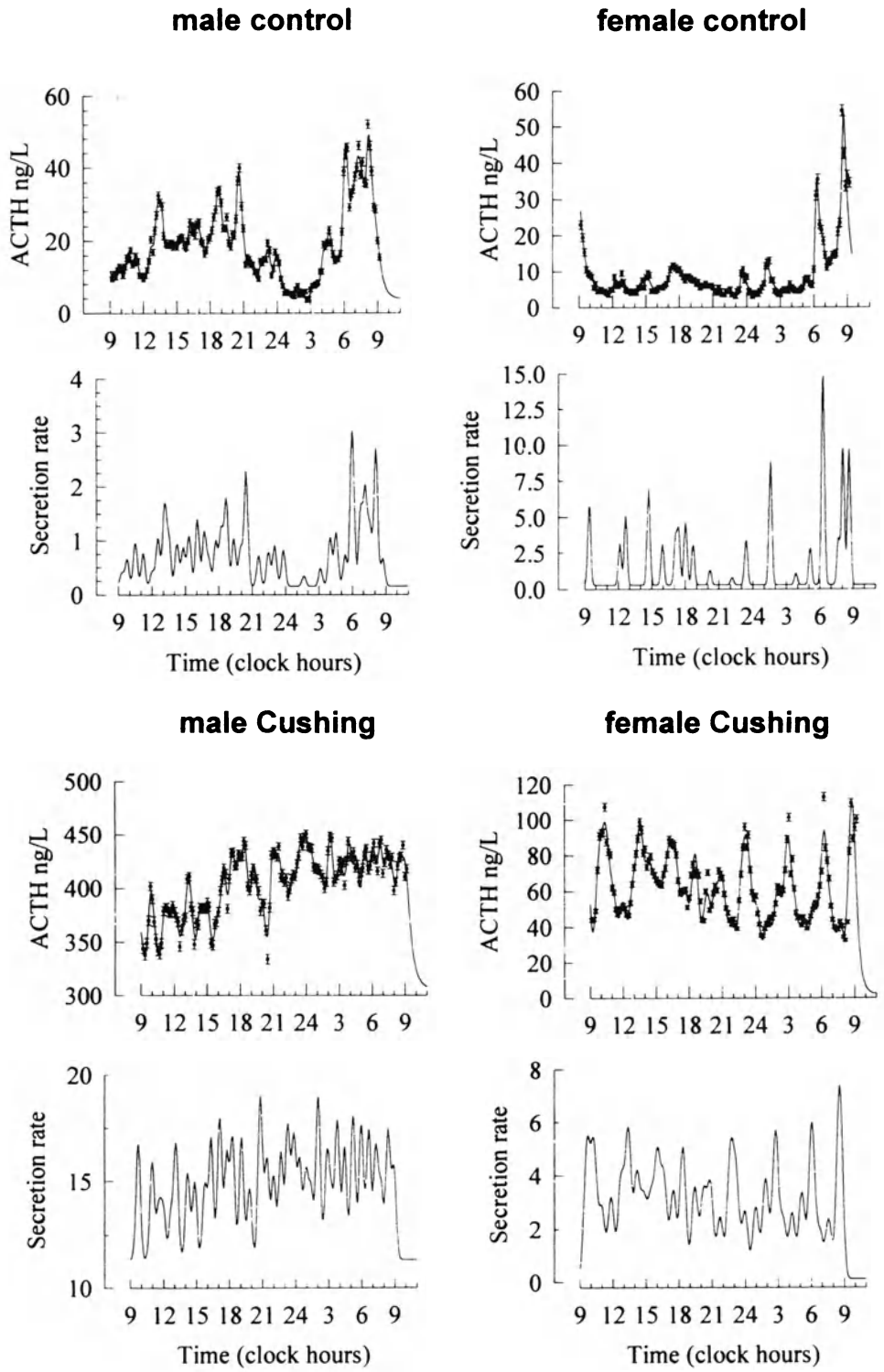

Fig. 5. Twenty-four hour plasma ACTH concentration (upper) profiles in two normal adults (*Panel A*) and two patients with Cushing's disease because of ACTH-secreting pituitary tumors (*Panel B*). Tumoral secretion (lower) of ACTH is marked by lower amplitude, higher frequency, and variable-duration hormone release episodes yielding greater disorderliness of the overall release pattern, which is superimposed upon an elevated basal (nonpulsatile) hormone release rate. [Adapted with permission from Van den Berg G, Frolich M, Veldhuis JD, and Roelfsema F, (1996). Combined amplification of the pulsatile and basal modes of adrenocorticotropin and cortisol secretion in patients with Cushing's disease: evidence for downregulation of the adrenal glands. *J Clin Endocrinol Metab* 80:3750–3756.]

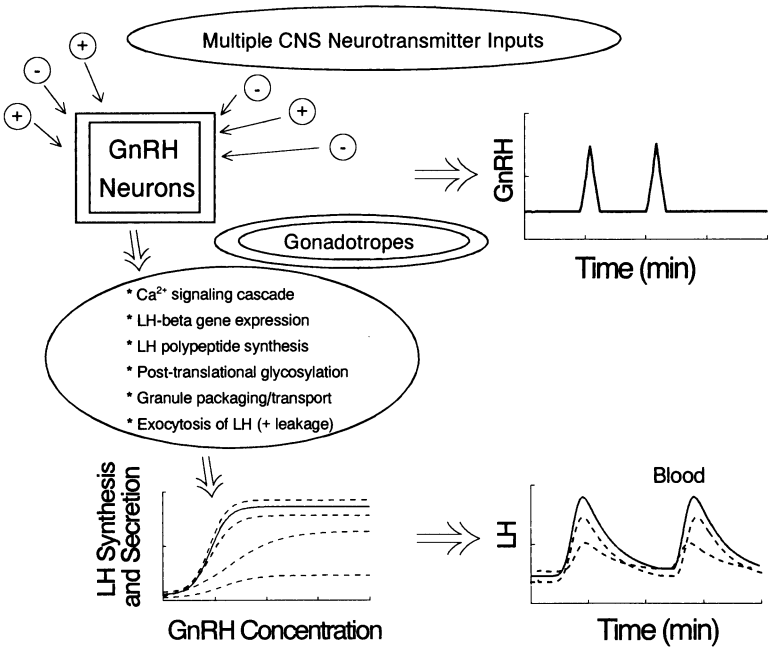

Fig. 6. Schema of the relationship between hypothalamic release of GnRH pulses and time-delayed multistep activation of LH biosynthesis, packaging, and secretion by anterior pituitary gonadotrope cells. This pulse-signal activated pathway is modulated further by (not shown): (a) intrapituitary activin, follistatin, and inhibin [and possibly alpha-subunit and prolactin or other lactotroph product(s)]; and (b) systemic feedback signals (e.g., estradiol, testosterone, and probably inhibin B in the human, etc.). The notion of an interfacing dose-response function for GnRH's action on LH release is implicit.

itary sites in the supervision of episodic (ultradian) pituitary hormone release. Electrochemical stimulation studies confirmed such inferences, and the isolation of multiple specific secretagogues within discrete brain regions extended the mechanistic basis for pituitary governance by the hypothalamus. The hypothalamus serves as a master integrator of multiple CNS neurotransmitter inputs, which are largely specific to each *neuroendocrine axis*, as well as relevant feedback signals (e.g., sex steroids from gonadal cells). The "final" (net) balance of output of relevant hypothalamic neural stimulatory and inhibitory signals into the pituitary portal microvasculature ultimately directs pituitary hormone secretion. In turn, recent studies reveal that *the pituitary gland secretes to the brain*, e.g., GH acts by feedback on key hypothalamic sites to stimulate somatostatin release, thereby limiting its own secretion after a time delay.

Although the intuitive concept of a "hypothalamic pulse generator" has been suggested, the exact neural basis for coordinated hypothalamic neural discharge of bursts of releasing factors remains relatively elusive. For example, LH (and FSH), GH, TSH, ACTH, and PRL are all released in coherent secretory bursts (putatively) activated by respective hypothalamic pulse-generator mechanisms, but the various neuronal ensembles and the regulation of their episodicity is minimally understood to date. Electrophysiological correlates of the GnRH and GHRH "burst-generator system" can be identified in the *mediobasal hypothalamus* (and/or arcuate nucleus region in the primate), but the exact structural anlage of these multiunit activity (MUA) electrical discharges is not known. In the case of immortalized GnRH neurons (GT-1/GT-7 cells), a higher frequency (1–3 spikes/h) low-amplitude release activity is suggested in vitro, with feedback inhibition by GnRH, dopamine, and PRL, and conversely stimulation by glucagon-like peptide (GLP-1) and ciliary neurotrophic factor (CNF-1): Fig. 10. However, whether such *transformed GnRH neurons* that show autonomous rhythmicity recapitulate the coupling physiology expected in the more complex in vivo network of neural and glial elements in which GnRH neurons are embedded is not known. Similar deficits in our knowledge exist for putative growth hormone releasing hormone (GHRH), TRH, and CRH neuronal ("pulse-generator") ensembles, which presumably ultimately convey episodicity of secretion to GH, TSH, and ACTH. On the other hand, oxytocin and vasopressin pulsatility associated with the posterior pituitary gland has been somewhat better elucidated electrophysiologically. Importantly, most pituitary hormones are now known to be controlled not only by a primary hypothalamic regulator, but also by one or more *cosecretagogues or neuromodulators* (e.g., galanin, neuropeptide Y, dopamine, etc.).

2.2. Pituitary Gland

An important contemporary physiological precept is that hypothalamic signals must act upon variably

Table 2
Differential Actions on Target Tissues of Continuous vs Pulsatile Agonist Delivery

Agonist	*Target tissue/hormone*	*Response*
GnRH	gonadotropes/LH and FSH (all species)	more rapid LH than FSH down-regulation by continuous GnRH exposure (1)
GH	liver second-messengers (rat)	STAT5 pathways inhibited by continuous GH (2)
ACTH	adrenal cortex (human)	aldosterone, but not cortisol, is downregulated by continuous ACTH (3)
LH	testis, Leydig cell (ram)	testosterone secretion not downregulated by continuous LH (4)
Testosterone	GnRH/LH unit (human, ram)	continuous delivery of testosterone is more suppressive of LH (5)
CRH	ACTH/cortisol (CRH-deficient human)	ACTH axis output is restored by pulsatile CRH treatment in CRH deficiency (6)
CRH	ACTH and cortisol (CRH-deficient mouse; human)	continuous CRH maintains diurnal ACTH and cortisol rhythms (7,8)
Insulin	liver and fat tissues	pulsatile insulin injection is more hypoglycemic than continuous insulin (9)

(1) Southworth MB, Matsumoto AM, Gross KM, Soules MR, Bremner WJ. The importance of signal pattern in the transmission of endocrine information: pituitary gonadotropin responses to continuous and pulsatile gonadotropin-releasing hormone. *J Clin Endocrinol Metab* 1991; 72:1286–1289.

(2) Udy GB, Towers RP, Snell RG, Wilkins RJ, Park SH, Ram PA, Waxman DJ, Davey HW. Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. *Proc Natl Acad Sci USA* 1997; 94:7239–7244.

(3) Tsigos C, Chrousos GP. Physiology of the hypothalamic-pituitary-adrenal axis in health and dysregulation in psychiatric and autoimmune disorders. *Endocrinol Metab Clin North Am* 1994; 23:451–466.

(4) Chase DJ, Schanbacher B, Lunstra DD. Effects of pulsatile and continuous luteinizing hormone (LH) infusions on testosterone responses to LH in rams actively immunized against gonadotropin-releasing hormone. *Endocrinol* 1988; 123:816–826.

(5) Zwart A, Iranmanesh A, Veldhuis JD. Disparate serum free testosterone concentrations and degrees of hypothalamo-pituitary-LH suppression are achieved by continuous versus pulsatile intravenous androgen replacement in men: a clinical experimental model of ketoconazole-induced reversible hypoandrogenemia with controlled testosterone add-back. *J Clin Endocrinol Metab* 1997; 82:2062–2069.

(6) Vgerinos PC, Schurmeyer TH, Gold PW, Tomai TP, Loriaux DL, Sherins RJ, Cutler Jr, GB, Chrousos GP. Pulsatile administration of human corticotropin-releasing hormone in patients with secondary adrenal insufficiency: restoration of the normal cortisol secretory pattern. *J Clin Endocrinol Metab* 1986; 62:816–821.

(7) Muglia LJ, Jacobson L, Weninger SC, Luedke CE, Bae DS, Jeong KH, Majzoub JA. Impaired diurnal adrenal rhythmicity restored by constant infusion of corticotropin-releasing hormone in corticotropin-releasing hormone-deficient mice. *J Clin Invest* 1997; 99:2923–2929.

(8) Schultze HM, Chrousos GP, Gold PW, Booth JD, Oldfield EH, Cutler Jr. GB, Loriaux DL. (1985) Continuous administration of synthetic ovine corticotropin-releasing factor in man: physiological and pathophysiological implications. *J Clin Invest* 1985; 75:1781–1785.

(9) Matthews DR, Naylor BA, Jones RG, Ward GM, Turner RC. Pulsatile insulin has greater hypoglycemic effect than continuous delivery. *Diabetes* 1983; 32:617–621.

responsive, and locally modulated, target cells within the anterior pituitary gland. For example, GnRH actions on the gonadotrope cell population are dependent on *developmentally mature gonadotrope* cell populations, whose secretion of LH and FSH is controlled further by at least:

1. Their instantaneous *receptive or refractory state* (being unresponsive after downregulation and refractory shortly after an active GnRH impulse),
2. Paracrine lactotroph-cell and possibly (paracrine and autocrine) alpha-subunit modulation,
3. Paracrine and autocrine actions of activin, inhibin, and follistatin, and
4. The intrapituitary microcirculation.

Thus, GnRH superagonists and high-frequency (or continuous) GnRH stimuli downregulate ultradian LH pulsatility; *hyperprolactinemia* likely inhibits pulsatile hypothalamic GnRH release via brain feedback effects to enlist suppression by dopamine; GnRH stimulates FSH release in part by altering follistatin-activin interactions within the anterior pituitary gland; and, *intrapituitary tumors or hemorrhage* may impair

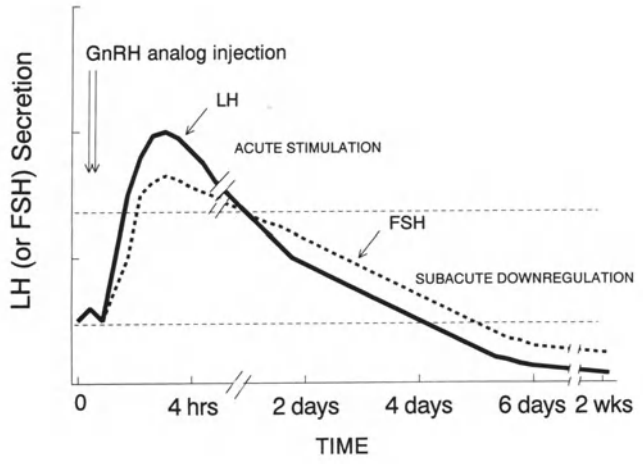

Fig. 7. Nominal time-course of gonadotropin secretory response in the adult human to unvarying GnRH (superagonist) engagement with pituitary gonadotropes. LH (and FSH) secretion is marked by transient/short-term (30 min–1.5 d) stimulation, followed by progressive and sustained downregulation of responsiveness. Downregulation is mediated both GnRH-receptor and postreceptor (intracellular signaling) mechanisms. The horizontal dotted lines define normal LH (and FSH) levels.

GnRH and/or LH delivery via the intrapituitary circulation or impair gonadotrope metabolic function.

2.3. Feedback Regulation and Network Functions

A unique feature of physiological neuroendocrine axes is *feedforward and feedback* connections that maintain *within-axis hormonal homeostasis*. For the male hypothalamo-pituitary-gonadal axis, GnRH *feeds forward* on LH via an agonistic dose-response function, which is modified by age, sex-steroids, developmental status, and disease. LH in turn feeds forward on testicular Leydig cells to drive testosterone secretion via a second independent control function, which varies in health and disease. Testosterone (and its aromatized product, estradiol) *feeds back* on both hypothalamic GnRH and pituitary LH secretion via relevant inhibitory dose-response curves: Figure 11. Studies in the healthy aging human (male) reveal that those *feedback linkages* are disrupted even when 24-h mean serum LH and testosterone concentrations remain in the normal young-adult range. The ultradian release of LH and testosterone in older men is distinguished quantitatively by selected attenuation of LH secretory burst amplitude, and by uncoupling of both feedforward (LH's acting within 20–60 min to drive testosterone) and *feedback* (testosterone's inhibition of LH secretion approximately 8–120 min later) *control*. Thus, the *network* concept of combined *time-delayed* feedforward and feedback regulatory interactions within a normally functioning neuroendocrine axis unmasks more subtle pathophysiology than that evident by inspection of individual or mean (24 h) serum hormone concentrations.

Other axes, such as the that embodied by GHRH/somatostatin-GH-IGF-I, or CRH/AVP-ACTH-cortisol, feedforward and feedback, can also be viewed as *control systems*. Clinical and laboratory experiments indicate for example that sex hormones impact the network function of the GH-IGF-I axis, thereby mediating certain of the gender distinctions in the ultradian pulsatility of GH in the male and female.

2.4. Axes Interactions

Far less is known about between-axes than within-axis interactions. However, *bi-axis* and *tri-axis* interactions are well-appreciated clinically. For example, the somatotropic (GH) and gonadotropic (LH) axes interact significantly in puberty. With awakening (via currently unknown neuroendocrine mechanisms) of GnRH secretion in early puberty, there is a consequent rise in gonadal sex hormones. Concurrently, pulsatile GH secretion increases 1–5–8-fold in amplitude, there is greater disorderliness of the GH release process, and 24-hour rhythmicity of serum GH concentrations is amplified: Fig. 12. These GH secretory changes are induced by graded doses of testosterone in boys with delayed or arrested puberty, but in the human are not reproduced by nonaromatizable androgens (e.g., anabolic steroids or 5α DHT), suggesting that in boys—as in girls—estrogen is the proximate activator of the GH-IGF-I axis in puberty.

A *tri-axial* interaction likely occurs among the gonadotropic, somatotropic, and corticotropic (ACTH-adrenal) axes in response to the stress of nutrient deprivation. Fasting in young (mid-luteal phase) women suppresses the daily LH secretion rate, while increasing 24-h GH and cortisol secretion (Fig. 13). Serum concentrations of leptin (a nutritional signaling peptide produced by fat cells that acts on hypothalamic regulatory sites, such as GnRH-, TRH-, and somatostatin-secreting neurons in the rat) fall in fasting women. Although leptin infusions in the rat reverse many neuroendocrine alterations in fasting animals, the exact metabolic signals that concurrently control LH, GH, and ACTH secretion in this complex tripartite *adaptive* response to nutritional restriction in the human are not known. Thus, further knowledge of clinical *integrative physiology* will be important in this arena.

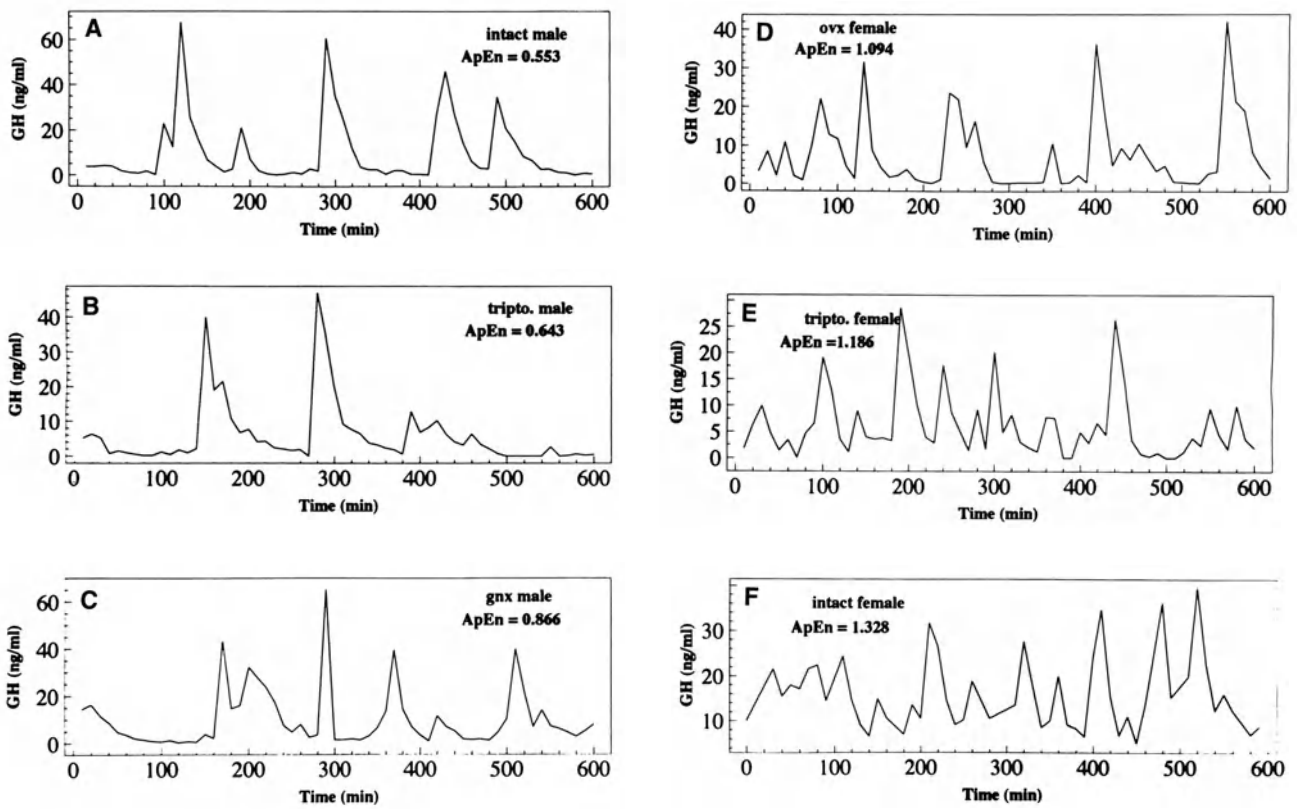

Fig. 8. Gender contrasts in the ultradian (pulsatile) modes of GH secretion in the adult male versus female rat. The intact male animal (left upper most subpanel) shows prominent, high-amplitude, infrequent (approximately, 3.3 hourly) serum GH peaks with undetectable GH concentrations in the interpeak valleys. In contrast, GH pulsatility in the intact female (right lower most panel) is visibly and quantifiably more irregular, of reduced amplitude, and associated with elevated interpulse nadir serum GH concentrations. GnRH agonist ("tripto.") treatment or castration ("gnx male," and "ovx female") evokes a graded hierarchy of intermediate GH release patterns. Such gradations in the orderliness of hormone release patterns can be quantified by the approximate entropy (ApEn) statistic. [Adapted with permission from Pincus SM, Gevers E, Robinson ICA, Roelfsema F, Hartman ML, and Veldhuis JD, (1996). Females secrete growth hormone with more process irregularity than males in both human and rat. *Am J Physiol* 270:E107–E115.]

Table 3
Gender Distinctions in GH Action:
Pattern-Dependent Pulsatile (Male-Like) vs Continuous (Female-Like) GH Actions on Target Tissues

Pulsatile GH (male)	*Continuous GH (female)*
linear growth	hepatic EGF and LDL receptors
body weight	hepatic sulfatase
muscle IGF-I	CBG
liver 2C11 hydroxylase	desensitze STAT 5a
liver 5-alpha reductase	glutathione-S-transferase
STAT 5b	etc.
etc.	

Adapted with permission from Gaussian A, Veldhuis JD. Pathophysiology of the neuroregulation of GH secretion in experimental animals and the human. *Endocrine Rev*, in press.

4. CONTRIBUTION OF ULTRADIAN RHYTHMS TO CIRCADIAN HORMONE VARIATIONS

As schematized in Fig. 14, 24 h variations in serum concentrations of (pituitary) hormones (*see* Table 1) could arise in principle from *three primary dynamics*; namely, nyctohemeral variations in (a) secretory burst mass/amplitude, (b) secretory pulse frequency, and/or (c) basal (nonpulsatile) hormone release rates. One or more of these three mechanisms would be pertinent for any given pituitary hormone, e.g., amplitude control for ACTH; amplitude and frequency control for GH and LH; and amplitude and interpulse (basal) hormone secretion for TSH and PRL (Table 4). In contrast to marked ultradian amplitude and frequency variations diurnally, changes in hormone half-life across 24 h rarely contribute substantially to serum

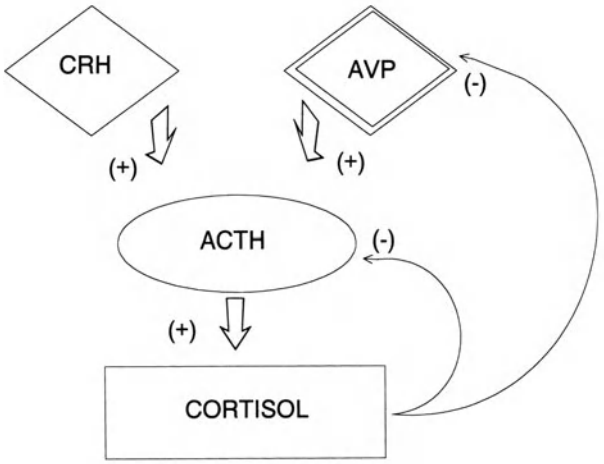

Fig. 9. Core feedforward (CRH and AVP's acting on ACTH, and ACTH's stimulating cortisol) and feedback (cortisol's inhibiting CRH and AVP, as well as ACTH) connections illustrated for the corticotropic axis. CRH denotes ACTH (corticotropin)-releasing hormone, and AVP defines arginine vasopressin. Dose-responsive interconnections are exerted after time delays in this network concept.

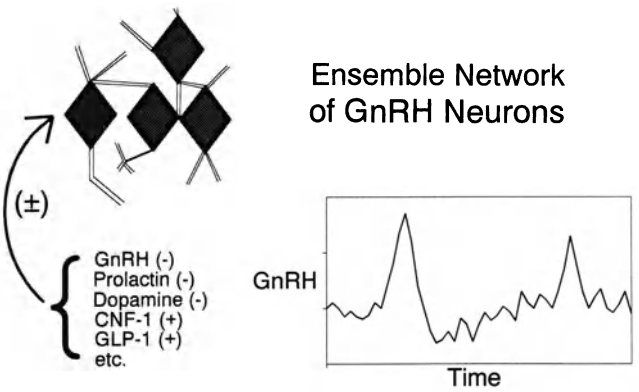

Fig. 10. Schema of intrinsic but regulatable GnRH neuronal pulsatility, as inferred from in vitro studies of immortalized GnRH neuronal populations. Transformed mouse GnRH neurons are susceptible to feedback inhibition by GnRH itself (autonegative feedback), dopamine, and PRL (among other effectors) and feedforward stimulation by glucagon-like peptide (GLP-1) and ciliary-neurotrophic factor (CNF-1) in in vitro perifusion studies.

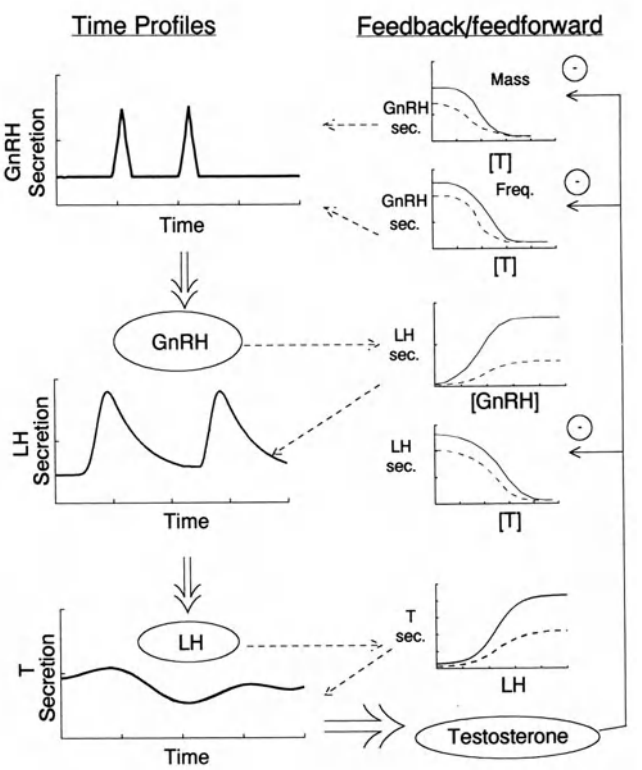

Fig. 11. The male hypothalamo-pituitary-gonadal axis viewed as a combined feedforward (double-open arrows) and feedback (single interrupted line) control system with anticipated time delays, and nonlinear interfacing dose-response curves. The pulsatile variations in GnRH and LH release and homeostatic control mechanisms are embedded theoretically not only in the individual behavior of three major control loci (GnRH neurons, pituitary gonadotropes, and gonadal Leydig cells), but also their relevant (nonlinear) interactions. [Adapted with permission from Keenan DM, Veldhuis JD, (1998). A biomathematical model of time-delayed feedback in the human male hypothalamic-pituitary-Leydig cell axis. *Am J Physiol* in press.]

hormone variations. For example, the 24-h serum GH rhythm arises from as much as 30–100-fold variations in GH secretory burst amplitude in young men over the day and night. Variations in ACTH and cortisol secretory burst amplitude and/or frequency (or its reciprocal, interpulse interval) over 24 h in young men are shown in Fig. 15.

Insights into how the foregoing 24 h control of hormone burst amplitude and/or frequency (or basal release rates) is mediated will require a better understanding of physiological linkages between circadian inputs and ultradian pulsatility control mechanisms. For example, given a dominant role for the *suprachiasmatic nucleus* (SCN) in governing various circadian activities, including temperature, locomotor activity, sleep/wake cycles, etc., a logical construct would embody circadian-ultradian coupling via SCN-hypothalamic communications. This expectation is likely the case for the circadian modulation of the surge-like release of LH on the afternoon of proestrus in the rodent, but is less clearly articulated for the ACTH, GH, TSH, and PRL axes. Although 24 h variations in hypothalamic CRH gene expression occur in the rat, the relevance of hypothalamic control of circadian ACTH periodicity via CRH variations is not fully established. In humans, continuous CRH infusions will

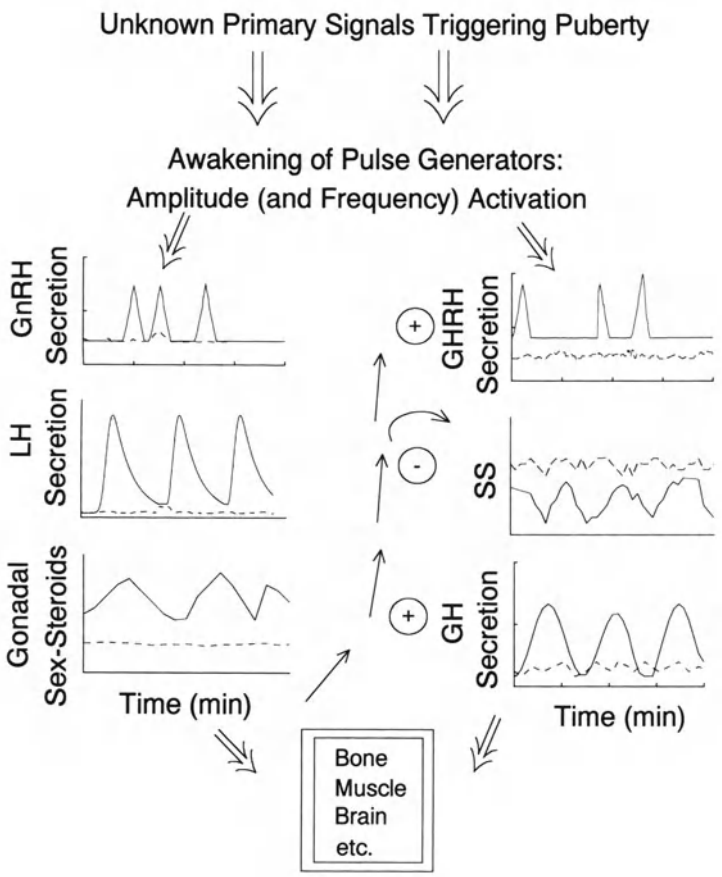

Fig. 12. Somatotropic (GH) and gonadotropic (GnRH-LH) bi-axes interactions in puberty via feedforward actions of gonadal sex-steroids on the GHRH/somatostatin (SS)-GH neuroendocrine unit. Testosterone acts directly on relevant sites and/or exerts effects via its (aromatization) product, estradiol-17β or its (5-alpha reduced) metabolite, 5α-dihydrotestosterone (5α DHT). In the human, the principal actions of testosterone on the GH axis centrally are via estrogen, whereas in the rat 5α DHT is a potent mimic of testosterone. GH and sex-steroids also interact on relevant target tissues, e.g., bone, muscle, brain, liver, etc. GHRH denotes GH-releasing hormone. [Adapted with permission from Veldhuis JD, Iranmanesh A, Rogol AD, and Urban RJ. (1995). Regulatory actions of testosterone on pulsatile growth hormone secretion in the human: studies using deconvolution analysis, in: Adashi EY and Thorner MO, eds. Somatotropic Axis and the Reproductive Process in Health and Disease. Springer-Verlag, New York, pp. 40–57.]

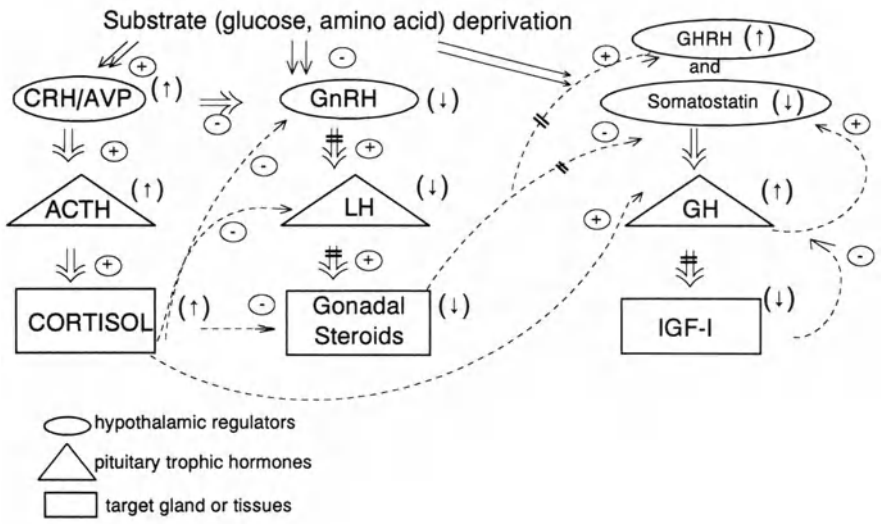

Fig. 13. Proposed neuroendocrine *tri-axis* responses to fasting in the human: augmented CRH-ACTH-cortisol feedforward-driven secretion; reduced GnRH-LH-sex steroid secretion [and restricted TRH-TSH release (the latter not shown), both of which are restored by leptin infusions in the rat]; and amplified GH secretion (which is inhibited by food-restriction in the rat, and restored by leptin, illustrating major species differences in some metabolic-stress responses). Heightened cortisol secretion antagonizes activity of the GnRH-LH-gonadal axis at multiple levels (double bars). Starvation thus results in withdrawal of sex-steroid actions on GHRH-somatostatin, and also blocks GH's stimulation of (liver) IGF-I production. Suppressed FSH and PRL secretion occur also (not shown), but to a less marked degree. Unpublished schema.

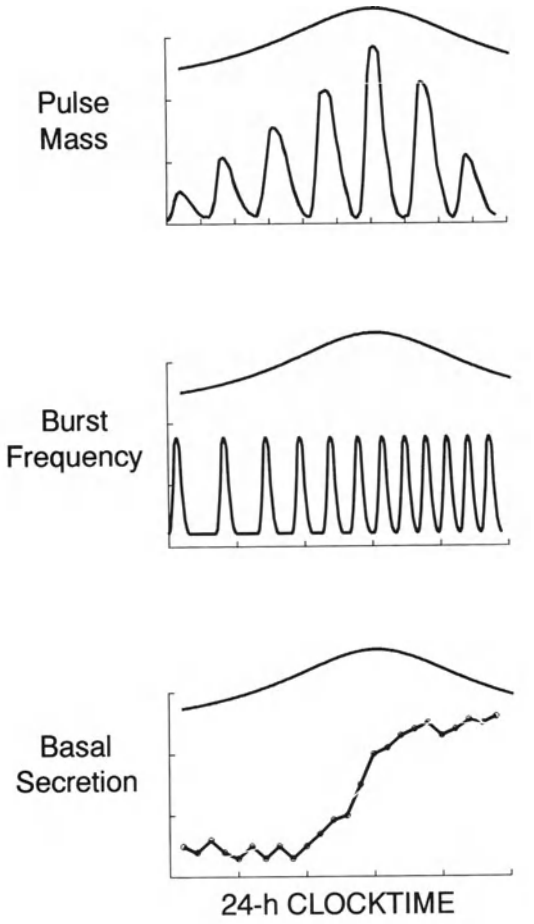

Fig. 14. Theoretical ultradian mechanisms mediating the pulsatile-circadian linkages for pituitary hormone secretion, *viz.* 24 h variations in ultradian pulse amplitude and/or pulse frequency, and circadian regulation of basal (nonpulsatile) hormone release. [Adapted with permission from Veldhuis JD, Johnson ML, Iramanesh A, and Lizarralde G. (1992). Rhythmic and nonrhythmic modes of anterior pituitary hormone release in man, in: Touitou Y, and Haus E, eds. *Biological Rhythms in Clinical and Laboratory Medicine*, Springer-Verlag, New York, pp. 277–291.]

Table 4
Ultradian (Pulsatile) Mechanisms SubServing 24-h Variations in Serum Concentrations of Pituitary Hormones in Healthy Men

	Secretory Burst Amplitude	*Interburst ("basal") Secretory Rate*	*Secretory Burst Frequency*
Hormone			
LH	+ (12)	–	–
FSH	± (6)	–	–
Prolactin	+ (30)	+ (27)	–
TSH	+ (28)	–	+ (13)
GH	+ (82)	–	+ (23)
ACTH	+ (60)	–	–
Beta-endorphin	+ (18)	–	+ (22)

+ Denotes a significant 24-h variation.
– Denotes no significant 24-h variation.
Parenthesis indicate the percentage variations in each measure.

Adapted with permission from Veldhuis JD, Johnson ML, Iramanesh A, Lizarralde G. Rhythmic and non-rhythmic modes of anterior pituitary hormone release in man. In: Touitou Y, Haus E, eds., *Biological Rhythms in Clinical and Laboratory Medicine,* New York: Springer-Verlag, 1992; 277–291.

maintain 24 h serum ACTH and cortisol rhythmicity; repeated fixed-dose pulses of GnRH over 24 h preserve LH and testosterone nyctohemeral variations in boys and men; conversely, continuously delivered testosterone feedback is considerably more effective in suppressing output of the human male GnRH-LH unit than the same daily amount of pulsatile androgen; and, invariant doses of GHRH infused every 90 min for 3 d sustain 24 h serum GH rhythms. Thus, intrapituitary mechanisms, other cosecretagogues or inhibitors of pituitary secretion, and/or distal target-tissue feedback variations may generate or modulate the *circadian patterns* recognized for the amplitude and/or frequency of ultradian pituitary-hormone secretory bursts. Further study will be needed to clarify the *neuroendocrine linkages between circadian rhythms and ultradian pulsatility*. Strong diurnal rhythmicity is preserved throughout puberty, e.g., in the GH axis, but at variable amplitude, as shown in Figure 16.

5. OTHER CLINICAL DISORDERS OF ULTRADIAN RHYTHMICITY

Selected *clinical disorders* of reversibly suppressed ultradian (pulsatile) LH (and presumptively GnRH secretion) are outlined in Table 5. Outstanding as etiologies of reversible "hypothalamic hypogonadotropism" are *psychological stress* (e.g., officers in training school, air traffic-control training), *physical stress* (e.g., extensive burns, physical overtraining or exertion at the marathon-level), *weight loss* (e.g., malnutrition, anorexia nervosa), or *drug-induced suppression* of GnRH output (e.g., use of opiates, such as heroin or methadone). Such abrogation of ultradian GnRH and LH signaling can fully shut down the male and female reproductive axes. Whenever gonadotrope and gonadal cells are not directly damaged or impaired by the illness, associated

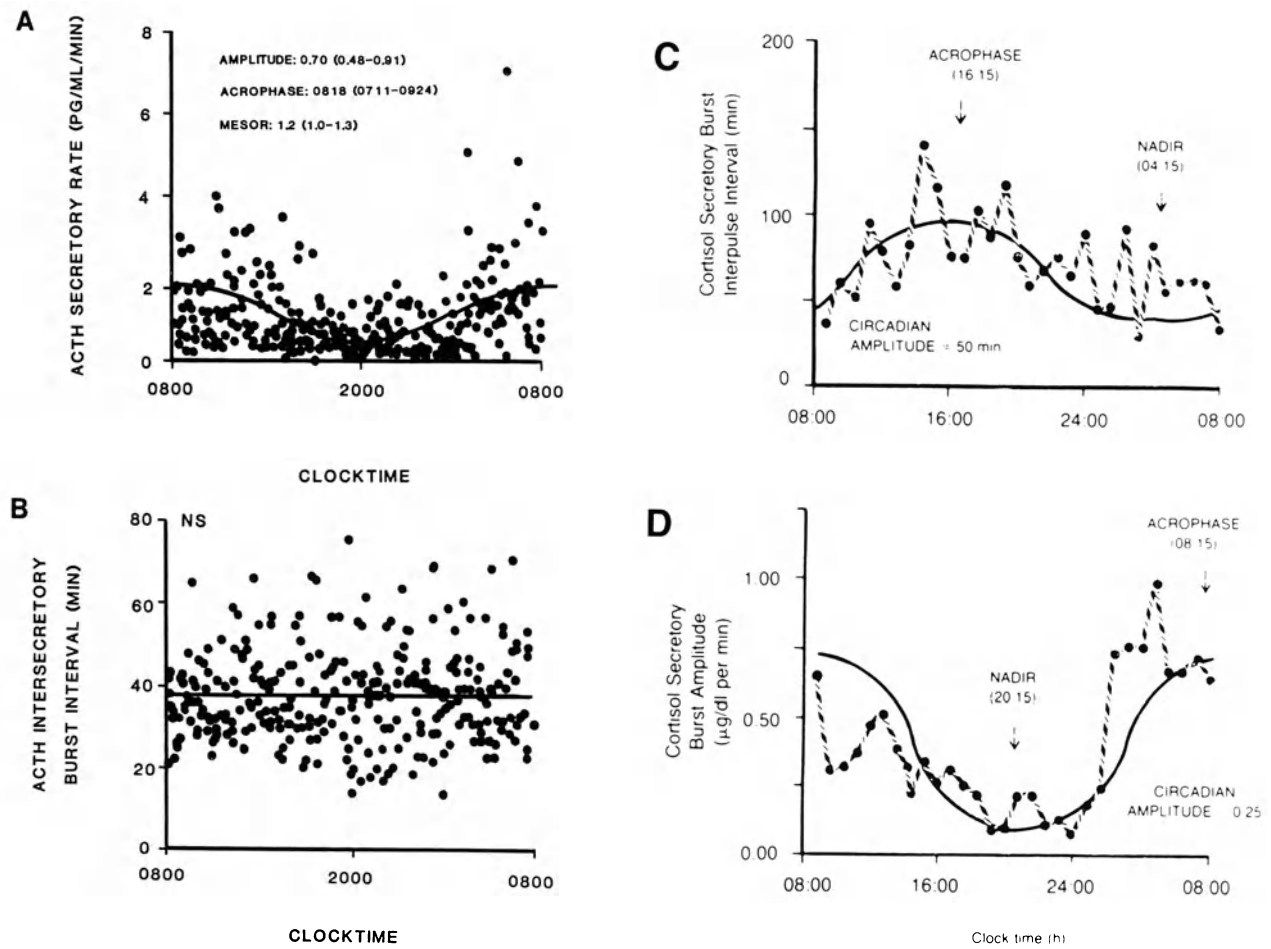

Fig. 15. Twenty-four hour rhythms of ACTH (*Panels A and B*) and cortisol (*Panels C and D*) interpulse intervals *versus* secretory burst rate (amplitude or mass) in healthy young men. ACTH interburst interval (and hence, its reciprocal, pulse frequency) is independent of time of day. In contrast, there is a day–night variation in both ACTH and cortisol secretory burst amplitude (rate) and the mass secreted per burst. The *amplitude* of the 24 h rhythm is one-half the difference between zenith and nadir. The *acrophase* is the time of maximal value in the rhythmic variation. The *mesor* is the mean value about which the rhythm oscillates over 24 h. ACTH data are individual pulse measures and cortisol results are hourly means, from eight healthy young men. Data are presented with permission from Veldhuis JD, Iranmanesh A, Johnson ML, Lizarralde G, (1990). Amplitude, but not frequency, modulation of ACTH secretory bursts gives rise to the nyctohemeral rhythm of the corticotropic axis in man. *J Clin Endocrinol Metab* 71:452–463, and Veldhuis JD, Iranmanesh A, Lizarralde G, Johnson ML, (1989). Amplitude modulation of a burst-like mode of cortisol secretion subserves the circadian glucocorticoid rhythm in man. *Am J Physiol* 257:E6–E14.]

stress, or other concomitant pathophysiology, then *i.v. or s.c. pulsatile GnRH therapy* will reconstitute LH (and FSH) release and gonadal sex-steroid secretion, e.g., as demonstrated in women with weight loss owing to anorexia nervosa, patients with inborn GnRH deficiency (Kallmann's syndrome), and healthy postpartum women with lactation-(hyperprolactinemia) associated amenorrhea.

Reversible suppression of ultradian LH, GH, and thyrotropin (TSH) release also is recognized in profound acute illness; e.g., because of multiple trauma, postoperatively following major surgical procedures (cardiovascular, neurosurgical, abdominal), and/or extensive burns. In such patients, prolonged infusions of relevant secretagogues (GnRH in pulses, GHRH and TRH continuously) will reinstate significantly, although not always fully, pulsatile GH, LH, and TSH release, and restore otherwise reduced serum concentrations of IGF-I, IGF-I BP-3, acid labile subunit (all markers of GH axis activity), testosterone and thyroxine toward normal. The role (if any) of such restorative hypothalamic-peptide therapy in life-threatening illness is still under clinical investigation. Such experiments point to stress-adaptive inhibition of multiple hypothalamic regulatory centers (e.g., GHRH-, TRH, and GnRH-secreting neuronal

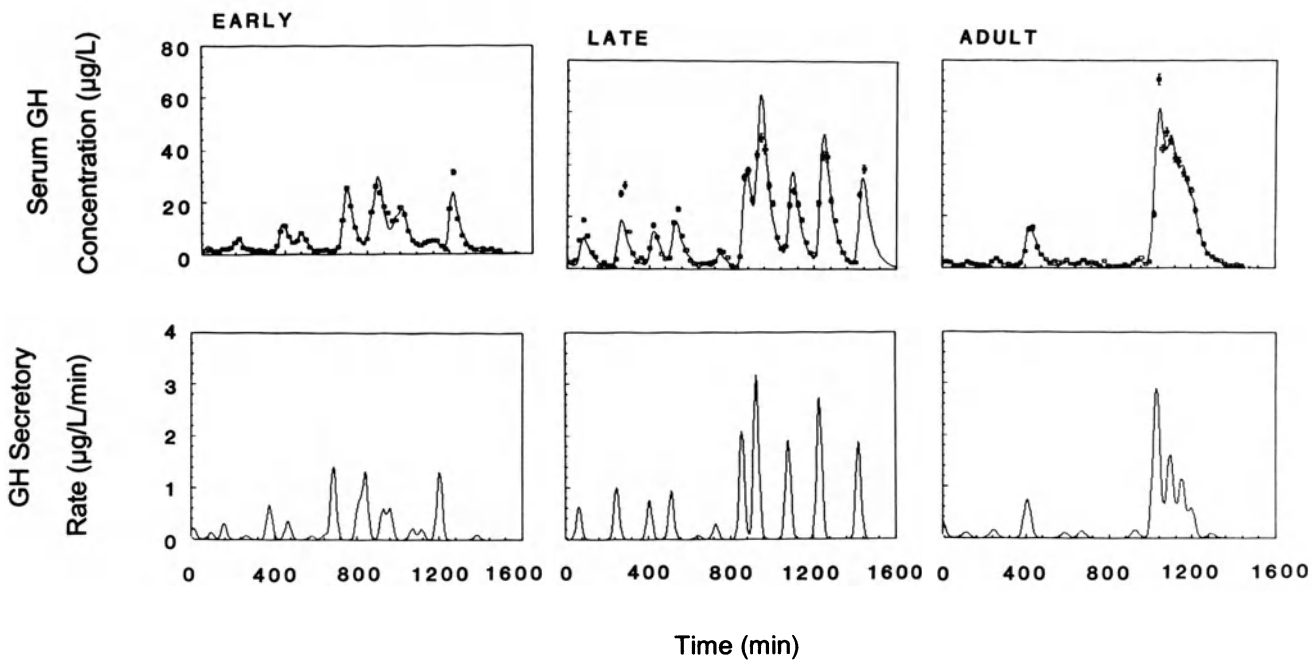

Fig. 16. Daily (24 h) serum GH concentration profiles (upper subpanels) and calculated GH secretion rates (lower subpanels) in three individual human males (early and late puberty, and a young man). There is preservation of 24 h rhythmicity of serum GH concentrations across puberty *via ultradian amplitude-specific variations.* [Adapted with permission from Martha Jr. PM, Goorman KM, Blizzard RM, Rogol AD, and Veldhuis JD, (1992). Endogenous growth hormone secretion and clearance rates in normal boys as determined by deconvolution analysis: relationship to age, pubertal status, and body mass. *J Clin Endocrinol Metab* 74:336–344.]

Table 5
Human Hypothalamo-Pituitary Hypogonadotropic States

I. *Fixed Deficiency of GnRH*
  A. Congenital: Kallmann's syndrome
  B. Hypothalamic lesions:
    (i) tumors: craniopharyngioma, hamartoma, glioma, etc.
    (ii) vascular: contusion, infarction, vasculitis
    (iii) infiltrative: granulomatous disease (e.g., histoplasmosis, sarcoidosis, etc.)
    (iv) others: metastatic lesions, arachnoid cysts, etc.
II. *Reversible GnRH Deficiency*
  A. Weight loss, starvation, type I diabetes mellitus, etc.
  B. Stress (physical, psychological, metabolic)
  C. Opiates (e.g., heroin, methadone)
  D. Exercise (near-exhaustive levels)
  E. Exogenous (anabolic) sex steroids
III. *Intrapituitary Disease*
  A. Para- or intrasellar mass (e.g., meningioma, aneurysm, etc.)
  B. Adenomas (e.g., prolactinoma, acromegaly)
  C. Ischemic infarction (e.g., pituitary apoplexy)
  D. Abscess (e.g., tuberculosis)
  E. Metastatic carcinoma (e.g., adenocarcinoma, melanoma, etc.)

Adapted with permission from Reyes-Fuentes A, Veldhuis JD. Neuroendocrine physiology of the normal male gonadal axis. In: Veldhuis JD, ed., *Endocrinology and Metabolism Clinics of North America,* Philadelphia, PA.: W.B. Saunders, 1993:93–124.

Table 6
Selected Neuroendocrine Analytical Tools
for Assessing In Vivo Hormone Ultradian Rhythmicity

Analytical Strategy	*Application*	*Example*
1. Discrete peak detection	count serum hormone concentration pulses and calculate amplitude	DETECT CLUSTER PULSAR ULTRA etc. (1,2)
2. Fourier analysis	estimate sinusoidal (regularly occurring) rhythms	Insulin secretion(3)
3. Deconvolution analysis	calculate secretory rates and enumerate bursts	DECONV (4) PULSE (5)
4. Approximate entropy	quantitate the pattern orderliness (or process randomness in the data	GH patterns in male versus female (6); LH in older vs young men (7)
5. Stochastic differential equation model	incorporates feedback functions and errors	LH secretion in females (8)

(1) Urban RJ, Evans WS, Rogol AD, Kaiser DL, Johnson ML, Veldhuis JD. Contemporary aspects of discrete peak detection algorithms: I. The paradigm of the luteinizing hormone pulse signal in men. *Endocrin Rev* 1988; 9:3–37.

(2) Evans WS, Christiansen E, Urban RJ, Rogol AD, Johnson ML, Veldhuis JD. Contemporary aspects of discrete peak detection algorithms: II. The paradigm of the luteinizing hormone pulse signal in women. *Endocrin Rev* 1992; 13:81–104.

(3) Bingley PJ, Matthews DR, Williams AJ, Bottazzo GF, Gale EA. Loss of regular oscillatory insulin secretion in islet cell antibody-positive non-diabetic subjects. *Diabetologia* 1992; 35:32–38.

(4) Veldhuis JD, Carlson ML, Johnson ML. The pituitary gland secretes in bursts: appraising the nature of glandular secretory impulses by simultaneous multiple-parameter deconvolution of plasma hormone concentrations. *Proc Natl Acad Sci USA* 1987; 84:7686–7690.

(5) Veldhuis JD, Johnson ML. Specific methodological approaches to selected contemporary issues in deconvolution analysis of pulsatile neuroendocrine data. *Meth Neurosci* 1995; 28:25–92.

(6) Pincus SM, Gevers E, Robinson ICA, Roelfsema F, Hartman ML, Veldhuis JD. Females secrete growth hormone with more process irregularity than males in both human and rat. *Am J Physiol* 1996; 270:E107–E115.

(7) Pincus SM, Mulligan T, Iranmanesh A, Gheorghiu S, Godschalk M, Veldhuis JD. Older males secrete luteinizing hormone and testosterone more irregularly, and jointly more asynchronously, than younger males: dual novel facets. *Proc Natl Acad Sci (USA)* 1996; 93:14,100–14,105.

(8) Keenan DM, Veldhuis JD, Yang R. Joint recovery of pulsatile and basal hormone secretion by stochastic nonlinear random-effects analysis. *Am J Physiol* in press.

systems), which otherwise control ultradian pituitary hormone secretion in the human.

6. EMERGING ISSUES

Whereas ultradian pulsatility is critical for the GnRH-LH secretory unit and strongly influences GH action on target tissues (e.g., in the rodent), much remains to be learned about the mechanisms of pulse generation by hypothalamic neuronal ensembles. The quantitation of mechanistically complex (variably admixed basal and pulsatile) neurohormone secretion patterns also remains difficult (*see* Fig. 17). How circadian outputs (whether neuronal or otherwise) couple to ultradian signals (variably at the levels of the hypothalamus, pituitary gland, and/or target tissues) will require further study. And, the network concept of integrated, but interacting, neuroglandular arrays (e.g., the hypothalamus/GnRH, pituitary gland/LH, and testis/testosterone axis) should be extended more formally with appropriate quantitative techniques implemented for network analysis, and with inclusion of relevant stochastic (random) elements (e.g., *see* Fig. 18). Last, in addition to the strictly pulsatile modes of neurohormone quantitated by discrete peak detection methods (Fig. 19A), or secretory pulse analysis (deconvolution analysis Fig. 19B), the subordinate (nonpulsatile) pattern orderliness of serial hormone measures can be quantified by (approximate) entropy (Fig. 19C). A more robust and comprehensive knowledge of integrated neuroendocrine regulatory mechanisms that operate in normal physiology will be an essential foundation for novel clinical insights into pathological states in women, men, and children,

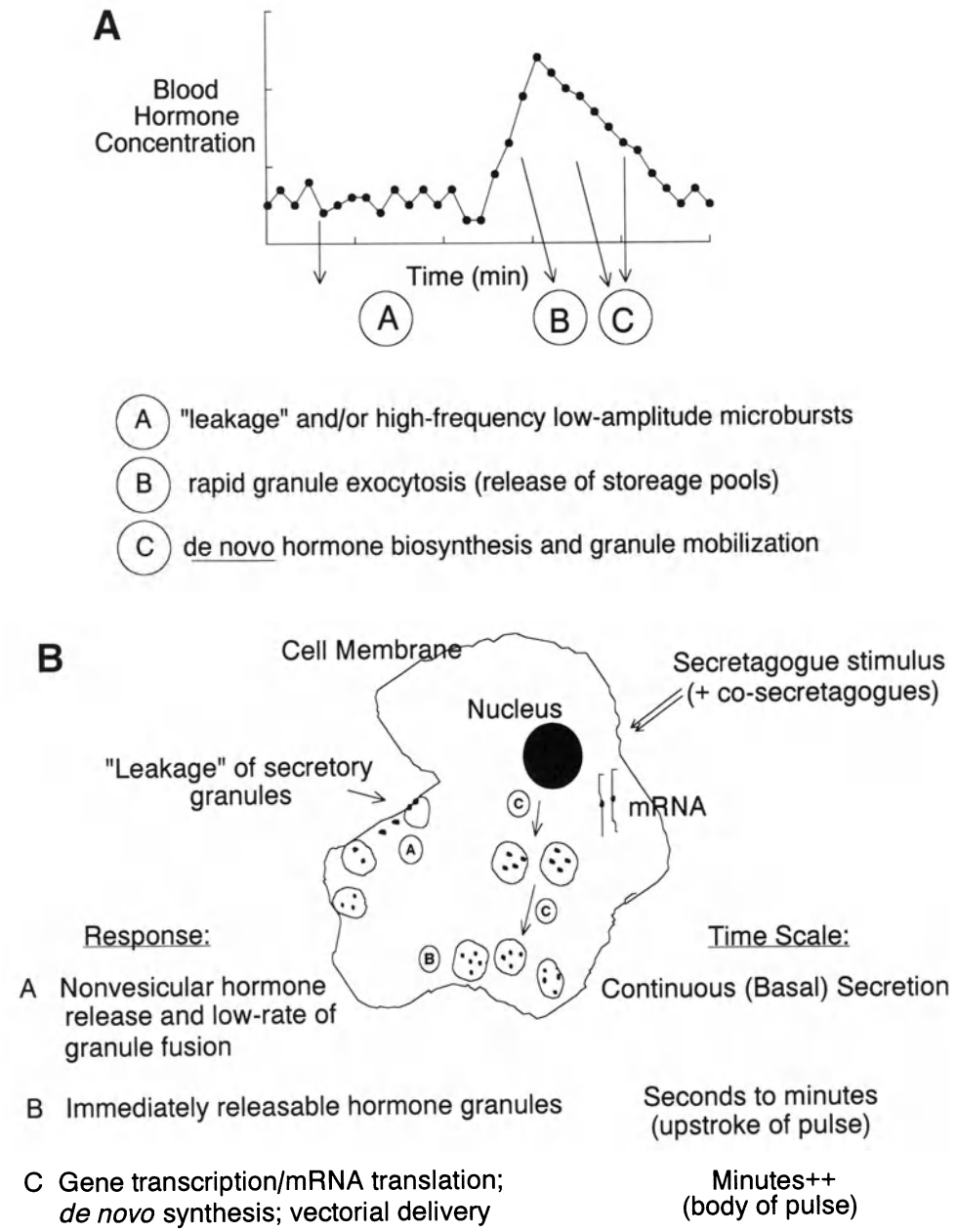

Fig. 17. Hypothetical construct of admixed basal and pulsatile (ultradian) neurohormone secretion (*Panel A*), and corresponding intuitive notion at the cellular and biochemical levels of underlying basis of admixed basal and pulsatile neurohormone secretin (*Panel B*).

as well as in domestic animals and endangered wild species.

7. SUMMARY PERSPECTIVE

All seven principal anterior pituitary hormones are secreted in a *pathophysiologically regulated* (ultradian) *pulsatile fashion.* Pulsatility is driven by relevant hypothalamic (co-)secretagogues and/or withdrawal of inhibitors, and further modulated at the pituitary level by autocrine and paracrine factors, and by extrinsic (systemic) feedback effectors. The GnRH-LH and GH-target tissue feedforward interactions are strongly ultradian pulse-signal dependent. Thus, pulsatile versus time-invariant neurohormone signaling mechanisms exert differential effects on selected target tissues, providing a biological diversity

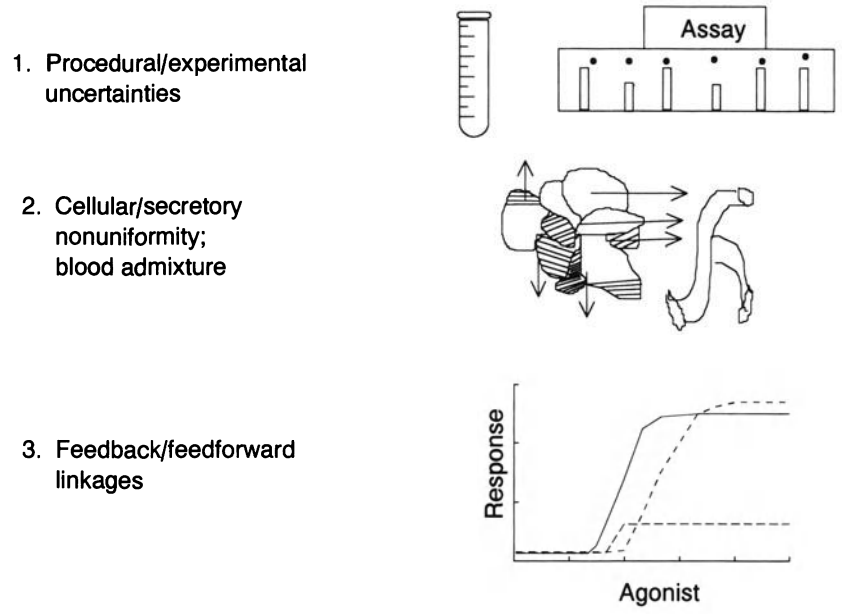

Fig. 18. Sources of experimental and biological uncertainties (stochastic elements) embedded in measurements of a pulsatile neuroendocrine feedback control system. [Adapted with permission from Keenan DM and Veldhuis JD, (1998). A biomathematical model of time-delayed feedback in the human male hypothalamic-pituitary-Leydig cell axis. *Am J Physiol* in press.]

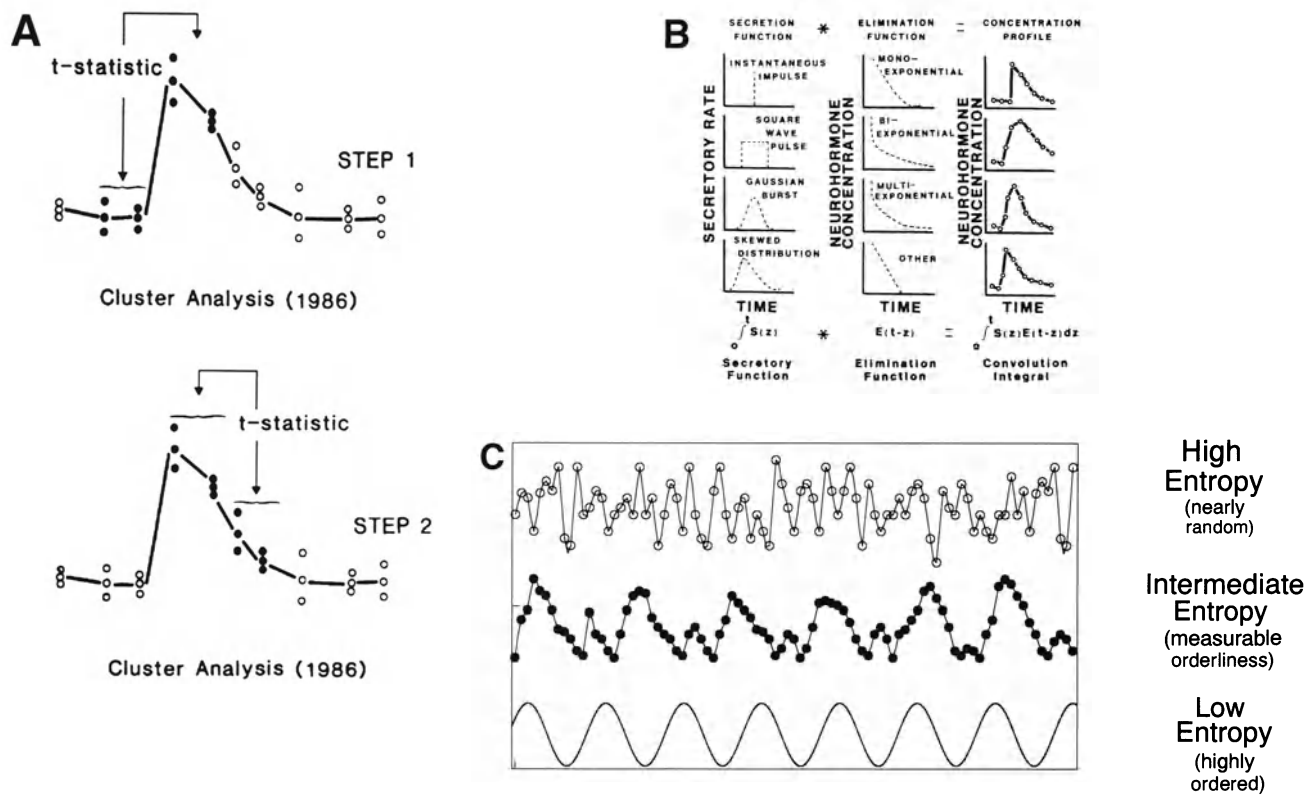

Fig. 19. Intuitive presentation of notions of **(A)** discrete peak detection, e.g., by Cluster analysis, **(B)** secretory burst analysis by mathematical deconvolution analysis, and **(C)** approximate entropy calculations, as complementary to pulse enumeration. [Adapted with permission from Veldhuis JD and Johnson ML, (1986). Cluster analysis: A simple, versatile and robust algorithm for endocrine pulse detection. *Am J Physiol* 250:E486–E493; Urban RJ, Evans WS, Rogol AD, Kaiser DL, Johnson ML, and Veldhuis JD, (1988). Contemporary aspects of discrete peak detection algorithms: I. The paradigm of the luteinizing hormone pulse signal in men. *Endo Rev* 9:3–37; and Veldhuis JD and Pincus SM, 1998. Orderliness of hormone release patterns: a complementary measure to conventional pulsatile and circadian analyses. *Eur J Endocrinol* 138:358–362.]

of responses achievable by the same effector molecule depending on its time course of delivery to the receptor. An insightful contemporary concept emerging from integrative neuroendocrine physiology is the interactive, time-delayed feedforward, and feedback nature of neuroendocrine axes, which more appropriately are viewed as *control systems* or *homeostatic networks*. Feedback-intact, and physiologically well adapted, neuroendocrine axes can maintain low-entropy (well organized) ultradian pulsatility, which is impaired in aging, and disrupted in disease. Finally, *external or internal stressors* may shift an interfacing feedforward dose-response function within the network (e.g., attenuation of LH receptor-mediated drive to testosterone biosynthesis in older men), alter feedback linkages (e.g., reduced IGF-I negative feedback in the GH axis in fasting), and/or disrupt circadian-ultradian coupling (e.g., stress-mediated loss of ACTH/cortisol circadian rhythmicity, but preserved ultradian pulsatility). Given this foundation, prospects in clinical and experimental neuroendocrinology are auspicious for uncovering new and informative regulatory mechanisms over the next decade.

SELECTED READINGS

Clarke IJ, Cummins JT. Pulsatility of reproductive hormones: physiological basis and clinical implications. Bailliere's Clin Endocrinol Metabol 1987; 1:1–21.

Conn PM, Crowley, Jr. WF. Gonadotropin-releasing hormone and its analogs. New Engl J Med 1991; 324:93–103.

Evans WS, Christiansen E, Urban RJ, Rogol AD, Johnson ML, Veldhuis JD. Contemporary aspects of discrete peak detection algorithms: II. The paradigm of the luteinizing hormone pulse signal in women. Endo Rev 1992; 13:81–104.

Keenan D, Veldhuis JD. Stochastic model of admixed basal and pulsatile hormone secretion as modulated by a deterministic oscillator. Am J Physiol: Regulatory, Integrative, & Comparative Physiology 1997; 273:R1182–R1192.

Lincoln DW, Fraser HM, Lincoln GA, Martin GB, McNeilly AS. Hypothalamic pulse generators. Rec Prof Horm Res 1985; 41:369–419.

Ordog T, Chen MD, Nishihara M, Connaughton MA, Goldsmith JR, Knobil E. On the role of gonadotropin-releasing hormone (GnRH) in the operation of the GnRH pulse generator in the Rhesus monkey. Neuroendocrinology 1997; 65:307–313.

Pincus SM, Mulligan T, Iranmanesh A, Gheorghiu S, Godschalk M, Veldhuis JD. Older males secrete luteinizing hormone and testosterone more irregularly, and jointly more asynchronously, than younger males: dual novel facets. Proc Natl Acad Sci USA 1996; 93:14,100–14,105.

Plotsky PM. Hypophysiotropic regulation of adenohypophyseal adrenocorticotropin secretion. Fed Proc 1985; 44:207–213.

Tannenbaum GS. Neuroendocrine control of growth hormone secretion. Acta Paediatr Scand Suppl 1991; 372:5–16.

Tsigos C, Chrousos GP. Physiology of the hypothalamic-pituitary-adrenal axis in health and dysregulation in psychiatric and autoimmune disorders. Endocrinol Metabol Clin North Amer 1994; 23:451–466.

Urban RJ, Evans WS, Rogol AD, Kaiser DL, Johnson ML, Veldhuis JD. Contemporary aspects of discrete peak detection algorithms: I. The paradigm of the luteinizing hormone pulse signal in men. Endo Rev 1988; 9:3–37.

Van Cauter E, Honinchx E. Pulsatility of pituitary hormones. In: Schulz H, Lavie P, eds. Ultradian Rhythms in Physiology and Behavior. Springer-Verlag 1986 Publ., Berlin, Germany.

Veldhuis JD. Pulsatile hormone release as a window into the brain's control of the anterior pituitary gland in health and disease: implications and consequences of pulsatile luteinizing hormone secretion. The Endocrinologist 1995b; 5:454–469.

Veldhuis JD. The neuroendocrine regulation and implications of pulsatile GH secretion: gender effects. The Endocrinologist 1995a; 5:198–213.

PART V NEUROENDOCRINE PATHOLOGY AND DISEASE

27 Diagnosis and Treatment of Hypothalamic Disease

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and Nina Rosa de C. Musolino, MD

CONTENTS

1. INTRODUCTION

Anatomo-functional studies on the hypothalamus have faced major difficulties, because this organ cannot, as far as function is concerned, be considered like the cerebral cortex, where the homunculus could be mapped, with usually well-determined brain regions corresponding to somatic or visceral afferent or efferent projections, according to an established map. This may be because of the proximity of the hypothalamic nuclei to afferent and efferent paths of the cortex, thalamus, limbic system, and spinal cord; all occurring in a rather limited region, measuring 1.5 × 1.5 × 1.3 cm, weighing approximately 2.5 g and divided into three zones (periventricular, medial and lateral) and four regions (preoptic, supraoptic, tuberal, and mamillary.) In Table 1, we describe the several hypothalamic regions or nuclei involved in different functions, according to studies conducted both in human beings and other animals.

The hypothalamic syndromes have several etiologic possibilities and clinical manifestations. Among 60 patients reviewed by Bauer as suffering from hypothalamus affecting disorders (documented by autopsy), neuroophthalmic involvement occurred in 78% of cases, pyramidal or sensitive deficits in 75%, headache in 65%, extrapyramidal cerebellum signs in 62%, vomiting in 40%, precocious puberty in 40%, diabetes insipidus in 35%, hypogonadism in 32%, sleepiness in 30%, dysthermia in 28%, and obesity or edema in a further 25%. Most of these patients had two or more of such symptoms concurrently. However, some disorders are clinically represented as restricted hypothalamic manifestations, such as precocious puberty or gelasmus convulsive crisis in the case of hamartomas.

Systemic signs and symptoms (therefore, not in the hypothalamus) should be sought in diseases such as sarcoidosis, histiocytosis, infections, porphyria, metastatic tumors, and lymphomas. Seldom are such diseases restricted to the hypothalamus. Acute lesions, such as trauma or vascular accidents, generally lead to consciousness impairment, hyperthermia, and diabetes insipidus, which may be transient should the patient survive, whereas the more chronic diseases lead to cognitive impairment and endocrine disorders, usually irreversible. Table 2 depicts the main causes of hypothalamic syndromes.

From: *Neuroendocrinology in Physiology and Medicine*, (P. M. Conn and M. E. Freeman, eds.),

Table 1
Hypothalamic Regions

Function	*Nucleus or Region*
CRH, TRH, Dopamine, Somatostatin, GnRH	Paraventricular
Somatostatin, Dopamine, Proopiomelanocortin, GHRH	Arcuate
Vasopressin (ADH)	Supraoptic and Paraventricular
Thirst	Anterior and Lateral Preoptic Hypothalamus
Gain and Loss of Heat (Afferent Path)	Anterior Preoptic Hypothalamus
Gain of Heat (Efferent Path)	Posterior and Medial Hypothalamus
Loss of Heat (Efferent Path)	Posterior and Lateral Hypothalamus
Sleep	Anterior Hypothalamus
Wakefulness	Posterior Hypothalamus
Sympathetic stimulus	Posteromedial Hypothalamus
Parasympathetic stimulus	Anterior Preoptic Hypothalamus
Emotion	Ventromedial, Medial, Posterior, Caudal and Lateral Hypothalamus
Memory	Ventromedial, Mamillary and Medial-Dorsal
Urination and Evacuation	Medial Tuberal Region
Gastrointestinal Motility	Anterior Preoptical Hypothalamus and Posterior Dorsolateral Region

Table 2
Etiology of Hypothalamic Syndromes

Craniopharyngioma
Germ Cell Tumor
Hamartoma
Meningioma
Glioma
Chordoma
Pituitary Tumor
Metastatic Tumor
Dermoid and Epidermoid Tumor
Granulomatous/Inflamatory Diseases
- Sarcoidosis
- Histiocytosis X
- Tuberculosis

Radiation Lesion
Traumatic Lesion
Other: Meningitis, Hydrocephalus, Laurence–Moon–Biedl and Prader–Willi Syndrome, Aneurysm, Arteriovenous Malformation, Wernicke's Disease, Aracnoid Cyst

2. LESIONS LEADING TO HYPOTHALAMIC DYSFUNCTION

2.1. Craniopharyngioma

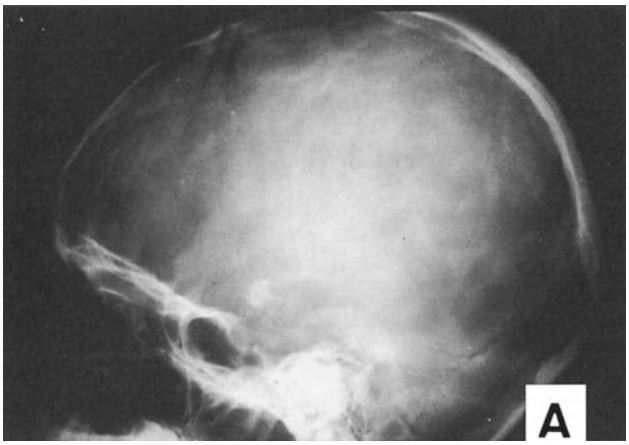
Craniopharyngiomas are neoplasms in the hypothalamus-pituitary region, considered to arise from Rathke's pouch remnants. Although histologically benign, they may present malignant behavior according to their location and growth rate. By and large craniopharyngiomas account for approximately 2.5% of brain tumors, and for 5 to 10% of children's brain neoplasms. They can be cystic, solid, or mixed, either calcified or not. (Fig. 1A and B).

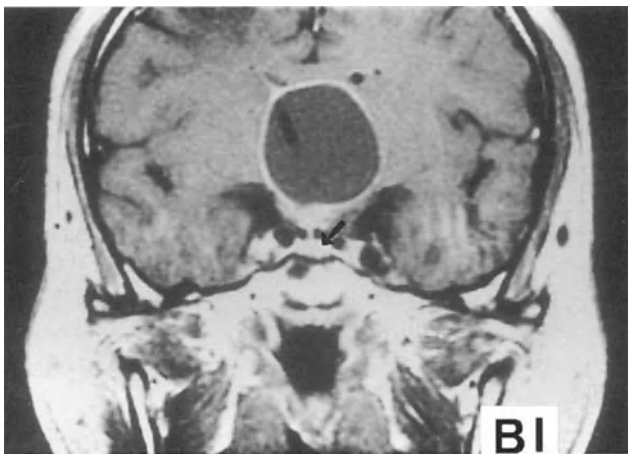
Symptoms at onset depend both on the location and size of the lesion and patient age. Thus, such tumors may either lead to variable neurological and endocrine symptoms (Table 3) or just be an incidental finding on an imaging examination.

The incidence of partial or total hypopituitarism is high as from diagnosis. Figure 2 illustrates the frequency of such a deficiency in the several hypothalamus-pituitary sectors in patients bearing craniopharyngiomas, at diagnosis. Thus, patients diagnosed as having craniopharyngioma should have their hormonal profile assessed in order to receive proper hormonal replacement, particularly glucocorticoid and thyroid hormone, whose aim is to reduce surgical treatment risks.

Image assessment should be performed by magnetic resonance imaging (MRI) associated with computed tomography imaging with no contrast agent, to allow better identification of calcification.

Initial craniopharyngioma treatment is almost invariably surgical. Ideally, the lesion should be completely removed (Fig. 3). However, as such procedure may be associated with high morbidity and mortality,





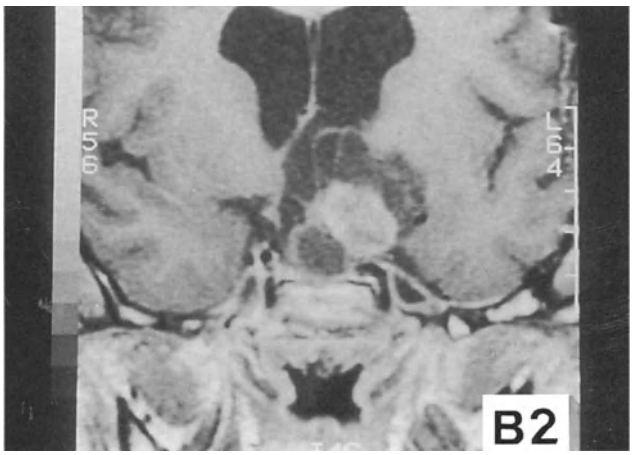


Fig. 1. Craniopharyngioma imaging: **(A)** Plain skull radiography showing suprasellar calcification. **(B)** MRI (T1 weighted coronal view enhanced by gadolinium). **(B1)** predominantly cystic suprasellar lesion. A normal pituitary gland is observed (arrow). **(B2)** mixed (solid / cystic) sellar and suprasellar tumor.

Table 3
Clinical Presentation in 29 Patients with Craniopharyngioma*

Headache	80%
Visual impairment	60%
Delayed puberty	44%
Growth arrest	40%
Diabetes insipidus	23%
Intracranial hypertension	23%

*Neuroendocrine unit, Division of Neurosurgery, Hospital das Clinicas, University of S.Paulo.

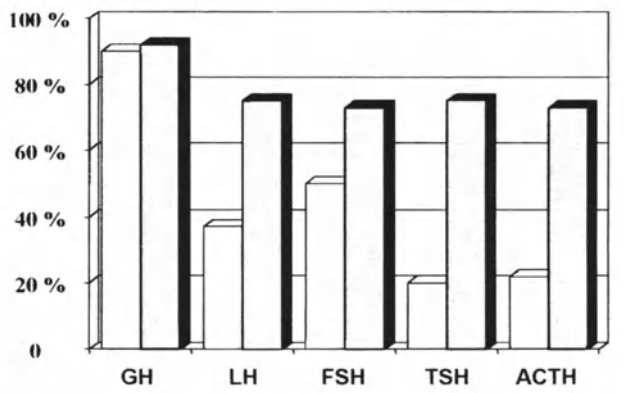


Fig. 2. Hormonal failures (%) before (□) and after (■) surgical treatment for craniopharyngiomas (Neuroendocrine Unit, Division of Neurosurgery, University of S.Paulo).

it is not unusual to combine partial resection with a postsurgical radiation therapy. Surgical approach may either be transsphenoidal or transcranial, with or without endoscopy aid. Both the choice of approach and treatment outcome depend mainly on the craniopharyngioma initial features. Young age (under 5 yr), presence of hydrocephalus, and giant tumors are associated with a higher incidence of surgical and hypothalamic complications.

Hormonal impairment is quite frequent after surgery, associated or not with radiation therapy (Fig. 2). Thus, patients should be reassessed following treatment. However, it is a well-known fact that some children show normal growth despite growth hormone (GH) deficiency after surgery or radiation therapy. The mechanism accounting for catch-up growth is uncertain. It is assumed that improved feeding, with resulting weight gain, may lead to increased serum insulin levels, a hormone that could be implicated in such effect; other growth factors may also be involved. The importance of this effect has led to the discussion about indicating GH replacement on clinical or laboratory basis.

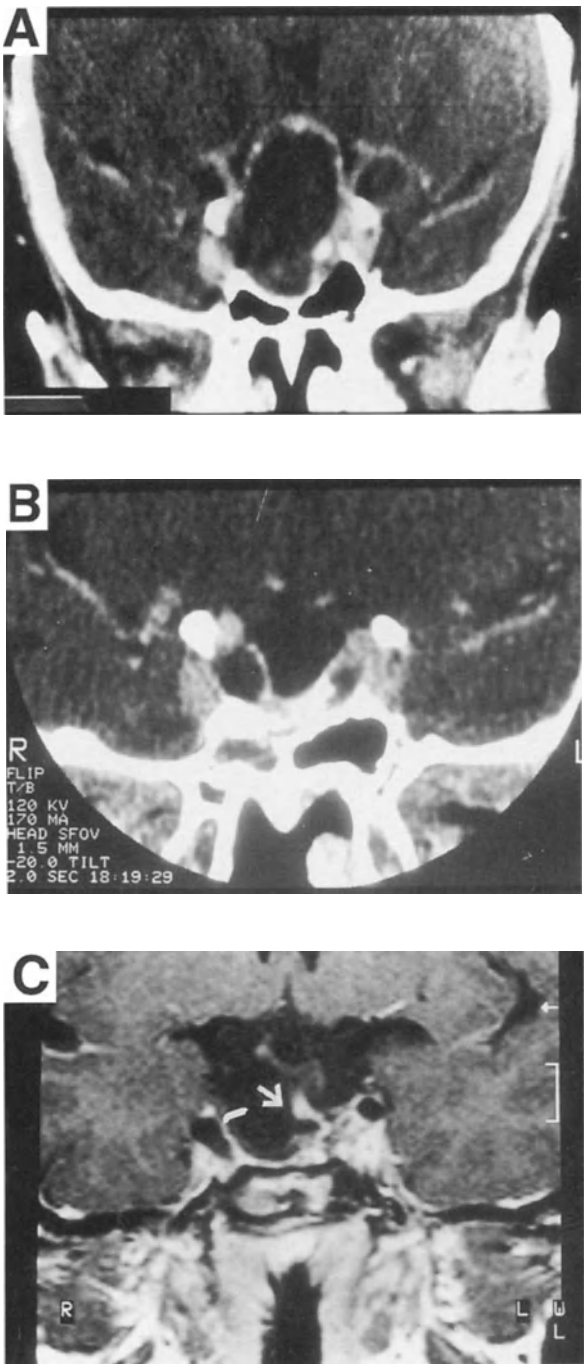


Fig. 3. CT coronal views of a cystic craniopharyngioma before **(A)** and shortly after surgery **(B)**. **(C)** MRI coronal view, 5 yr after surgery showing no tumor remnants and stalk displacement (arrow).

2.2. Germ Cell Tumors

Germ cell tumors account for 1.8% of primary brain tumors before 20 yr of age in the United States and 6.5% of all brain tumors in Japan; with incidence peaks in the second and third decades of life, and male prevalence. Incidence of such tumors is 65% for germinomas, of a better prognosis, 5% for those tumors of nongerminomal cells as embryonal carcinoma, 5% for choriocarcinoma (worst prognosis), 7% for yolk sac tumor, and 18% for teratoma (either mature or immature). These tumors, which may be mixed, represent totipotent germ cells that differentiate into germ cells (germinoma), embryonic cells (embryonal carcinoma and teratoma), and extraembryonary elements as yolk and trophoblastic sac (choriocarcinoma). Cells originating germ cell neoplasms in the brain are unknown. Such cells may be located in the suprasellar or pineal region and sometimes a tumor is found in both sites. Teratomas and choriocarcinomas occur more often in childhood, whereas germinomas, embryonal carcinomas, tumors of the yolk sac, and immature teratomas occur more often in adolescents and young adults, with a male gender prevalence.

Mixed tumors account for 50–60% of tumors. If suprasellar, pure germinomas are the tumors most often found, whereas the pineal ones are usually mixed.

The most frequent found clinical setting is that of diabetes insipidus, visual loss, and hypopituitarism, sometimes combined with hypothalamic syndromes of dysfunction of appetite control and adipsia, hydrocephalus, somnolence, and Parinaud sign. Neurological abnormalities are more typical in nongerminal tumors, whereas the endocrine ones are more frequently related to germinal tumors. Approximately, 5% of the latter develop precocious puberty, which may occur because of median eminence compression or hCG production in boys.

Beta-hCG and alpha-fetoprotein can be found both in the blood and cerebrospinal fluid of patients with germ cell tumors. Alpha-fetoprotein is produced by yolk sac and fetal liver, being inhibited at birth, only to reappear in patients hosting hepatocellular or germ cell tumors with either a yolk sac or an embryonal carcinoma component. Beta-hCG is produced by giant syncytiotrophoblast cells, likely to be found in patients with choriocarcinoma and nontrophoblastic tumors, as lung and stomach adenocarcinomas, hepatocellular carcinoma, and malignant melanoma. High level of alpha-fetoprotein indicates the presence of germ, nongerminomal cells within the tumor, whereas an increase in beta-hCG may occur both in germinoma and in nongerminomal neoplasms. Germinomas and teratomas are typically negative to both markers. Both markers may be useful to control therapeutic success, tending to increase again if the tumor recurs.

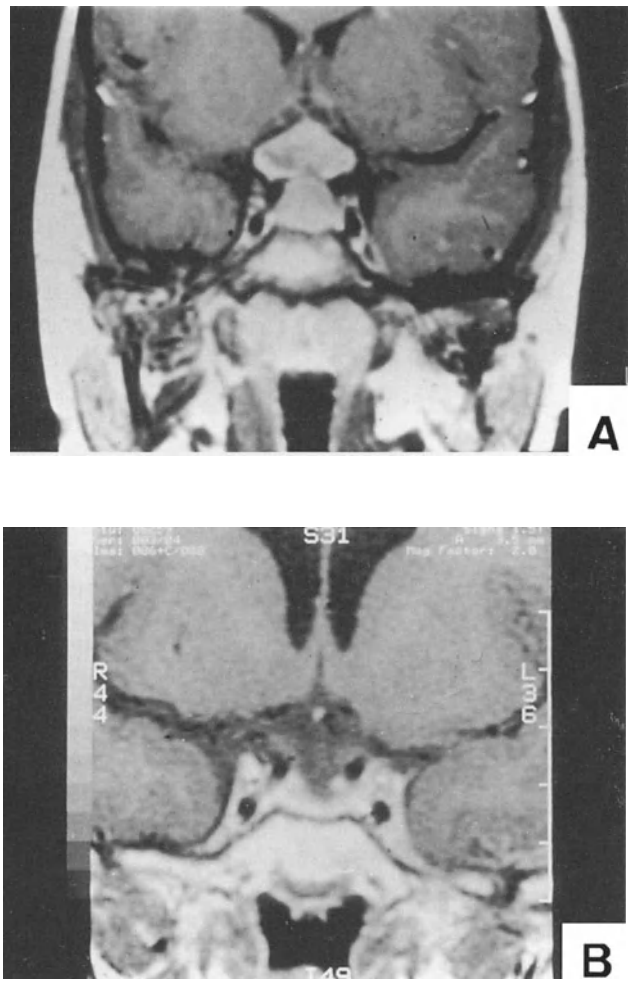


Fig. 4. MRI (T1 weighted coronal view enhanced by gadolinium) of a seven years old girl with a biopsy proved germinoma, before **(A)** and after **(B)** radiotherapy. Important tumor reduction is observed.

Some authors advocate the use of an initial radiation therapy trial with a therapeutic dose of about 2000 cGy, based on the high germinoma sensitivity to such therapeutic modality. Others insist on obtaining histologic diagnosis, by performing a tumor excision or biopsy alone in germinomas, followed by adjunctive radiation therapy (Fig. 4) or chemotherapy.

2.3. Hamartoma

Hamartomas are benign tumors composed of a blend of neurons, astrocytes, and oligodendrocytes, which may occasionally contain myelinated nerve fibers. Most of them are less than 1.5 cm and are found in the posterior hypothalamus (Fig. 5C and D). They occur more often before two years of age and express gonadotropin-releasing hormone (GnRH), releasing such hormone in a pulsatile manner, leading to an actual precocious puberty in about 90% of patients (Fig. 5A and B). Some cases lead to precocious puberty even with no GnRH immunoexpression, presumably by compression with median eminence stimulation or interruption of GnRH inhibitory factors. Such cases also show a gonadotropin positive response following acute injection of the hypothalamic peptide. Moreover, hamartomas may lead to convulsions, particularly of the gelasmus type, psychomotor delay, and emotional instability. Pallister–Hall syndrome consists of hypothalamic hamartoblastoma; face-skull, heart, renal, lung, and fingers abnormalities; imperforate anus; and hypopituitarism with absence of both the anterior and posterior pituitary gland. When the clinical setting points to precocious puberty, as a rule GnRH analogs administration has been the treatment of choice. Surgical management is currently uncommon, as these tumors are slow-growing and resection is usually followed by high morbidity-mortality rates. Total excision may control convulsive crisis.

2.4. Meningioma

Meningiomas account for one fourth of all brain tumors. Peripituitary meningiomas have their dural origin in several sella-adjacent areas. Meningiomas originate in meningoendothelial cells and arachnoid villi. Female gender prevails and estrogen and progesterone receptors have been shown in some of these tumors. Such evidences may be the link between the presence of meningioma and higher prevalence of breast cancer in the same patient, as well as neurological impairment in meningioma patients during pregnancy or menstruation. Visual loss is the most prevailing symptom, Foster–Kennedy syndrome may be found in 7.2% of cases, i.e., one eye amaurosis with ipsilateral optic disk atrophy and contralateral papilledema. Endocrine abnormalities occur in 22% of patients, and mostly involve the anterior pituitary gland. Diabetes insipidus occurs in only 4.8% of patients.

In radiological semiology, hyperostosis at the meningeal point of origin may appear on plain skull radiography and computed tomography (CT); in some cases, calcifications may be found. Both on CT and MRI marked contrast enhancement is found and the meningioma surface is often nodular, what is depicted in the Fig. 6. On MRI sometimes the point of origin may be located, which can contribute to distinguish pituitary adenoma. In some cases copious meningioma vascularization may give rise to a blush on digital cerebral angiography.

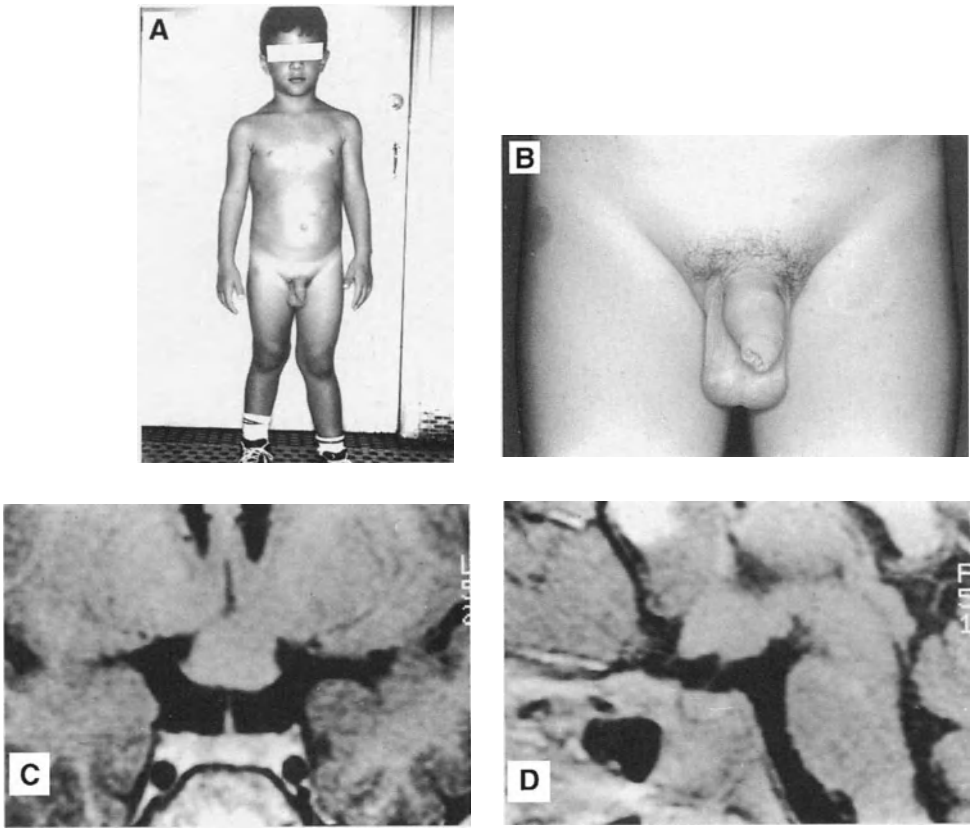


Fig. 5. Four-year-old boy with precocious puberty harboring hypothalamic hamartoma. Coronal **(A)** and sagital **(B)** views. Note the similarity of T1 weighted features between the mass and brain.

Surgery, preferably through a transcranial approach, is the treatment of choice; as such tumors are firm and highly vascularized, a transsphenoidal approach would compel the surgeon to proceed, in the majority of cases, through a normal pituitary gland, entailing further hormonal impairment.

2.5. Glioma

Optic and hypothalamic glioma accounts for 1–3% of brain tumors in children and adolescents. The three types of astrocyte-derived neoplasms, astrocytomas, anaplastic astrocytomas, and glioblastomas, can be found in the hypothalamus. Most typical is the pilocytic astrocytoma, arising on the third ventricle walls or optic paths in 75% of cases. The remaining 25% are intraorbital. Two types of pilocytic astrocytomas, adult and juvenile, are found. The former is usually firm and, although not reaching a large size, tends to be more invasive and aggressive than the latter. The juvenile type occurs before the age of two in about 40% of cases, and in 80% before ten years of age. About 20–33 percent of bearers of optic gliomas have neurofibromatosis. Gliomas of the posterior third ventricle more frequently present neurological and opthalmological disturbances, but other important neurological findings are cerebellar abnormalities and mental changes. Endocrine abnormalities are not frequent.

The best therapeutic choice for such tumors is controversial. Some authors advocate medical follow-up alone, because of benign course of such lesions, unless they cause neurological deficits. Others defend a conventional or stereotaxic radiation therapy, which would lead to growth inhibition or delayed tumor recurrence.

2.6. Chordoma

Chordomas are rare, locally invasive tumors, arising from intrabony notochord remnants. They are slow growing tumors and metastasis rarely occurs. Chordomas occur more often in the sacral-coccygeal region and about one third in the clivus (at sphenoid-occipital synchondrosis). On imaging assessment, osteolytic bony erosion and occasional calcification are found. Surgery has been the exclusive treatment, recurrence is the rule, with an average 5-yr survival.

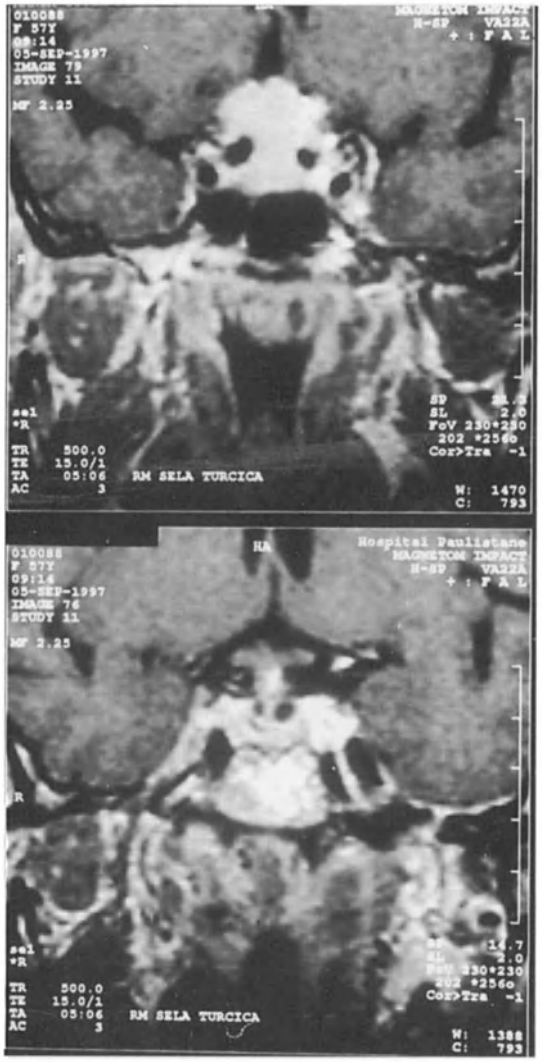


Fig. 6. Coronal view of MRI (T1 weighted) of a meningioma arising from the *tuberculum sella.*

This kind of tumor is frequently resistant to adjunctive therapy as chemotherapy or radiotherapy and rarely do they develop into sarcomatous tumors, with an aggressive natural history.

2.7. Pituitary Tumors

Pituitary adenomas account for 10% of brain tumors. According to their size, such tumors can be classified into microadenomas and macroadenomas. Macroadenomas can be expansive and invasive (Fig. 7), leading to hypothalamus compression and dysfunction. Pituitary carcinomas are exceedingly rare.

Prolactinomas are the most frequent pituitary adenomas, followed by "clinically nonfunctioning" and GH-producing adenomas. Apart from being rarer, ACTH-producing adenomas, which account for

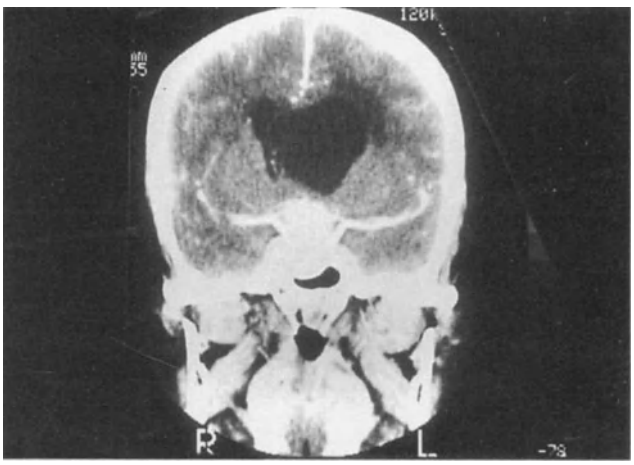


Fig. 7. CT scan coronal view of an expansive macroprolactinoma leading to intracranial hypertension (note the dilated lateral ventricules).

Cushing's disease, are most often microadenomas and even in its macroadenoma presentation rarely expand to the hypothalamus. Nevertheless, in the case of Cushing's disease treated by bilateral adrenalectomy, the ACTH-producing adenoma may become more aggressive and expansive producing what is known as Nelson's syndrome.

At clinical presentation, patients with expansive adenomas usually have visual disturbances due to optic chiasm compression. Headache owing to meningeal expansion and symptoms related to the compression of cranial nerves, should the cavernous sinus be affected, can also occur. The association of hydrocephalus with pituitary adenomas is rare but can be present in cases of large suprasellar expansion (Fig. 7). In addition to the neuroophthalmologic symptoms, further symptoms can be found depending on hormonal hypersecretion (hyperprolactinemic syndrome, giganto-acromegaly, Cushing's disease, or Nelson's syndrome) or hyposecretion (hypopituitarism owing to destruction or compression of the normal pituitary gland, hypothalamic nuclei responsible for pituitary function control, or pituitary stalk). The association of diabetes insipidus with nontreated pituitary adenoma is rare, and more common after surgical treatment.

The tumor is diagnosed and located with the aid of imaging methods, the most accurate of which is MRI. Such examination shows the tumor location and size, as well as its relation to adjacent structures as the optic chiasm, cavernous sinus, sphenoidal sinus, and hypothalamus. Cystic or hemorrhagic components of the tumor can also be depicted. CT is also useful, particularly in identifying calcifications and

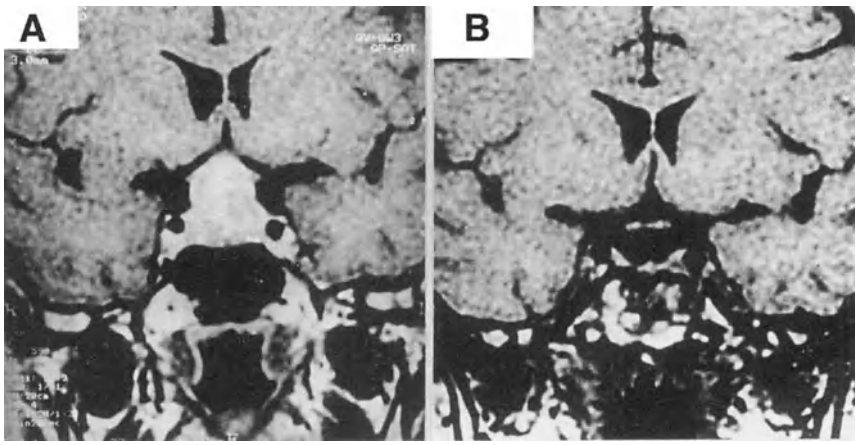


Fig. 8. MRI (T1 weighted coronal views) of an acromegalic patient: **(A)** before and **(B)** after transsphenoidal surgery. Note the complete removal of tumor mass.

the integrity or damage of bone structures, such as the sellar floor.

Hormonal assessment is necessary to determine hormone hypersecretion and status of the pituitary function. Prolactinoma diagnosis is given by high serum prolactin levels, almost invariably higher than 100 ng/mL. Lower levels can be related to clinically nonfunctioning tumors, which lead to hyperprolactinemia by pituitary stalk compression, hindering the dopamine flow to the normal pituitary gland. Hyperprolactinemia can also occur in association with growth hormone production in cases of acromegaly. Glycoproteic hormone-producing tumors (LH, FSH, and TSH) are rare and can lead to hypersecretion syndrome, as, for instance, hyperthyroidism. Acromegaly diagnosis (GH-producing tumors) is established by the finding of high levels of serum GH (basal or during GTT) and IGF-I, inadequate to the patient's sex and age. In the case of Cushing's disease associated with pituitary microadenoma, laboratory diagnosis is usually aided by tests of ACTH/cortisol suppression or stimulation, and in unclear cases, petrous sinus catheterization can confirm pituitary ACTH production. In Nelson's disease, the clinical assumption is confirmed by high ACTH serum levels.

Assessment of patients with expansive pituitary tumors includes a thorough ophthalmic examination with evaluation of visual fields.

The treatment of choice for pituitary adenomas is almost invariably surgical (Fig. 8), except for prolactinomas. Such cases are initially treated with dopaminergic agonists, which may lead to normalization of prolactinemia, tumor regression (Fig. 9), and recovery of the visual and neurological condition, depending on tumor expansion. Surgery for this type of tumor is indicated only for cases that do not respond to clinical treatment. For other adenoma types, surgery, either transsphenoidal or transcranial, depending on the extension of the tumor, is indicated. Compressive symptoms almost invariably improve following such treatment. Complete tumor resection will depend on the surgeon expertise and adjacent structures invasion.

In functioning adenomas, persistence of hormonal hypersecretion reveals incomplete tumor resection and further need of adjunct therapy. In clinically nonfunctioning adenomas, MRI after surgery will show the presence of tumor remnants. In the case of incomplete tumor resection, irradiation has been the most used treatment. In acromegaly, the use of somatostatin analogs may lead not only to reduction or normalization of GH and IGF-I, but also to tumor reduction; therefore, such agents can be used as primary treatment in patients when surgery is contraindicated or following partial surgical resection, either associated or not with radiation therapy.

Surgery will allow the histological diagnosis, and, by means of immunohistochemistry or in situ hybridization, the functional diagnosis of the tumor. Clinically nonfunctioning adenomas may express hormones, mainly gonadotropins.

Hypopituitarism treatment, of both pituitary and hypothalamus origin, should be carried out with proper hormonal replacement, as dealt with in a further chapter.

2.8. Metastatic Tumors

Metastases to the hypothalamus-pituitary region occur in approximately 3.5% of cancer patients. The posterior pituitary gland is the prevalent location for such metastases, probably because of their systemic circulation-dependent irrigation. Diabetes insipidus is the first manifestation in 70% of cases. Malignant tumors that mostly metastasize to this region are: breast (47%), lung (19%), gastrointestinal tract (6%), and prostate (6%) tumors. Figure 10 shows the CT of a patient with breast cancer and sellar and suprasellar

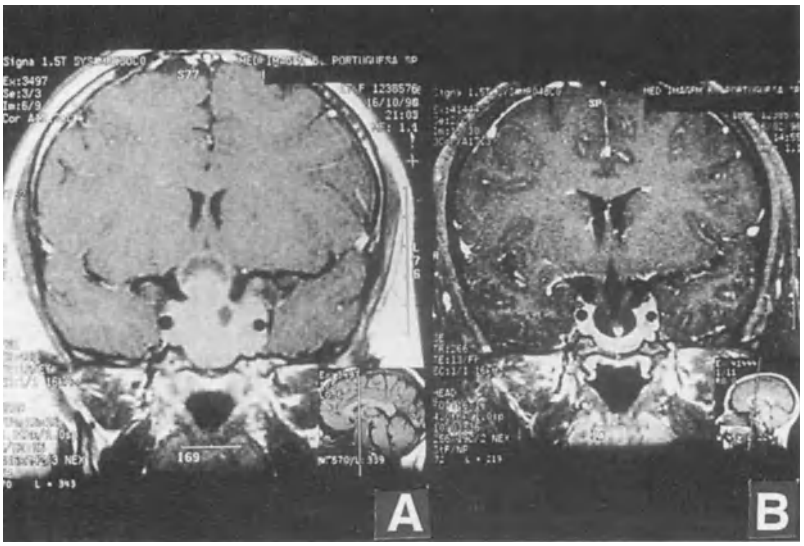


Fig. 9. Macroprolactinoma treated by bromocriptine. Coronal view of MRI (T1 weighted). **(A)** Before treatment: showing an huge invasive macroprolactinoma with suprasellar and parasellar extensions and sphenoid sinus invasion. **(B)** During treatment: note near complete disappearance of tumor mass with secondary empty sella.

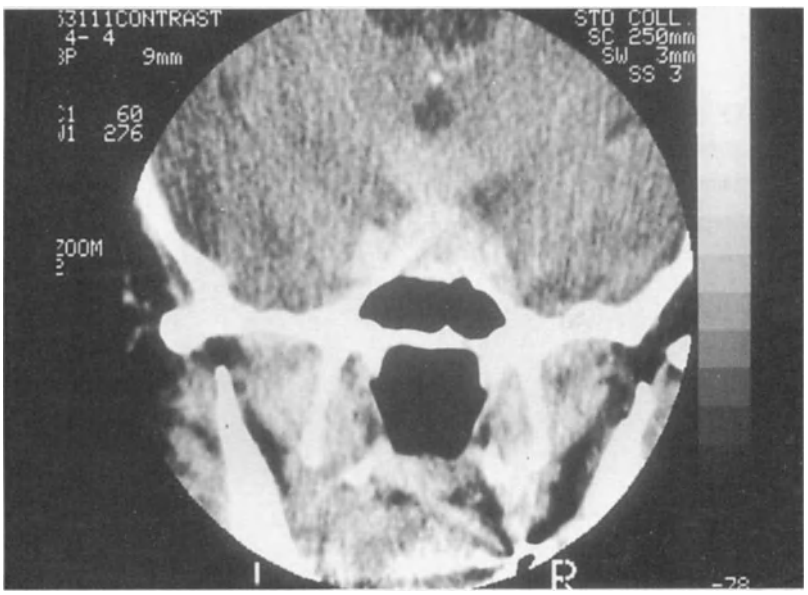


Fig. 10. CT scan coronal view of a breast metastatic tumor to the sellar region in a 42-year-old female patient.

metastases. Up to 25% of women with metastatic breast cancer have pituitary metastasis. Sometimes pituitary adenomas cannot be distinguished by means of the available imaging methods, but in other occasions bone destruction draws attention to the diagnosis of metastasis.

2.9. Dermoid and Epidermoid Tumors

Dermoid and epidermoid tumors are rare tumors, resulting from inclusion of epithelial elements at the time of neural tube closure. The epidermoid tumors are more common and contain a pearly fluid. The dermoid type contains dermic appendices such as hair follicles and sebaceous or sweat glands. Surgery is the treatment of choice.

2.10. Granulomatous/Inflamatory Diseases

2.10.1. Sarcoidosis

Idiopathic granulomatous disease, which may affect almost any organ. About 5% of cases involve the nervous system, 1.35% involve the central nervous system (CNS), and 0.53%, the hypothalamus-pituitary region, ranging from a mere meningeal thickening to actual circumscribed masses. The disease courses subacutelly or chronically, mimicking other granulomatous lesions or neoplasms There is no gender predominance and hilar adenopathy occurs in 66% of cases on chest roentgenogram; it may present with lytic lesions of the skull. Diabetes insipidus (37.5%) and cranial nerves palsy are common findings and,

sometimes, dysthermias, somnolence, hypodipsia, and obesity may also occur. Hypothalamic hypopituitarism is frequent. Cerebrospinal fluid shows a slight lymphocitic pleocytosis and moderate increase in protein content and gamma globulin. The glucose content is reduced in some patients. Sarcoid granulomas must be searched by biopsy in other tissues (uveal tract, skin, lungs, and bones). Some of the deficits improve with glucocorticoids. Prednisone, in daily doses of 40 mg is given for 2 wk, followed by 2-wk periods in which the dose is reduced until a maintenance dose from 10 to 20 mg is reached. Therapy should be continued for at least 6 mo, and in many cases for several years.

2.10.2. Histiocytosis X

Term referring to a group of histiocytic alterations, ranging from bone eosinophilic granuloma to disseminated, generally lethal, disease of soft tissues. Sometimes the course is that of an inflammatory disease, others that of a neoplastic one. In Hand–Schüller–Christian disease multifocal eosinophilic granulomas occur, often affecting the skull. It is the mostly involved condition considering hypothalamus-pituitary located lesions, and leads to the triad exophthalmos, lytic lesions in membranous bones, and diabetes insipidus, the latter occurring in about one third of patients. Some cases are associated with the presence of hypothalamic antibodies, probably as a result of hypothalamic lesion; hypopituitarism and hypodipsia are also found. Additionally, we can find enlarged lymph nodes, spleen, or liver. Biopsy for any suspecious lesion, specially in the skin must be performed. The assessment should be repeated 1–2 times annually if negative. Low-dosing radiation therapy, corticotherapy, and chemotherapy have been used with disease control, however, often with no regression of either diabetes insipidus or hypopituitarism.

2.10.3. Tuberculosis

Hypothalamic *tuberculomas* are tumor-like masses of tuberculous granulation tissue that may produce symptoms of a space-occupying lesion. It rarely occurs in the majority of the countries, but in some underdeveloped countries they constitute from 5 to 30% of all intracranial mass lesions. Because of their proximity to the meninges, the spinal fluid often contains a small number of lymphocytes and increased protein, but the glucose level is often normal, unlikely of tuberculous meningitis, which presents with reduced spinal fluid glucose. Treatment includes isoniazid, rifampin, and a third drug, which may be ethambutal or pyrazinamide. Under the influence of these drugs, the *tuberculoma* may decrease in size and ultimately disappear; otherwise, excision may be necessary.

2.11. Radiation-Induced Lesions

In the short term, brain irradiation may lead to vomiting, drowsiness, and focal motor signs. Later lesions occur more often 1–3 yr following applications, sometimes appearing much later. Intellectual deterioration, leukoencephalopathy, and endocrinopathy are included. The pituitary, as compared to the hypothalamic tissue, is more radiation-resistant. Brain irradiation before two years of age almost invariably leads to endocrinopathies, which are less common when the procedure is carried out after 11 yr of age. Adults are less likely to develop hypopituitarism after radiation therapy as compared to children and adolescents. Hypothalamic injury is proportional to the radiation dose used, and complications are reduced with greater fractionation of the total dose; endocrine insufficiency is time-dependent.

Radiation-induced tumors, mainly of the malignant glioma type, although rare, are often fatal.

2.12. Traumatic Lesions

The hypothalamus is a region vulnerable to lesions secondary to severe brain trauma. Necropsy studies in subjects who underwent fatal brain trauma have shown lesions of the posterior pituitary gland, pituitary stalk, and anterior pituitary gland, as well as capsular bleeding. Both microscopic and macroscopic hypothalamic hemorrhage were found in 42% of the examined corpses. The typical hypothalamic trauma patient is young, male, victim of a car accident, whose first shock organ is the head. Such patients usually suffer a frontal, temporal, or skull base fracture, and show clinical evidence of lesion of the second, third, fourth, and sixth cranial nerves, with a frequently prolonged coma. Diabetes insipidus may be associated with the clinical setting in the acute phase. Anterior pituitary hormonal deficits may appear some weeks or months following trauma and are often underdiagnosed. In the acute phase of prolonged coma, dysthermias and hemodynamic instability occur more often, frequently aggravated by dehydration resulting from polyuria, increase in insensitive losses because of hyperthermia or even an adrenal failure secondary to damaged CRH production cells. In case of hypotension, corticosteroid agents should always be administered. Hormonal deficit, which may

occur in hypothalamic trauma, as a rule follows the usual sequence of events, the somatotrophic sector being most commonly affected, followed by the gonadotrophic, thyrotrophic, lactotrophic sectors, and, finally, the corticotrophic axis. Precocious puberty secondary to hypothalamic trauma has been described and may distort a GH-deficit condition in childhood.

3. HYPOTHALAMIC SYNDROMES

3.1. Dysthermias

3.1.1. Hyperthermia

Acute lesions of the anterior hypothalamus and preoptic region may result in an up to 41°C (105.8°F) temperature rise, tachycardia and loss of consciousness, usually for less than 2 wk. The mechanisms of heat production are maintained, whereas those of heat dispersion show failure. A clinical clue to differentiate infectious fever from inflammatory fever is a less accelerated heart rate in the hypothalamic hyperthermia. In case of highly elevated hyperthermia, we should consider the malignant neuroleptic syndrome, more related to drugs of higher antidopaminergic potency. Muscle contractions occur and may lead to rabdomyolisis, and as a result, high serum CPK levels and renal failure. A possible aggravating factor of this syndrome is the inhibition of sweating, owing to anticholinergic effects of neuroleptics. The conscience level ranges from agitation and stupor to coma. It lasts from 5 to 10 d, killing 20–30% of the affected patients.

Chronic hyperthermia develops in lesions of the tuberal-infundibulum region. They may result from loss of heat dispersion mechanisms, stimulation of the maintaining mechanisms, or increase in the activation threshold of dispersion mechanisms. The general health status is often maintained and long-term hyperthermia occurred in 10% of Bauer's cases. It may respond to sedative or anticonvulsive agents, but not to salicylates.

3.1.2. Hypothermia

Hypothermia can occur in either a continuous or a paroxysmal way. The condition results from injury to heat-producing mechanisms or establishment of a newer, lower set-point. The continuous form is rare and may be because of anterior hypothalamus injuries. Patients usually maintain the heat dissipation mechanisms. In paroxysmal cases, crisis of a fall in body temperature may occur, whose frequency may range from daily up to a decade-interval. The onset is abrupt with no triggering event or environmental change. Duration ranges from minutes to days, and body temperature may fall to 32°C or lower. Tearing, asterixis, ataxias, cardiac arrhythmias, arterial hypotension, hypoventilation, and loss of consciousness can occur. The model is that of a lower set-point. Hypothermia has been associated to third ventricle steatoma, gliosis, and cellular loss in the premamillary and arcuate nucleus regions, and corpus callosum agenesis.

Although several features of paroxysmal hypothermia suggest an epileptic crisis modifying the hypothalamus thermostat, most patients fail to respond to anticonvulsants.

3.1.3. Poikilothermia

Poikilothermia is defined as a body temperature oscillation of at least 2°C on account of environmental temperature changes. In this dysthermia, found most frequently in humans, mechanisms of both heat production and heat dissipation are damaged. Its severity varies, according to the type and degree of the hypothalamus lesion.

Poikilothermia results from destruction of posterior hypothalamus or rostral midbrain. Hypothalamic lesion should be bilateral in order to poikilothermia to occur. Many patients fail to realize their condition and show no signs of discomfort. As room temperature is usually lower than normal body temperature, patients are hypothermic most times, and only children (of a higher metabolism) are rarely hyperthermic in warm environments.

3.2. Feeding Disorders

Human beings maintain the same body weight for years. Should a small caloric gain or loss occur daily, in a very short time, we would have deep weight variations. Somehow, signs that modulate either food intake or metabolism are sent, however, there is a difference between food intake and temperature regulation. Temperature is very similar among individuals, whereas body weight is quite different and is, to a larger extent, influenced by other factors such as stress, food appreciation, exercise and several other environmental and genetic factors. The control of food intake might act not as a single set-point, but as several set-points coexisting. Such control can be helped by the fat storage mechanism, in which the larger the amount of nutrient intake, the lower the food conversion into fat, maintaining a stable storage even with a nutrient intake variation. However, if

such intake is persistently high, the mentioned set-point may rise.

Caloric expenditure also tends to be quite stable, as shown in studies of animals on *ad libitum* diet. The animals may gain weight when feeding is imposed or when extremely tasty food is provided, and they may loose weight if calories are limited. Under such conditions, the relation between energetic expenditure and body weight changes: slim animals require fewer calories to keep their weight and the opposite occurs with the heavier ones.

Food intake seems to be controlled by two hypothalamic nuclei: ventromedial nucleus, whose destruction leads to hyperphagia, and lateral hypothalamus, which causes aphagia when destroyed. Based on such findings, the former is believed to be the nucleus of satiety and the latter, the nucleus of hunger. There are, however, controversies as to the existence of simple nuclei controlling specific functions in the hypothalamus.

Several humoral signs such as blood glucemic levels, insulin, glucagon, serotonin, cholecystokinin, neuropeptide Y, and leptin, seem important in the regulation of the feeding behavior, and are discussed further in this book.

Although the compulsive eater stands for an acknowledged clinical disorder, actual hyperphagia has been well documented in hypothalamic lesions.

3.2.1. Hyperphagia and Obesity

Mohr in 1840 first described hypothalamic lesions causing obesity and Hogner further localized the critical zone to the basal hypothalamus. Hyperphagia and obesity affect 25% of patients with hypothalamic disease, although rarely as initial manifestations of hypothalamic dysfunction. Reeves and Plum described a young woman who developed marked hyperphagia and obesity associated with aggressive behavior placated only by giving an 8000 kcal per day diet. At autopsy, she was found to have a hamartoma precisely and completely destroying the ventromedial hypothalamus. A young man studied by Anderson was found to have only partial ventromedial destruction because of encephalitis, and he had developed mild obesity. Apparently, the degree of ventromedial destruction influences the degree of obesity in man and in animals. The bilateral destruction of ventromedial nuclei causes obesity both in human beings and in other animals. Patients with such lesions usually have CNS neoplasms, 60% of which being craniopharyngiomas. Only 6% have inflammatory or granulomatous processes, 5% posttrauma lesions, and 2% leukemic infiltration. Hyperphagia and obesity are also associated with syndromes as Laurence–Moon–Bardet–Biedl and Prader–Willi.

As already mentioned regarding hyperthermia, hypothalamic hyperphagia is followed by other symptoms related to disorders of this region, such as diabetes insipidus, drowsiness, convulsions, hypodipsia, and anterior pituitary hypofunction. Antisocial behavior and improper aggression are usually shown in hypothalamic obesity.

In some cases, the satiety set-point seems to be reset. This occurs more often following head injury, and an exaggerated gain in body weight occurs in the first six months, with a trend towards late stabilization and, sometimes, loss of weight, reaproaching pretrauma level. The syndrome can also result from tumors in the region, which, when enlarged, may worsen a preexistent hyperphagia. What establishes the new set-point remains unexplained. When one encounters a patient with hypothalamic obesity, the presence or absence of hyperphagia at that particular point in time depends on whether or not the subject has attained his new set-point body weight. Some authors have commented on a tendency toward centripetal fat accumulation with true hypothalamic obesity. These patients have a more severe hyperinsulinemia when compared to the exogenous obese of same weight, probably a result of vagus liberation, already documented in animals with ventromedial nucleus injury.

3.2.2. Hypothalamic Cachexia

Only few adults have shown the association of cachexia with other hypothalamic symptoms. Kamalian described a woman who had a progressive wasting illness accompanied by hypophagia, but in spite of high-caloric tube feedings she continued to lose weight. At autopsy, the underlying illness of multiple sclerosis had produced both new and old plaques involving the lateral hypothalamic regions. In Bauer's series, 18% of cases had significant weight loss, 8% bulimia, and 7% anorexia. The destruction of the ventromedial nucleus and the lateral hypothalamus leads to anorexia, which also occurs in isolated lateral hypothalamic lesions. Rapid weight loss, muscular hypotrophy, activity reduction and hypophagia occur, leading to cachexia and death; it is more often the result of neoplasms.

3.2.3. Kleine–Levin's Syndrome

Kleine–Levin's syndrome is most common in male adolescents, and is characterized by repeated episodes

of hypersomnia, hyperphagia, hiperactivity when awake, and behavioral disorders, particularly hypersexuality. Compulsive eating is not necessarily linked to bulimia. Crises last from days to weeks, and resolve with no sequelae. During the symptomatic period the night sleep is of poor efficiency, sleep is fractionated and 3, 4, and REM (rapid eye-movement) sleep stages are reduced. A 24-h hormonal sampling performed every 20 min has shown elevation of TSH and prolactin mean values and reduction of GH and cortisol in the symptomatic period, which strengthens the theory of dopaminergic tonus reduction in such patients. The etiology is unknown, however, some patients have a previous viral infection. The disease usually remits in the third decade of life.

3.2.4. Anorexia Nervosa

There is evidence supporting a hypothalamic dysfunction component in this disorder. Patients may present with hyperprolactinemia, poikilothermia, diabetes insipidus, and hypothalamus-anterior pituitary dysfunction described below. It most often affects middle or upper class young women, who have a distorted self-image, considering themselves as obese, and making exaggerated feeding restriction, many times associated with an intensification of physical exercises. They commonly develop a bulimic behavior, vomiting after meals; other times, they take diuretics or cathartic agents on their own.

The endocrine abnormalities are diverse, with amenorrhea as major prevalence, sometimes preceding weight loss, and persisting even after the reestablishment of normal weight. Gonadotropins are hyposecreted, both spontaneously and following GnRH stimulus. After weight reestablishment, the patients develop a "second puberty," with nocturnal LH pulses and reappearance of gonadotropin release to GnRH.

The serum level of IGF-1 is often low and the basal GH may be normal or high, as in the undernourished. A paradoxical response to both glucose and TRH may be present, with normal response to GHRH. Such abnormalities revert to normal with weight reestablishment.

As far as thyroid function is concerned, such patients behave as in the syndrome of euthyroid sick disease, and the physician should be aware for the differential diagnosis with true hypothyroidism, because thyroid reposition in anorectic patients is not recommended.

There is evidence supporting a reduced cortisol clearance, with high serum levels leading to low ACTH levels. There may be no response to 1 mg dexamethasone suppression and hyporesponsiveness to CRH injection. These abnormalities found in the dynamics of the hypothalamus-pituitary-adrenal axis are also found in patients with major depression and whether such hypothalamic abnormalities reflect a hypothalamic etiology of the syndrome or whether these manifestations are mere epiphenomena of a primarily psychiatric syndrome remains to be determined.

The treatment of anorexia nervosa patients aims, at first, at their renutrition, many times demanding hospitalization with enteral or even parenteral diet. An individual or group psychotherapy work is concurrently started, conducted by professionals having expertise in such a disease. Medical treatment lies basically in the use of antidepressive medications.

3.3. Sleep Disorders

3.3.1. Hypersomnia

Hypersomnia is the most common disorder, the prevalence in hypothalamic dysfunction being 30%. In acute lesions, the most involved regions are periaqueductal gray matter, mamillary bodies, and the activator reticular system. When the posterior hypothalamus is affected, hypothermia and irritability are associated. The most common causes of hypersomnia are neoplasias, mainly craniopharyngioma and germ cell tumors and about 40% of patients are also obese.

3.3.2. Insomnia

Insomnia may occasionally mean hypothalamic lesion in the anterior and preoptic region and sometimes (especially in hypothalamus cystic tumors) it is accompanied by daytime sleepiness. Lesions of the tuberal region may have clinical presentation similar to akinetic mutism.

3.4. Behavioral Disorders

Hypothalamus appears to affect behavior in three spheres: it coordinates the motor, autonomic, and endocrine components of behavior; it produces the behavior appropriate to the affective state; and it influences the intensity of each behavior. Behavior has a complex supra tentorial, limbic, and hypothalamic regulation. The latter seems to integrate the appropriate emotional expression. Aggressiveness, emotional lability, and destructive antisocial behavior form a spectrum of emotional disorders involved in ventromedial nucleus lesion. Although there are reports of hypersexual behavior with compulsive

masturbation, hyperphagia and hallucinations, most sexual dysfunctions of hypothalamic origin are of the hypogonadic type. The already described Klein–Levin syndrome, of which etiology is speculated to be a hypothalamic functional abnormality, affects mostly male adolescents with recurrent episodes of hypersomnia, annoyed wakenings, incoherent speech, hallucinations, forgetfulness, masturbation, and compulsive eating. These symptoms are accompanied by a feeling of indolence and headache. The episodes occur at intervals of 3–6 mo and last for 5–7 d, sometimes even for weeks. Spontaneous cure occurs quite often at late adolescence or early adult life.

3.5. Diencephalic Epilepsy

Convulsion hypothalamic etiologies are rare. Seizures include coordinated patterns of intense autonomic hyperactivity. During a typical crisis, the child stops his/her activities, starts laughing, groaning, and presents uni- or bilateral clonic movements of the ocular, eyelid, or mouth muscles. Consciousness is maintained, unless the crisis is followed by a major or *petit mal* convulsion. Gelasmus or laughter crises may be found in patients with tuberal hamartomas. These hamartomas, if totally excised, may be followed by complete control of the crisis. Partial excisions usually fail to lead to the same result.

3.6. Authonomic Dysfunction

Stimulus of the suprasympathetic region in the anterior preoptic hypothalamus leads to a vagus response with miosis, hypotension, and bradycardia, and an increase in the visceral blood flow to the disadvantage of a lower muscular blood flow. Yet, when the postero-medial hypothalamus, area of the sympathetic nervous system, is stimulated, a typical fight and flight reaction occurs with mydriasis, tachycardia, and tachypnea, and an increase in arterial tension, hair erection, and visceral blood flow reduction and muscle blood flow increase, as if preparing the subject to fight or flight.

The autonomic dysfunction secondary to hypothalamic lesions may lead to symptoms such as lower threshold to cardiac arrhythmia, arterial hypertension, gastric and duodenal erosions and hemorrhages (there are reports of gastric acidity and pepsin increase in anteromedial hypothalamus lesions) and, rarely, acute pulmonary edema.

3.7. Diencephalic Syndrome of Childhood

Described by Russell in 1951, this syndrome occurs in about 80% of low-grade gliomas of the hypothalamus or optic paths. Children seem normal at birth, but at the end of their first year, they start losing weight and showing hyperactivity signs, with no growth impairment. They seem to be always alert because of eyelid retraction. Although appetite is maintained, nystagmus, vomiting, tremors, and optic atrophy may evolve. The night–day cortisol cycle is lost and a paradoxical GH response to glucose load may develop, as well as high basal levels. The children usually die before two years of age, but those who survive longer maintain appetite, gain weight and become obese. The pleasant personality is gradually replaced with irritability and drowsiness and precocious puberty may develop.

3.8. Diencephalic Glucosuria

Hyperglycemia and glucosuria may follow hypothalamic lesions in the tuberal-infundibular region, mostly reported following basal skull fractures, intracerebral hemorrhages, or surgeries involving the third ventricle floor. In patients with skull trauma, high-glucose levels are a relatively common finding, secondary to factors leading mainly to insulin resistance, such as stress, infections, dexamethasone use, infusion of rich glucose parenteral solutions. However, lesions in the above mentioned regions seem to pose a greater risk to hyperglycemia development.

3.9. Thirst and ADH Secretion Disorders

The control of plasmatic osmolality and its major determinant, plasma sodium, results mainly from mechanisms of water conservation, mediated by ADH, and from thirst-induced water ingestion. Therefore, disorders that affect fluid balance are the main responsible for alterations of plasmatic osmolality and cause hyper- or hypotonic syndromes. The clinical manifestations of these disorders are consequences of alterations of cellular volume, particularly at the CNS level and of effective circulating volume.

3.9.1. Essential Hypernatremia and Adipsic Hypernatremia

Hypernatremia in adults rarely occurs because of excessive sodium ingestion. By and large it is secondary to a deficiency in water ingestion. In the absence of major liquid losses, chronic hypernatremia in conscious individuals with free access to water is a result of inappropriate lack of thirst.

Essential hypernatremia is secondary to thirst decrease associated to a readjustment towards higher osmotic threshold. Therefore, these patients are able to concentrate and dilute urine at an osmolal level

higher than that usually observed, and unlike patients suffering from adipsic hypernatremia, they are protected from extreme hypernatremia because they maintain some degree of osmoregulation.

In adipsic hypernatremia syndrome, thirst deficiency is caused by alterations of osmoreceptors and it is commonly associated to defects in the osmotically regulated ADH secretion. These patients present secretion of a small amount of ADH not related to plasmatic osmolality and may be exposed to both hypo- and hypernatremia.

3.9.2. Diabetes Insipidus (DI)

The deficiency of ADH secretion or of its renal action leads to decreased urinary concentration ability and excessive excretion of urine. The resulting syndrome, manifested by polyuria and polydipsia, is known as *Diabetes Insipidus* and may have different etiologies as shown on Table 4.

3.9.2.1. Etiologies: Central Diabetes Insipidus. Known as neurogenic, cranial, or hypothalamohypophyseal DI, it is manifested only when there is involvement of at least 80% of ADH-secreting hypothalamic neurons. The removal of the neurohypophysis may not result in DI, because proper amounts of ADH may be released to systemic circulation from the stump of the hypophyseal stem. This explains why tumors in the anterior pituitary compressing the neurohypophysis are rarely concurrent with DI.

The hypothalamic involvement in DI accounts for the largest number of cases. The estimated incidence of central DI is 1:25,000 cases, and occurs equally in both sexes.

Familial neurohypophyseal DI is a rare autosomal dominant disorder, linked to AVP-neurophysin gene mutations. Symptoms appear at birth or some years later and circulating ADH levels may temporarily vary from undetectable to normal. Some cases of sporadic congenital central DI have already been described.

The association of central DI with *Diabetes Mellitus*, optical atrophy, and neurological deafness (DIDMOAD) is the clinical picture of the Wolfram syndrome, an autosomal recessive condition.

The idiopathic central DI, which accounts for approximately 30% of the acquired central DI cases, has its onset in infancy. This diagnosis may be made after excluding organic lesions of the hypothalamohypophyseal region. Patients suspected to have this diagnosis must be periodically followed-up in order to detect intracranial lesions that may appear many years after the onset of the clinical picture of DI. The presence of ADH-secreting antineuron antibodies is observed in approximately one-third of these patients.

Brain lesions resulting from accidents or neurosurgical procedures are the most common cause of central DI. Some statistics have shown central DI as a complication of up to 75% of suprasellar surgeries for removal of craniopharyngiomas in children. In adults the incidence is lower. DI occurrence after transsphenoidal surgery is approximately 10–20%. Persistent DI develops only after a sufficiently high lesion in the supra-optico-hypophyseal tract, causing bilateral neuronal degeneration of supra-optic and paraventricular nuclei. In surgeries of the pituitary fossa, transitory DI may occur. Postoperative DI may present a three-phase course: (a) *acute phase:* characterized by polyuria right after surgery, persisting for four to five days; (b) *interphase:* characterized by regression of polyuria as a result of an autonomous release of ADH by degenerated neurons, and lasts for 5–7 d; and (c) *permanent DI:* polyuria is definitively established. It is important to recognize these three phases in order to prevent fluid intoxication that may occur during interphase if hypotonic fluid infusion, initiated in the acute phase, continues.

Several lesions in the hypothalamohypophyseal region are concurrent with central DI and must be investigated by means of neuroophtalmologic and neuroradiologic diagnostic procedures (Table 4). DI seldom occurs during normal pregnancy, secondary to a placental production of vasopressinase. Polyuria generally starts in the third quarter and spontaneously disappears in the immediate puerperal period.

3.9.2.2. Nephrogenic Diabetes Insipidus. Nephrogenic DI is secondary to hyporesponsiveness of the renal tubular cells to ADH action, resulting in renal excretion of persistently hypotonic urine in the presence of normal plasma or high ADH levels. It may result from different causes (Table 4).

3.9.2.3. Primary Polydipsia. Excessive water ingestion with total body water expansion and hyposmolality is the first event in primary polydipsia. The resulting drop in ADH plasma concentration causes urinary dilution and polyuria, thus protecting the individual from hyperhydration. Inappropriate thirst may occur because of a psychiatric disorder (psychogenic DI) or to an abnormality in thirst mechanism (dipsogenic DI).

In psychogenic DI, water ingestion may start suddenly and tends to fluctuate from one day to another.

Dipsogenic DI may occur secondary to diseases involving the CNS, at hypothalamus level, such as

Table 4
Etiology of *Diabetes Insipidus*

Central
Congenital
Autosomal dominant
Autosomal recessive (Wolfram syndrome)
Sporadic congenital DI
Acquired
Head trauma
Postoperative
Associated to injuries in the hypothalamohypophyseal region
Primary tumors: suprasellar cysts, craniopharyngiomas, astrocytomas, germinomas, meningiomas, hamartomas, hypophyseal adenomas, tumors of the hypophyseal stem, suprasellar tumors, gliomas
Metastatic tumors: lungs, breasts, leukemia and lymphomas
Granulomatosis: sarcoidosis, histiocytosis X, Wegener's granulomatosis, tuberculosis, syphilis
Vascular: sickle cell disease, cerebral aneurysm, vasculitis, cerebral trombosis or hemorrhage, Sheehan's syndrome
Other lesions: empty-sella syndrome, intracranial hypertension and infections
Pregnancy (transient)
Idiopathic
Nephrogenic
Familial
Acquired
Renal diseases: chronic renal failure, polycystic disease, acute tubular necrosis, after obstructive uropathy, post transplantation, chronic pyelonephritis, amyloidosis.
Systemic diseases with renal involvement: multiple myeloma, sickle cell anemia, sarcoidosis, Sjögren's syndrome, Fanconi's syndrome
Metabolic: hypocalemia and hypercalcemia
Drugs: lithium, demeclocycline, alcohol, amphotericin, glyburide, glibenclamide
Primary polydipsia
Psychogenic
Dipsogenic

sarcoidosis, tuberculosis, vasculites, and tumors, or it can be an idiopathic defect associated or not to abnormalities of ADH osmoregulation. A possible explanation for dipsogenic DI is the lowering of thirst threshold. Therefore, thirst remains responsive to osmotic influences, but it occurs in plasmatic osmolalities lower than those that usually trigger thirst in normal individuals.

3.9.3. Clinical Picture

3.9.3.1. Central and Nephrogenic Diabetes Insipidus. The primary symptom of DI is persistent polyuria (24-h diuresis higher than 30 mL/kg), and urine is hyposmolal in relation to plasma (uOsm lower than 300 mOsm/kg or urinary density lower than 1010). The excreted volume of urine in a 24-h period may vary from few liters, in the cases of partial deficiency in ADH secretion or incomplete resistance to ADH action in the kidney, to a maximum of 18 L, in absolute ADH deficiency or ADH complete resistance conditions. Nycturia is almost always present. The symptoms of hypertonic dehydration, irritability, mental confusion progressing or not to coma, hyperthermia, and hypotension occur if access to water is not ensured, such as in the cases of hypodipsia because of impairment of thirst osmoregulation or in cases of unconsciousness.

Most of central DI cases present polyuria of acute onset; on the contrary, polyuria secondary to alterations of the renal mechanism of urine concentration is insidiously established. The major dilation of the urinary tract may progress to hydroureter and hydronephrosis in patients with DI with onset in childhood. Finally, primary neurological symptoms in individuals with DI because of intracranial lesions may be prominent.

3.9.3.2. Primary Polydipsia. The primary symptom is polydipsia together with polyuria. The polydipsia of psychogenic etiology may be episodic. Diuresis may exceed 18 L in a 24-h period and nycturia is almost never present.

3.9.4. Differential Diagnosis of Polyuria

DI must be differentiated from diabetes mellitus and other forms of diuresis due to excess of solute. Hyperglycemia and glycosuria confirm the diagnosis of diabetes mellitus. Clinical history is essential in order to identify other causes of diuresis due to excess of solute, as for example, recovery from acute renal failure, postobstructive diuresis or after solute administration as sodium, mannitol, or contrasts.

The differential diagnosis among the three types of DI is relatively simple when there is complete involvement. Thus, for example, a polyuria developed after pituitary surgery and decreased with ADH administration does not need further tests in order to confirm the diagnosis of central DI. Nevertheless, the clinical picture quite often does not help in the differential diagnosis because the syndrome occurs either with incomplete abnormality or associated with diseases that may cause different subtypes of DI. For instance, tuberculous meningitis may be associated with central and dipsogenic DI, and sarcoidosis may cause the three types of DI (central, nephrogenic, and dipsogenic). Some psychiatric patients presenting psychogenic DI may be taking lithium or may have suffered cranioencephalic traumatism. In such cases, it is necessary to use other criteria to establish DI diagnosis.

Diagnostic evidence is based on renal capability to excrete hypertonic urine after osmotic stimulus. The simplest way to produce hypertonicity of body fluids is fluid restriction. The absolute value of urinary concentration obtained through this test depends on the presence of ADH, and on the capability to stimulate renal cells sensitive to ADH and on the hypertonicity level of renal medula.

In patients with mild polyuria, water deprivation may start in the night before the test. However, in order to be observed, patients with severe polyuria are submitted to water restriction only on the day of the test. During the assessment, fluid ingestion is completely suspended and patients are prevented from smoking.

Weight and vital signs (blood pressure and heart rate) are recorded every hour, and urine and blood samples are collected to determine uOsm, pOsm, and electrolytes. When two consecutive values of uOsm vary less than 30 mOsm/kg or when there is loss of 3–5% of body weight, 5U of aqueous pitressine or 1 μg of desmopressine (dDAVP) is administered by SC or IV route when the patient has already emptied all bladder content. Sixty minutes later another sample must be collected to determine uOsm. This test must be interpreted as follows.

In normal individuals fluid deprivation results in uOsm 2–4 times greater than pOsm and the subsequent exogenous administration of ADH results in an increase lower than 9% in uOsm. Endogenous ADH levels are high and cause maximum antidiuresis.

Patients with primary polydipsia who present medullar hypotonicity may discretely concentrate urine after water restriction. However, these patients present maximum increase in endogenous ADH and an increase lower than 9% in uOsm after exogenous ADH. Patients with primary polydipsia who decrease their water ingestion and receive dDAVP some days before the test may restore medullar hypertonicity and present normal response.

Patients with complete central DI do not present an increase in uOsm higher than pOsm during fluid restriction, but they respond to exogenous ADH administration with an increase higher than 50% in uOsm. Those patients with partial central DI may present some degree of increase in uOsm during water restriction, however increase of uOsm of at least 10% after ADH administration is also observed.

Finally, the water restriction test in individuals with complete nephrogenic DI does not cause an increase in uOsm higher than pOsm, even after ADH administration. In the partial defect, exogenous ADH administration results in some increase in uOsm.

Many times water restriction test does not make the discrimination of the three DI types possible, because the percentages of uOsm increase after exogenous ADH administration may not differentiate primary polydipsia from partial defects of secretion or action of endogenous ADH.

DI diagnosis may also be established by means of therapeutic test with AVP or its analogue dDAVP. Thirst abolition, polydipsia, and polyuria triggered by administration of these compounds with no excessive fluid retention suggest diagnosis of central DI. On the other hand, in nephrogenic DI, absence of effect of these hormones in normal doses is observed. Nevertheless, therapeutic tests may bring some difficulties in its interpretation when some patients present abolition of polyuria with delayed or not very potent effect over thirst and polydipsia. In these cases, there is excessive fluid retention and development of hyponatremia and hyposmolality.

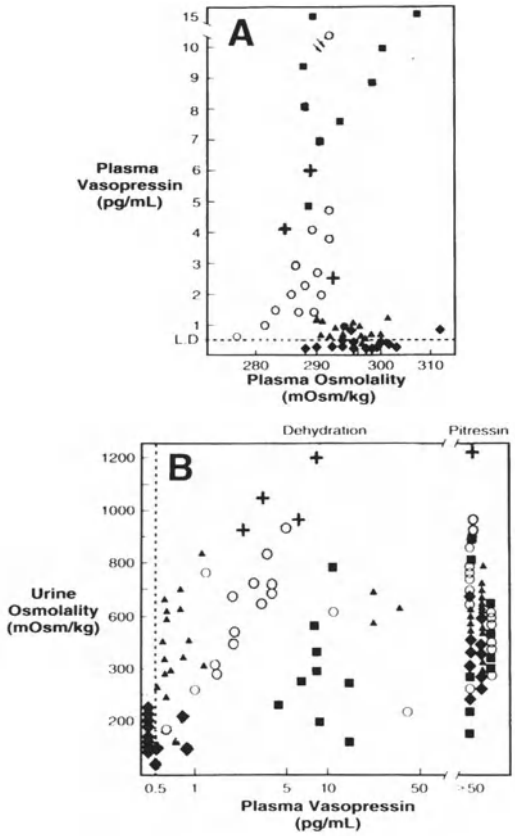


Fig. 11. Diabetes insipidus. Relationship of plasma vasopressin to plasma osmolality **(A)** or urine osmolality **(B)** during fluid deprivation/hypertonic saline infusion in patients with central (▲ or ◆), nephrogenic (■), or dipsogenic (○). (From Robertson GL. Diabetes insipidus. In: Dluhy RG, ed. *Clinical Disorders of Fluid and Eletrolyte Metabolism: Endocrinology and Metabolism Clinics of North America*, vol. 24. Philadelphia, PA: W. B. Saunders, 1995; 3:566 [**waiting for permission of W.B. Saunders Company**].)

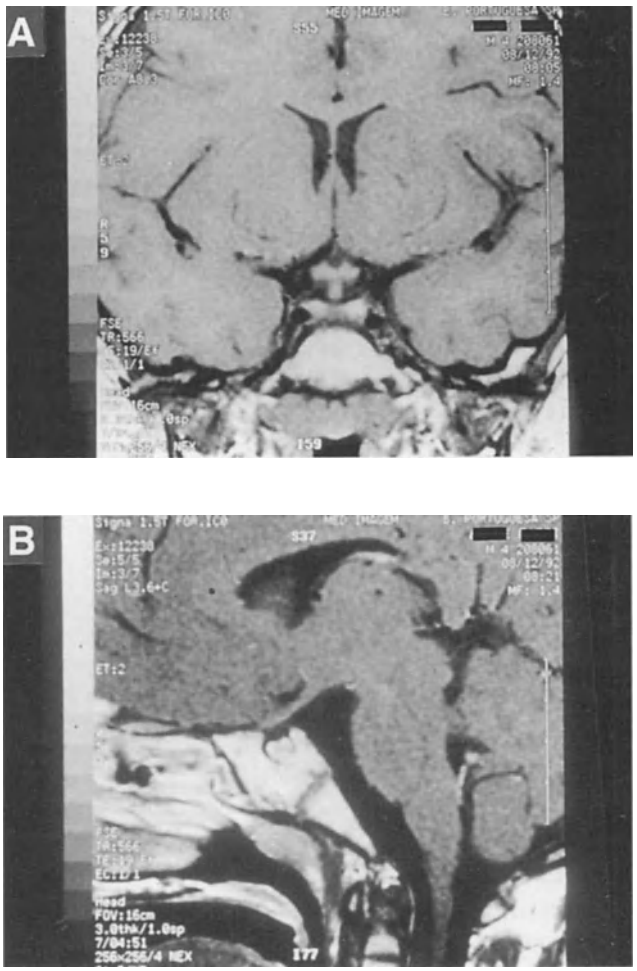


Fig. 12. A seven-year-old boy with central diabetes insipidus. **(A)** MRI coronal view (T1 weighted) showing a thickened stalk. **(B)** MRI sagital view (T1 weighted) with disappearance of the typical posterior pituitary signal.

The quantitative determination of plasmatic ADH concentration related to pOsm and uOsm may improve accuracy of the differential diagnosis of the different types of DI (Fig. 11). Normal or high ADH concentrations in the presence of diluted urine suggest nephrogenic DI. Basal concentrations, below 1 pg/mL impair the establishment of diagnosis and it is necessary to determine ADH during water restriction test. In central DI, ADH is inappropriately low in relation to pOsm, whereas in primary polydipsia (as well as in nephrogenic DI) the ADH/pOsm ratio is normal. The infusion of saline solution may be used in order to study ADH release in patients with no limitations of cardiac reserve. This procedure consists of measuring pOsm and ADH plasmatic concentration during infusion of hypertonic saline solution (3%), at 0.1 mL/kg/min, during two hours. Patients with primary polydipsia or nephrogenic DI present ADH release in response to hypertonicity. Patients with central DI do not release ADH or do it inappropriately.

Recently, the urinary excretion of aquaporine 2 has been suggested as a tool for the differential diagnosis of DI. In central and nephrogenic DI, aquaporine 2 excretion was shown to be low and increases after AVP administration, only in the former.

Another important tool in the differential diagnosis of DI is the absence of the typical T1 weighted hyperintensity of neurohypophisis in central DI (Fig. 12 B).

3.9.5. Treatment

3.9.5.1. Central DI with Preservation of Thirst and Consciousness. When treating polyuria in these cases our aim is to give comfort to the patient, to improve his/her life quality and to prevent urinary tract distention. Nontreated children may have sleep and feeding disor-

ders that result in growth delay and learning problems. Water must be always handy, however, the patient must not be forced to drink if not thirsty.

The treatment of choice is desmopressin (dDAVP), a synthetic nonapeptide: 1-deamin-8-D-arginine vasopressin. There is much individual variation concerning required dosage and administration interval, which seems to be mostly independent on the size of the patient. The administration of dDAVP depends basically on the measured 24-h urinary volume and on the therapeutic regimen used. We should first prescribe the lowest possible dosage to make the patient slightly polyuric (2–2.5 L/24 h), and to reduce the risk of water intoxication, especially in posttrauma or postoperative DI, in which inappropriate secretion of ADH may follow DI within a few days. In adults, this is easily obtained through nasal dosages of 2.5–5 μg (0.025–0.05 mL) every 12 h, or 0.1–0.2 mg of oral preparation, twice or thrice a day. The maximum intranasal dosage should not exceed 40 μg and in children the initial nasal dosage depends on age: newborns: 0.25 μg; infants: 0.5–1.0 μg and children: 2.5 μg. If the first dosage does not produce a proper antidiuretic effect for at least 8 h, dosing will be gradually increased.

During follow-up, the need to increase dosage or to shorten interval will depend on monitoring of 24-h diuresis. Side effects such as abdominal cramps, headache and nasal congestion are rare.

Although dDAVP is the drug of choice, there are alternative drugs in some cases mainly if 24-h diuresis is lower than 4–5 L. Three drugs have been used to increase ADH effect—chlorpropamide, carbamazepine, and clofibrate. Chlorpropamide is the most efficient, alone or combined with dDAVP, in 125–500 mg dosage. Its major inconvenience is the risk of hypoglycemia, especially if the patient suffers from hypopituitarism. Even if properly treated, the patient must remain under observation because hypoglycemia may occur again in the same day because of long half-life of the drug, that is 36 h. Carbamazepine may cause a severe adverse effect, aplastic anemia; however, if the patient needs an anticonvulsant in the postoperative period of a neurosurgery, carbamazepine must be considered. Clofibrate is usually not very efficient. Other analog substances, such as pitressin and lysine-vasopressin, are no longer used because of side effects, half-life, and pain at administration.

3.9.5.2. DI with No Preservation of Thirst or Consciousness. If the patient is confused, in coma, or recovering from anesthesia, the main step to be taken is to establish a strict control of fluid gains and losses, because in DI the major loss is free water. The fluid balance must be performed, including insensitive losses and gain of endogenous water, based on the last serum sodium level measured, which, if high, indicates a need of water replacement by gavage or as a glucose solution at 5%. In these cases, serum sodium must be determined two or three times in 24 h.

The treatment of patients with essential hypernatremia depends basically on an increase in fluid ingestion, but the control of patients with adipsic hypernatremia is extremely difficult. The administration of a set fluid ingestion results in wide fluctuations in plasmatic osmolality because of daily variations of fluid losses. In these cases, fluids should be ingested according to modifications in body weight. If ADH secretion is insufficient, a fixed dosage of dDAVP must be administered. Sodium and plasmatic osmolality, if possible, must be regularly checked in order to ensure not wide fluctuations in fluid balance.

3.9.5.3. Inappropriate ADH Secretion. A prospective study demonstrated prevalence of hyponatremia (serum sodium <130 mEq/L) up to 2.5% in hospitalized patients. Hyponatremia is not only frequent, but also it is associated to a high level of morbidity and mortality when symptomatic. Some authors point out that such hyponatremia is because of an increase in total body water content, dependent on increase in fluid ingestion when ability to eliminate water is reduced. The suppression of ADH secretion avoids hyponatremia in normal subjects who ingest up to 18 L of water within 24 h, and the kidneys eliminate all exceeding water as maximally diluted urine. However, a syndrome of inappropriate secretion of ADH (SIADH) is characterized by maintained ADH release usually not related to osmotic stimulus and fluid ingestion. The patient is either normal or hypervolemic.

Situations not defined as SIADH are related to release of ADH by adequate nonosmotic stimulus, such as in hypovolemia of adrenal failure and hypothyroidism or in hypervolemia with damage to arteriolar filling in severe heart failure and cirrhosis. It is worthwhile to remember that these situations are frequently associated with lower sodium supply to diluting segments of Henle loop.

Such requirements may be found in hyponatremic patients because of the use of certain drugs that may directly stimulate secretion of ADH or potentialize its action in the collecting tubule, resulting in SIADH. This situation may occur during treatment with many

cytostatic, anesthetic, analgesic, and antidepressive drugs and in the treatment of diabetes mellitus with chlorpropamide. Vincristin and cyclophosphamide are drugs that may cause these side-effects, regardless of nausea induced by them. Hyponatremia resulting from diuretics may be a physiological response of ADH secretion to hypovolemia.

3.9.6. Pathophysiology

Administration of ADH, together with fluid ingestion with no restrictions, lead to hyponatremia, urinary concentration, antidiuresis, and weight gain of about 3 kg. Approximately three days later, body weight and sodium concentration are closer to a steady state and natriuresis, the so-called sodium escape, occurs. High levels of natriuretic atrial peptide also contribute to natriuresis. When fluid restriction is prescribed, hyponatremia is usually corrected, body weight is reduced and urinary sodium excretion is decreased, even if ADH administration persists. Therefore, we may conclude that natriuresis, observed after three days of ADH administration, results from volume expansion related to fluid retention, with reduction in salt reabsorption in the proximal tubules. It is likely that with the chronic use of ADH there is a partial escape in response to action of the drug. Because of sodium depletion, natriuresis lowers to sodium ingestion level, and in this phase of SIADH urine may be poor in sodium. Because secretion of aldosterone is stimulated by hyponatremia, the secretion of this mineralocorticoid may also contribute to reduce renal loss of sodium in patients with hyponatremia and expanded volumes. Hyponatremic patients with SIADH present a disorder in thirst osmoregulation because they continue to present thirst at levels of evident hyposmolality. There is also renal loss of substances such as uric acid, and its excretion varies directly with effective circulating volume and with sodium excretion rates. As a consequence, hypouricemia is frequent in SIADH.

An investigation about osmoregulation of ADH secretion in 25 patients, who satisfied the criteria above mentioned concerning SIADH and presented several diseases, showed that there are four different patterns of ADH release (Fig. 13). The first pattern (type A) is characterized by large fluctuations in plasmatic ADH concentration, which occurs in a completely randomized way and does not keep any relation to modifications in plasmatic osmolality. The A pattern accounts for approximately 35% of patients with SIADH. A second group, consisting of about one third of the cases, presents a readjustment in

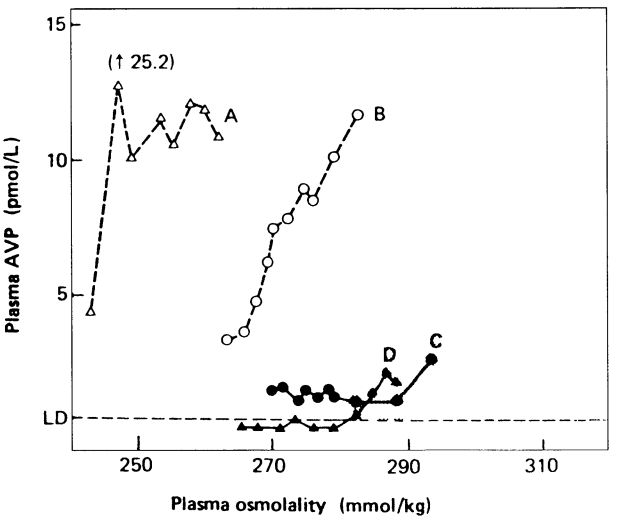


Fig. 13. Patterns of ADH response to hypertonic saline solution infusion in a group of hyponatremic patients with SIADH. A = erratic release; B = readjusted osmostat; C = ADH leakage; D = normoregulated ADH (From Baylis PH. Vasopressin and its Neurophysin. In: DeGroot LJ, ed. *Endocrinology*, vol 1. Philadelphia, PA: W. B. Saunders, 1989:224 [**waiting for permission of W.B. Saunders Company**].)

osmostat to the left (type B). In these patients, ADH is responsive to modifications of plasmatic osmolality, but the threshold for ADH release and thirst is subnormal. These patients are able to perform osmoregulation of water excretion retaining the ability to dilute and concentrate urine in an osmolality that is lower than normal. Because similar shifts to the left are observed in hypovolemia and hypotension, we suspect the cause may be a lesion in the afferent baroregulating paths. In some cases, ADH secretion cannot be entirely suppressed and the hormone "leaks" in low plasmatic osmolalities (type C). However, when plasmatic osmolality is increased, there is a normal response of ADH release. The last group (type D), represented by less than 10% of the patients, has completely normal osmoregulated secretion of ADH. Nevertheless, patients meet SIADH criteria because they fail to excrete a certain water load and are unable to maximally dilute urine. It is not known whether this abnormality is because of increased renal sensitiveness to extremely low amounts of ADH or to other antidiuretic factor.

3.9.7. Etiology

In many circumstances SIADH remains as a presumptive diagnosis because clear information on the main points of the syndrome is missing. The small cell lung carcinoma is probably the most common

neoplastic cause of the syndrome. In a few well-documented cases, ADH has been shown in tumoral extracts, suggesting that the tumor is the source of ADH. However, not every patient with SIADH associated with neoplastic diseases present an ectopic production of ADH. In these cases, an excessive neurohypophyseal ADH secretion has been observed. Other studies have suggested that abnormal forms of ADH are secreted by some tumors as observed in extracts of chromatographed tissues in positions where molecular weight is higher than that of ADH.

Trauma may lead to an "interphase" of ADH secretion. Possible presence of this interphase has been recently demonstrated in experimental animals with partial lesions of the hypophyseal stalk of SIADH without DI, because the injured stalk is able to avoid DI, and ADH is released. In the postoperative period, anesthesia, narcotic and sedative drugs, pain, intestinal manipulation, nausea, and circulatory adjustments may reduce water excretion for one or several days. In a recent study, the most common cause of hyponatremia in the postoperative period was excessive administration of glucose solutions. In newborns, especially those premature, SIADH is relatively common because of hypoxic cerebral lesion, frequently along with hemorrhage, pneumonia, pneumothorax and atelectasis, meningitis, patent arterial duct connection, and positive pressure ventilation.

3.9.7.1. Clinical and Laboratorial Diagnosis. The clinical picture is a combination of signs and symptoms of the primary disease (Table 5) and of hyposmolality. The most important signs and symptoms originate from neuromuscular and CNS. By and large, the first signs and symptoms to appear are anorexia, apathy, confusion, headache, asthenia, abdominal colics, and cramps in the limbs. In moderate cases, the clinical picture includes nausea, vomit and any other kind of neurological abnormality, ranging from depressed deep tendon reflexes to pathological reflexes, bulbar or pseudobulbar paralysis, and psychotic behavior. In the most severe cases, seizures, coma, and death may occur. Severity depends more on the speed of onset than on sodium levels. Chronic hyponatremia differs from acute in human beings regarding two major factors:

(a) approximately half of the patients with chronic hyponatremia are asymptomatic, even when serum sodium level is lower than 125 mEq/L;
(b) mortality rate is nearly zero in asymptomatic patients and it is about 10–15% in symptomatic patients.

Table 5
Causes of ADH excess (SIHAD)

Central Nervous System Diseases	*Drugs*
Meningitis	Cytostatic
Encephalitis	Anesthetic
Trauma	Analgesic
Ischemic-hypoxic lesion	Sedative
Tumor	Anti-depressive
Guillain–Barré syndrome	Sulfonylureas
Ventriculoatrial shunt obstruction	Causing nausea
Acute intermittent porphyria	
Thrombosis of skull basis sinus	*Neoplasias*
Hemorrhage (intracerebral, subarachnoid)	
Malformation	*Postoperative state*
Lung Diseases	*Trauma*
Pneumonia	Burns
Tuberculosis	
Neoplasm	*Endocrine failure*
	Thyroid
Decrease in left atrium filling	Adrenocortical
Positive pressure ventilation	
Pneumothorax	*Idiopathic*
Atelectasis	
Asthma	
Arterial duct connection	

In chronic hyponatremia there is solute extrusion from cerebral cells, mainly of NaCl an KCl, by membrane transportation processes different from Na-K-ATPase, making osmotic balance between cerebral cells and the extracellular medium to be performed with lower increase in cerebral volume. On the other hand, if there is fast hyponatremia correction, the cerebral cells depleted of solute will have a reduced volume. It is important to note that because of sodium escape, as previously mentioned, edema is seldom part of SIADH clinical picture.

True hyposmolality is always because of one out of two entirely different disorders: fluid retention or sodium depletion. The clinical history, underlying disease and physical findings, help in differentiating both causes. Primary sodium depletion is invariably associated to extracellular medium contraction and fluid retention is associated to its expansion. Such depletion is the result of either extrarenal (gastrointestinal, surgery, and inflammation) or renal losses (natriuretic peptides, mineralocorticoid deficiency, natriuretic medication, or salt-losing nephropathy). In those cases where clinical distinction between contraction and expansion is difficult, urea and plasmatic protein dosage will be high in sodium depletion. In

SIADH, plasmatic urea is initially low. In a later stage, urinary sodium may be lower or absent, but the syndrome may be distinct because the patient is unable to retain a sodium load, unless fluid ingestion is restricted. SIADH cannot be excluded in plasmatic hyposmolality associated to a maximally dilute urine, because it may occur in the previously mentioned type B.

3.9.8. Treatment

The primary disease must be treated first, paralleled with correction of hyponatremia. The way of approaching hyposmolality will depend on some previously mentioned factors, such as speed of onset, hyponatremia intensity, and mainly after characterization of clinical symptoms. Characterizing a patient as symptomatic or asymptomatic may be difficult, especially if the primary disease is neurologic and symptoms and signs may be confused with water intoxication. When plasmatic sodium is higher than 125 mEq/L, very seldom do patients become symptomatic. In the asymptomatic patients, the first step to be taken is fluid restriction in order to generate a negative fluid balance, which is generally attained with approximately 700–800 mL per day. Therefore, natriuresis decreases and an increase in sodium supply may be initiated. Three or five additional grams of sodium are initially administered, totaling a salt ingestion of approximately 15 g per day. Furosemide (40–80 mg/day) may be added to this regimen. Compliance with fluid restriction is many times difficult because of inappropriate thirst, and sometimes further therapeutical measures are needed, such as use of drugs that cause nephrogenic DI, as lithium and demethylchlortetracyclin (demechlocyclin). The action of these drugs may take up to six weeks to fully develop. Lithium carbonate is potentially more toxic and may lead to renal tubular acidosis, cardiotoxicity and thyroid hypofunction; the individual response is variable and, if used, lithium carbonate must be administered in a 600–1200 mg/day dosage and adjusted according to lithemia. Demechlocyclin must be used with caution in patients with hepatic failure because of risk of nephrotoxicity because of drug accumulation. It may be administered in a 3–5-mg/kg dosage, every 8 h. The drug of choice may be the ADH new specific analogue antidiuretic, OPC-31260. It has been tested in rats and humans with important ADH antagonism and few undesirable effects.

Symptomatic patients, with plasmatic sodium lower than 125 mEq/L, need immediate therapy because they are the ones who present the highest risks. Studies with animals show that hyponatremic females present higher mortality than males, which seems to be originated from a lower ability of females concerning mechanisms of volume regulation and production of high-energy phosphate. In human beings, it is not known whether female gender is a factor of bad prognosis.

Symptomatic hyponatremia, with serum sodium below 120 mEq/L, requires immediate treatment. Normal or hypertonic saline solution combined with furosemide is used. Urine induced by this diuretic presents osmolality lower than that of the plasma, and natriuresis determined by it is beneficial, because it decreases risks of extracellular volume expansion. Speed of correction of hyponatremia is a controversial topic in the literature as already mentioned. We consider that correction should be performed in a speed of approximately 0.5 mEq/L/h until serum sodium achieves 120 to 125 mEq/L. Cases of higher risks may be corrected with a speed of increase in sodium of 1–2 mEq/L/h. It seems important to avoid sodium increment greated that 25 mEq/L in 48 h. The major concern as to quick correction is in the central pontin myelinolysis (CPM), a demyelinization of the pons, with destruction of the myelin sheaths, sparing axon and nucleus. It also may occur in other white matter brain areas. Clinical characteristics include flaccid quadriplegia or paraplegia, facial paresis, dysphasia, dysarthria and coma. By and large patients with CPM present further risk factors such as alcoholism or malnutrition.

SELECTED READINGS

Berl T, Schrier RW. Disorders of water metabolism. In: Schier RW, ed. Renal and Eletrolyte Disorders. New York: Little Brown, 1997: pp. 1–71.

Dirks PB, Rufka JT, Becker LE, Hoffman HJ. Intracranial germ cell tumors: classification, diagnosis, and mangement. In: Youmans JR, ed. Neurological Surgery. Philadelphia, PA: W.B. Saunders, 1996: pp. 2530–2541.

Fujisawa G, Ishikawa S, Tsuboi Y, Okada K, Saito T. Therapeutic efficacy of non-peptide ADH antagonist OPC-31260 in SIADH rats. Kidney Int 1993; 44:19–23.

Girard J, Pampalone-Siervo A. Intracranial space-occupying lesions. In: Brook CGD, ed. Clinical Paediatric Endocrinology. Oxford, UK: Blackwell Scientific, 1990: pp. 266–277.

Kupfermann I. Hypothalmus and limbic system: Peptidergic neurons, homeostasis, and emotional behavior. In: Kandel ER, Schwartz JH, Jessell TM, eds. Principles of Neural Science. New York: Appleton & Lange and Simon & Schuster Business and Professional Group, 1991: pp. 735–760.

Melmed S. Tumor mass effects of lesions in the hypothalamus and pituitary. In: DeGroot LJ, ed. Endocrinology. Philadelphia, PA: W.B. Saunders, 1995: pp. 458–466.

Reichlin S. Neuroendocrinology. In: Wilson JD, Foster DW, Kronenberg HM, Larsen PR, eds. Williams textbook of Endocrinology. Philadelphia, PA: W.B. Saunders, 1998: pp. 165–248.

Robertson GL. Diabetes Insipidus. In: Dluhy RG, ed. Endocrinology and Metabolism Clinical of North America. Philadelphia, PA: W.B. Saunders, 1995: pp. 549–572.

Saito T, Ishikawa S, Sasaki S, et al. Urinary excretion of aquaporin-2 in the diagnosis of central diabetes insipidus. J Clin Endocrinol Metab 1997; 82:1823–1827.

Witte RJ, Mark LP, Daniels DL. Radiographic evaluation of the pituitary and anterior hypothalamus. In: DeGroot LJ, ed. Endocrinology. Philadelphia, PA: W.B. Saunders, 1995: pp. 467–486.

28 Hypothalamic Involvement in Disorders of Pituitary Hormone Secretion

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CONTENTS

1. INTRODUCTION

The hypothalamus, a relatively small structure located below the thalamus and above the pituitary, is a complex endocrine tissue involved in synthesizing the posterior pituitary hormones, production, and secretion of anterior pituitary releasing and inhibiting hormones, and thus plays a critical role in pituitary hormone regulation. Hypothalamic peptides, including somatostatin, growth hormone (GH)-releasing hormone (GHRH), GH-releasing peptide (GHRP), thyrotropin-releasing hormone (TRH), corticotropin-releasing hormone (CRH), gonadotropin-releasing hormone (GnRH), and dopamine have direct and immediate effects on hormonal secretion from pituicytes, and any structural or functional hypothalamic pathology, congenital or acquired, may result in severe disturbance of normal pituitary function.

This chapter describes the hypothalamic disorders directly involved in disruption of pituitary hormone production and secretion. Both the anterior (adenohypophysis) and the posterior (neurohypophysis) pituitary may be affected, one or several pituitary hormones may be involved, and disorders of hypo- or hypersecretion may occur. As the hypothalamic-pituitary unit is tightly connected and both organs may be involved in contiguous similar pathologies, the exact level of endocrine involvement is not always apparent, and combined hypothalamic and pituitary involvement may be diagnosed. This chapter discusses diagnosis and treatment of specific hypothalamic disorders resulting in pituitary endocrine malfunction. Related issues are covered in Chapter 27.

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2. HYPOTHALAMIC TUMORS

Craniopharyngioma. Craniopharyngiomas are benign tumors derived from remnants of Rathke's pouch that center on the pituitary stalk, and usually involve the suprasellar cisterns. The tumors are large and locally invasive, hypothalamic involvement is common, and half of patients suffer from anterior pituitary dysfunction. Tumors are cystic and many have calcifications evident on skull X-rays and CT scanning. These tumors constitute about 3% of all brain tumors. Half of patients present before age 20, usually with signs of increased intracranial pressure, including headache, vomiting, papilledema, and hydrocephalus. Other symptoms are visual field abnormalities (very common in adults), personality changes and cognitive deterioration, cranial nerve damage, sleep difficulties, and weight gain.

Partial or complete pituitary hormone deficiency is very common. Growth retardation because of GH deficiency is found in more than 40% of affected children. Panhypopituitarism, hypogonadotrophic hypogonadism, central hypothyroidism, and diabetes insipidus are also common endocrinologic disorders. Hormonal testing reveals that ACTH deficiency is also very common. Hyperprolactinemia may result from pituitary stalk compression and/or decreased dopamine secretion because of hypothalamic destruction. Treatment includes radical resection of the tumor (transcranial and transsphenoidal approach), and postoperative radiation of the residual tumor. This usually results in long-term survival and even cure.

Pituitary Macroadenomas. Invasive large macroadenomas, especially prolactin (PRL)-secreting tumors in males, and nonfunctioning adenomas, may present with extensive suprasellar extension into the hypothalamic area, resulting in severe mass effects including headaches, visual field defects, cranial nerve (III, IV, and VI) compression, bone erosions, and also hormonal hyposecretion. Gadolinium-enhanced MRI allows precise visualization of the pituitary gland, its stalk, the hypothalamus, and surrounding structures, including the suprasellar cisterns, cavernous sinuses, sphenoid sinus, and optic chiasm. The improved radiologic evaluation of the pituitary and hypothalamus has allowed better definition of pathological processes and masses in the hypothalamic-pituitary region. A complete neuroophthalmologic assessment is an important part of the evaluation and management of invasive pituitary masses. As these large pituitary tumors may invade and destroy critical areas of the hypothalamus involved in neuroendocrine regulation, combined hormonal deficiencies of both pituitary hormones and hypothalamic releasing and inhibiting factors may complicate the clinical picture. Commonly, mild hyperprolactinemia (in cases other than macroprolactinomas) results from loss of hypothalamic dopaminergic inhibitory tone. Combined hypogonadotrophic hypogonadism, GH deficiency, and even tertiary hypothyroidism and ACTH deficiency are part of the endocrine manifestations in these cases, whereas diabetes insipidus is not commonly encountered. As with pituitary masses with no extrasellar involvement, dynamic tests with GnRH, GHRH, CRH, and TRH are characteristically blunted, and do not produce significant pituitary hormone secretion.

Treatment of these invasive tumors usually includes debulking by transphenoidal and/or transcranial surgery of the pituitary and hypothalamic masses. Usually the removal is incomplete, but sufficient to reverse the mass effect symptoms. However, visual field defects may be irreversible, and long-standing pituitary and hypothalamic damage results in permanent panhypopituitarism that requires pituitary hormone replacement. This includes thyroid hormones, glucocorticoids, and sex hormones and/or gonadotropins to restore fertility. Recently, the role of GH replacement in adults with acquired GH deficiency has been elucidated. GH replacement in adults may improve body composition, increase muscle volume and strength, improve psychosocial achievement, and alter disordered lipid profiles. Prolactinomas are always treated with dopamine agonists (with or without surgery), and radiotherapy is given to patients with large residual nonfunctioning tumor mass remaining after surgery.

Metastatic Tumors. Metastases to the hypothalamic-pituitary area are not rare (up to 12% of autopsied cancer cases) and are more frequently found in the hypothalamus, pituitary stalk, and the posterior lobe. The primary source tumors are usually breast, bronchogenic, and colonic carcinomas. Hypothalamic metastases usually occur in association with metastases to other sites, but occasionally may be the presenting lesion. Most metastases are asymptomatic and less than 10% result in diabetes insipidus and rarely in the syndrome of inappropriate ADH secretion (SIADH). Hyperprolactinemia is also common, but anterior pituitary failure is rare.

CT scanning or MRI of the hypothalamus in symptomatic patients will demonstrate a mass (often more than one lesion). Treatment is usually palliative, but surgical decompression and radiotherapy may provide symptomatic improvement.

Other hypothalamic tumor masses, including hamartomas (may present as precocious puberty), germ cell tumors (commonly present with diabetes insipidus, hypopituitarism, and visual field defects), and suprasellar meningiomas (may cause hypogonadism, hypothyroidism, and diabetes insipidus) are not common, and hypothalamic-pituitary endocrine dysfunction can be the first presenting sign, even before localization of the tumor mass.

3. HYPOTHALAMIC INFILTRATIVE DISORDERS

These rare disorders, including sarcoidosis, histiocytosis X, amyloidosis, and hemochromatosis frequently involve the hypothalamus and posterior and anterior pituitary as a component of the systemic involvement in these diseases. Diabetes insipidus is a very common clinical manifestation (up to 50% of patients with hypothalamic sarcoidosis and histiocytosis) followed by growth retardation, hypogonadotrophic hypogonadism, and hyperprolactinemia. Specific treatment modalities, including high doses of glucocorticoids for CNS sarcoidosis, or chemotherapy and radiotherapy for histiocytosis, usually do not reverse the diabetes insipidus or other endocrine deficits.

3.1. Hypothalamic Irradiation

Therapeutic irradiation of the hypothalamic-pituitary region may result in long-term hypothalamic and pituitary dysfunction. Whole-brain irradiation for primary brain tumors and lymphoblastic leukemias, and irradiation to the head and neck for other neoplasms are associated with hypothalamic-pituitary hypofunction. Characteristically, children and adolescents are more susceptible to this damage, and the prevalence of hormonal abnormalities directly correlates with the time interval following completion of radiotherapy and the doses administered. Up to two-thirds of patients who receive a median of 5000 rads to the hypothalamus will develop hypothalamic-anterior pituitary hormonal abnormalities. GH deficiency is the earliest abnormality detected, and has usually been documented 10–15 yr after radiotherapy. ACTH deficiency and hypogonadotrophic-hypogonadism are less common, identified in 10–20% of patients after long intervals. Thus, patients exposed to radiotherapy of the brain or head and neck should undergo frequent anterior pituitary hormone evaluations, and receive appropriate replacement therapy.

4. HYPOGONADOTROPHIC HYPOGONADISM

4.1. Kallmann's Syndrome

The most common form of isolated gonadotropin deficiency, also known as olfactory-genital dysplasia, results from a defect in hypothalamic GnRH synthesis and secretion. Many patients demonstrate anosmia or hyposmia because of agenesis or hypoplasia of the olfactory bulbs. Most cases are sporadic, but they may also occur as an X-linked dominant or autosomal dominant trait with incomplete penetrance. Both the sporadic and familial types are less common in women than in men. The X-linked syndrome is owing to molecular defects in the *KAL* gene, which maps to chromosome *Xp*22.3. This gene encodes a protein of the fibronectin family that plays a critical role in neural chemotaxis and cell adhesion. It is believed that defects in the *KAL* gene prevent the migration of the GnRH neurons from the olfactory placode to the hypothalamus during fetal development, impairing their ability to deliver GnRH into the median eminence and hypophyseal portal system. Mutations of the *KAL* gene were also reported in patients affected only by anosmia. Other genetic defects may cause sporadic GnRH deficiency, including defects of the GnRH gene itself. Beside anosmia, Kallmann's syndrome may be associated with other somatic defects, including color blindness, optic atrophy, nerve deafness, craniofacial midline defects (cleft lip or palate), renal abnormalities, cryptorchidism, skeletal deformities, and various neurologic deformities.

Patients with Kallmann's syndrome display no pulsatile gonadotropin secretion at all, or very low-amplitude pulses, resulting in low levels of circulating sex steroids. Male patients present during adolescence when puberty fails to occur at all in the severe form of the syndrome, or is delayed and incomplete in the partial form. The penis remains small, and the scrotum is small, smooth, and hairless. The testes are soft and small, usually less than 2 mL in volume. Secondary sex characteristics are absent, muscular development is decreased, obesity is common, and mild gynecomastia is usually found. The skin is delicate and older subjects show the characteristic fine

wrinkling around the corners of the eyes and lips. Patients retain a childish high-pitched voice. The characteristic eunuchoidal proportions (the span exceeds the height and the upper-to-lower body segment ratio is <1) result from delay in the epiphyseal fusion of the long bones and their continued growth under the influence of GH. Kallmann patients lack the pubertal growth spurt, but reach a normal adult height because they continue to grow as epiphyseal fusion is delayed. Untreated adult patients have significant osteopenia. Patients do not display the postpubertal sex drive, and erections and nocturnal emissions are rare or absent. Affected males have azoospermia, low seminal plasma volume, and an infantile prostate. These patients are psychologically affected, demonstrating passive and introverted behavior. Female patients present with primary amenorrhea and absent secondary sex characteristics.

Serum testosterone in males and serum estradiol in females are low, and baseline serum LH and FSH may vary from undetectable to low-normal, but are always inappropriately low for the low levels of the sex steroids. The GnRH (25–100 μg, iv) stimulation test is of limited use in the distinction between hypothalamic and pituitary causes of hypogonadism, because patients with hypothalamic hypogonadism may have blunted FSH and LH response on initial testing because of secondary atrophy of the gonadotroph cells. However, repetitive administration of GnRH pulses may normalize gonadotropin responses in these patients, indicating pituitary integrity.

Puberty and secondary sex characteristics are attained with long-term treatment of testosterone or gonadotropin (human chorionic gonadotropin; hCG). Full fertility in male Kallmann's patients may be achieved with long-term gonadotropin treatment, or with small doses of GnRH given continuously by infusion pump every 90 min.

4.2. Laurence-Moon-Bardet-Biedl Syndrome

This syndrome is characterized by hypothalamic hypogonadotrophic hypogonadism, obesity, mental retardation, polydactyly, and retinal degeneration. This rare syndrome is transmitted as an autosomal recessive disorder. The occasional coexistence of obsesity and central diabetes insipidus with the hypogonadism suggests hypothalamic involvement, although no histopathologic abnormalities have been identified in the hypothalamus. Hypogonadism affects males (75%) more frequently than females (50%). Other associated abnormalities include renal malformations, nerve deafness, hyperlipidemia, glucose intolerance, and behavioral abnormalities. Digital abnormalities such as an extra digit in one or more extremities (hexadactyly), brachydactyly, and syndactyly are found in 75% of these patients. The retinopathy begins in early childhood, and by age 20–30 most patients are blind. Hormonal treatment includes vasopressin administration for diabetes insipidus, and sex hormones or gonadotropins for hypogonadism.

4.3. Frohlich Syndrome (Adipose Genital Dystrophy)

This hypothalamic disorder results in obesity and hypogonadotrophic hypogonadism. The syndrome may be caused by a wide variety of hypothalamic organic lesions, including encephalitis, tumors, demyelinating disorders, microcephaly, and Friedreich ataxia. These hypothalamic diseases result in decreased GnRH production and secretion, thus affecting anterior pituitary gonadotropin release.

4.4. Prader-Willi Syndrome

This syndrome includes muscle hypotonia, mental and developmental retardation, hyperphagia, obesity and adult-onset diabetes mellitus, multiple somatic anomalies involving the cranium, eyes, ears, hands, and feet, short stature, and hypogonadotrophic hypogonadism. The disorder is usually sporadic, and occasionally associated with chromosome 15 abnormalities. The endocrine defect is believed to reflect a long-standing GnRH deficiency, and chronic treatment with GnRH restores normal LH and FSH responses. However, no histopathologic abnormalities have been demonstrated in the hypothalamus. Other pituitary hormones, including TSH, ACTH, and PRL are normally secreted, but GH response to known secretagogs may be blunted.

4.5. Functional (Hypothalamic) Amenorrhea

Functional hypothalamic disorders are common etioligies of secondary amenorrhea. These nonorganic and reversible disorders may include stressful situations, psychological disturbances, severe diet and weight loss (anorexia nervosa), intensive exercise and physical activity (ballet dancers and marathon runners), and severe systemic diseases. In females, these result in gonadal insufficiency and anovulation. Affected males may complain of decreased libido and impotence. These disorders probably arise from temporary functional abnormalities in the hypothalamic GnRH producing neurons or because of a supra-

hypothalamic failure as the primary cause of the hypothalamic GnRH secretory malfunction. Exercise-induced or hypothalamic amenorrhea can delay normal sexual maturation for many years, and the estrogen deficiency may be associated with significant osteopenia. Serum estradiol is low and gonadotropins are low or inappropriately low. Treatment of the primary etiology (e.g., calorie replacement) or spontaneous recovery will usually restore menses.

5. GHRH/GH DEFICIENCY

GH deficiency (GHD) may be isolated or combined with deficiencies of other pituitary hormones. The incidence of isolated congenital GHD in children approaches 1:5000 to 1:10,000. Several types of hereditary GHD with different modes of inheritance have been described. Molecular defects include GH gene deletion (pituitary level) and lack of synthesis and secretion of GHRH at the hypothalamus. GHRH and GHRH receptor mutations (reported recently) in humans as a cause of GHD must be extremely rare, but the homozygous missense mutation in GHRH receptor of the *little* (*lit/lit*) mice results in a 10-fold decrease in somatotroph cell number and GH production. Excess hypothalamic somatostatin secretion has also been postulated as a cause for GHD, and decreased hypothalamic GHRP (the natural peptide) production or secretion is another attractive and possible cause of GHD.

Children with GHD are short and fail to grow at a normal rate. They tend to be overweight for their height, but are normally proportioned. These children have low stimulated GH levels and low IGFBP-3. IGF-1 levels are normally very low before age 3, and do not correlate with stimulated GH levels. As GHRH is probably secreted directly into the hypophyseal portal system, measurement of peripheral GHRH levels are of little meaning and cannot be used to diagnose GHRH deficiency. If the pituitary somatotrophs are first primed with intermittent GHRH pulses, the acute GHRH test may sometimes distinguish between hypothalamic and pituitary GHD, and GH response to subsequent GHRH administration may point to a hypothalamic lesion.

Currently, GH replacement with daily subcutaneous injections of recombinant hGH (rhGH) is started as early as possible in both hypothalamic and pituitary GHD, as total height gain is inversely proportional to pretreatment chronologic and bone age. Hoewever, in cases of hypothalamic GHD, the recently developed group of oral GH secretagogues (nonpeptide GHRP analogs) may, in the future, replace rhGH. The convenient oral administration of these potent stimulators of endogenous GH secretion and their more physiologic patterns of GH replacement have opened the field of GHD for long-term controled studies using these analogs.

6. HYPERPROLACTINEMIA

PRL secretion from the pituitary lactotrophs is under the hypothalamic inhibitory dopaminergic tone. This major PRL inhibitory factor acts by binding to the D2 dopamine receptor on lactotrophs to decrease PRL gene transcription, synthesis, and release. In contrast, the hypothalamic hormone, TRH, induces PRL transcription and secretion. Recently, a novel hypothalamic PRL-releasing peptide was cloned. This potent stimulator of PRL secretion that acts through a specific pituitary G-protein-coupled seven-transmembrane-domain receptor, may have a physiologic role in human PRL regulation.

Lesions of the hypothalamus and the pituitary stalk usually interfere with the neuroendocrine mechanisms that control PRL secretion and results in PRL elevation because of disinhibition of the tonic dopamine action on the pituitary lactotrophs. Characteristically, PRL levels are < 200 ng/mL in patients with hypothalamic hyperprolactinemia, compared with PRL levels higher than 200 ng/mL in macroprolactinomas. Hypothalamic/pituitary stalk lesions include large nonsecreting pituitary tumors, cranyopharyngiomas, meningiomas and other tumors, metastatic disease to hypothalamus, histiocytosis X, sarcoidosis, amyloidosis, irradiation damage, Rathke's cyst, surgical or traumatic damage to the hypothalamus, and idiopathic disordered hypothalamic regulation of PRL secretion (Table 1). Interestingly, most patients with hypothalamic hyperprolactinemia have intact function of other hypothalamic releasing factors, resulting in normal TSH-thyroid and ACTH-adrenal axis.

Evaluation of patients with hyperprolactinemia should include an MRI with gadolinium enhancement of the pituitary and hypothalamus and careful studies of the other hypothalamic-pituitary hormonal functions. PRL-secreting macroadenomas will generally respond to dopamine agonist treatment by normalization of PRL levels.

7. PRECOCIOUS PUBERTY

Sexual precocious puberty is considered to be present in boys presenting with secondary sex characteristics before age 9, and in girls before age 8. Isosexual

Table 1
Etiologies of
Hypothalamic/Pituitary Stalk Hyperprolactinemia

Tumors
Craniopharyngioma
Nonsecreting macroadenomas with suprasellar extension
Meningioma
Dysgerminoma
Metastases to hypothalamus
Histiocytosis X
Sarcoidosis
Amyloidosis
Irradiation damage
Suprasellar surgery
Traumatic damage / pituitary stalk section
Rathke's cyst
Idiopathic hypothalamic disordered PRL regulation

precocious puberty because of central mechanisms is called true or complete sexual precocity, is gonadotropin-dependent, and results from premature GnRH secretion. In contrast, "incomplete precocious puberty" is owing to primary gonadal or adrenal disorders, or tumors that produce hCG ectopically.

Central or hypothalamic precocious puberty occurs more frequently in girls (80%), and in many cases (70%) no defined cause is identified and idiopathic maturation of the hypothalamic-pituitary-gonadal axis probably accounts for these cases. Some patients may have a familial tendency toward earlier puberty onset. In boys with precocious puberty, only the minority have idiopathic sexual precocity.

Structural lesions of the central nervous system including neoplasms, inflammatory conditions, and lesions associated with irritative effects on the hypothalamus may result in premature pubertal maturation. Hypothalamic hamartomas may ectopically produce GnRH or decrease inhibitory effects on GnRH-secreting cells. Meningiomas and germinomas may directly secrete hCG. In McCune-Albright polyostotic fibrous dysplasia syndrome (cafe au lait spots, fibrous dysplasia of bones, and sexual precocity) some children have true gonadotropin-dependent precocious puberty. Other hypothalamic disorders that frequently cause precocious puberty include craniopharyngioma, neuroblastoma, glioma, arachnoid cyst, noncomunicating hydrocephalus, sarcoidosis, and head trauma.

The syndrome of true precocious puberty includes rapid skeletal growth, in boys—early increase in testes and penile size, secondary sexual development, aggressive behavior; and in girls—early breast development, sexual pubic hair, and menses before age 9.5.

CT and MRI scanning are used to diagnose intracranial tumors and other structural abnormalities of the hypothalamus. Measurements of basal LH (prominent LH pulses are detected initially), FSH, and hCG will distinguish premature hypothalamic GnRH secretion from tumors (meningioma, germinoma) that secrete hCG. Pubertal values of testosterone and estrogen are found in patients with hypothalamic precocious puberty.

Central precocious puberty is successfully treated with GnRH analogs (agonists) to inhibit the gonadotropin secretion and delay physical development. These agonists have increased GnRH receptor affinity and longer duration of action, thus acting as constantly infused GnRH. Initially, they stimulate pituitary gonadotropin secretion, but then downregulate the pituitary gonadotroph-GnRH receptors and decrease LH and FSH secretion. This leads to suppression of sex-hormone secretion, rapid growth arrest, decrease of testicular size, aggression, and erections experienced in boys, and decreased breast size in girls. Thus, monthly intramuscular administration of a depot preparation of GnRH agonist permits maintenance of effective gonadotropin suppression and clinical improvement.

8. HYPOTHALAMIC TUMORS SECRETING PITUITARY TROPHIC HORMONES

8.1. GHRH-Secreting Tumors

Eutopic hypothalamic GHRH-secreting tumors are a rare etiology of acromegaly (<1% of acromegalic patients). These rare hypothalamic tumors include hamartomas, gliomas, and ganglioneuromas, and directly induce pituitary somatotroph hyperplasia or GH-cell adenoma, resulting in excess pituitary GH secretion. Clinical manifestations of acromegaly because of GHRH-secreting tumors are similar to GH-secreting adenomas, including gigantism in children, and skeletal and soft-tissue overgrowth and visceromegaly in adults. Biochemical diagnosis is based on increased plasma IGF-1 levels and failure to suppress serum GH to <ng/mL in response to oral glucose load. Excess eutopic hypothalamic GHRH is probably secreted into the hypophyseal portal system and peripheral GHRH levels are not elevated. MRI

imaging of the hypothalamic region is mandatory to exclude the presence of hypothalamic GHRH-secreting tumor in acromegaly, especially when an enlarged pituitary with no distinct pituitary tumor mass is delineated on MRI.

The therapy of choice for hypothalamic GHRH-secreting tumors is surgical removal of the tumor if feasible, after accurate localization of the tumor mass. Pituitary surgery is ineffective in most cases, and GH hypersecretion is not suppressed. Octreotide and other somatostatin analogs may suppress GHRH secretion from hypothalamic tumors, while also decreasing pituitary GH secretion, and can be used to treat this unusual form of acromegaly.

8.2. CRH-Secreting Tumors

Rare hypothalamic tumors such as gangliocytomas have been described as a cause of chronic pituitary ACTH oversecretion, presenting as Cushing's syndrome. These patients exhibit pituitary corticotroph cell hyperplasia, but usually present with mild clinical features of hypercortisolism. These uncommon hypothalamic tumors may project away from the brain, to sites within the pituitary sella, have no attachments to the hypothalamus, but are still composed of hypothalamic-like neurons that secrete CRH, resulting in the rare syndrome of hypothalamic Cushing's disease. Surgical removal of the CRH-secreting tumor after prompt localization is the treatment of choice.

8.3. GnRH-Secreting Tumors

Central nervous system tumors including hypothalamic hamartomas, astrocytomas, ependymomas, and hypothalamic gliomas can cause precocious sexual development, either by ectopic production of GnRH or by removing the inhibitory tone imposed on the GnRH-secreting hypothalamic neurons.

9. PITUITARY INSUFFICIENCY OWING TO HYPOTHALAMIC DISORDERS

Hypopituitarism secondary to hypothalamic disease or stalk disorder characteristically includes vasopressin deficiency (diabetes insipidus) that is an unusual feature of primary pituitary disorders. Tertiary hypothyroidism because of TRH deficiency is clinically similar to primary hypothyroidism, but usually less severe. Low serum thyroxine (tri-iodothyronine levels are usually normal) associated with inappropriately normal-to-low serum thyrotropin levels are detected. Thyroxine treatment for hypothalamic hypothyroidism (Table 2) should be started only after CRH-ACTH deficiency is clearly excluded, or after initiation of glucocorticoid replacement. Otherwise, thyroxine administration may exacerbate features of cortisol deficiency.

Table 2
Hormone Replacement Therapy for Hypothalamic-Pituitary Dysfunction

CRH-ACTH deficiency—glucocorticoids (hydrocortisone, cortisone-acetate, prednisone, dexamethasone), one or two daily oral administrations.
TRH-TSH deficiency—thyroxine, one daily oral administration.
GnRH-gonadotropins deficiency—portable GnRH infusion pump (subcutaneous injection every 90–120 min);
Gonadotropins (hCG, weekly intramuscular administration; human menopausal gonadotropins, daily intramuscular administration; pure recombinant gonadotropins);
Testosterone (1–2 intramuscular administration of depot preparetion, monthly; transdermal patches)
Estrogen and progesterone (daily oral administration, cyclically or continuously; transdermal patches).
GHRH-GH deficiency—rhGH, daily subcutaneous administration;
GHRP analog, daily oral administration (still experimental).
PRL deficiency—no need for hormone replacement.
Vasopressin deficiency—desmopressin, 1–3 daily nasal or oral administrations.

Patients with CRH and corticotropin deficiency are less symptomatic compared to primary adrenal insufficiency, because mineralocorticoid secretion remains intact in most cases. However, damage to the CRH-ACTH axis can be life-threatening during infectious disease, trauma or after surgery, because these patients' stress response is limited. Thus, patients on glucocorticoid replacement therapy (Table 2) should increase their corticosteroid dose or may require intravenous treatment during severe illness or surgery. Interestingly, cortisol deficiency may mask central diabetes insipidus, which may become unmasked with cortisol replacement, required for effective free water clearance.

Hypogonadotrophic hypogonadism in premenopausal women with hypothalamic disease leads to menstrual abnormalities, and in men to decreased libido and sexual dysfunction. Fertility and pregnancy

may usually be achieved with gonadotropins or pulsatile therapy with GnRH (*see* Table 2). When fertility is not desired, normal sexual function, bone and muscle mass, and good general health are maintained in males by testosterone administration. Estrogen replacement in women prevents osteoporosis and premature atherosclerosis, and maintains libido.

10. SUMMARY

The hypothalamus and both the anterior and posterior pituitary gland, are anatomically and functionally closely related to each other. A unique portal vascular system directly connects the hypothalamus to the adenohypophysis, carrying hypothalamic regulatory factors to the pituitary. These factors are well characterized (i.e., CRH, GnRH, TRH, and others), but some other factors are still unknown (e.g., a novel PRL-releasing peptide was recently cloned) and their future cloning may identify novel pituitary regulatory roles played by the hypothalamus, and clarify several aspects of hypothalamic-pituitary function. Peripheral hormonal regulatory factors may have dual inhibitory effects on the pituitary and the hypothalamus.

Anatomically, the hypothalamus is a poorly defined region. Moreover, many disorders that affect the hypothalamus also involve the pituitary. Many of the disorders described in this chapter affect the pituitary both directly and indirectly through hypothalamic damage, and in most cases it is difficult and often impossible to localize the exact level of damage. Specific treatment modalities and replacement hormonal therapy are usually similar in cases of hypothalamic or pituitary disease. Nonendocrine hypothalamic dysfunction, present in cases when pituitary involvement is suggested, may in fact localize the damage to the hypothalamic level.

SELECTED READING

Arnold A, ed. Endocrine Neoplasms. Cancer Treatment and Research series. Norwell: Kluwer Academic Publishers, 1997.

Asa SL, Kovacs K, Tindall GT, Barrow DL, Horvath E, Versei P. Cushing's disease associated with an intrasellar gangliocytoma producing corticotrophin-releasing factor. Ann Intern Med 1984; 101:789–793.

Asa SL, Scheithauer BW, Bilbao JM, et al. A case for hypothalamic acromegaly: a clinicopathological study of six patients with hypothalamic gangliocytomas producing growth hormone-releasing factor. J Clin Endocrinol Metab 1984; 58:796–803.

Braunstein GD, Kohler PO. Endocrine manifestations of histiocytosis. Am J Pediat Hematol/Oncol 1981; 3:67–75.

Bray GA, Dahms WT, Swerdloff RS, Fiser RH, Atkinson RL, Carrel RE. The Prader-Willi syndrome: a study of 40 patients and a review of the literature. Medicine 1983; 62:59–80.

Edwards JA, Sethi PK, Scoma AJ, Bannerman RM, Frohman LA. A new familial syndrome characterized by pigmentary retinopathy, hypogonadism, mental retardation, nerve deafness and glucose intolerance. Am J Med 1976; 60:23–32.

Hinuma S, Habata Y, Fujii R, et al. A prolactin-releasing peptide in the brain. Nature 1988; 393:272–276.

Lieblich JM, Rogol AD, White BJ, Rosen SW. Syndrome of anosmia with hypogonadotrophic hypogonadism (Kallmann syndrome). Am J Med 1982; 73:506.

Melmed S, ed. The Pituitary. Cambridge: Blackwell Science, 1995.

Randall RV, Laws ER Jr, Abboud CF. Clinical presentations of craniopharyngiomas: a brief review of 300 cases. In Givens JR, ed. The Hypothalamus. Chicago: Year Book Medical Publishers, 1984.

Salvatori R, Hayashida CY, Aguiar-Olivera MH, et al. Familial dwarfism due to a novel mutation of the growth hormone-releasing hormone receptorgene. J Clin Endocrinol Metab 1999; 84:917–923.

Stuart CA, Neelon FA, Lebovitz HE. Hopothalamic insufficiency: the cause of hypopituitarism in sarcoidosis. Ann Intern Med 1978; 88:589–593.

Weintraub BD, ed. Molecular Endocrinology. Basic Concepts and Clinical Correlations. New York: Raven Press, Ltd., 1995.

Williams T, Frohman LA. Hypothalamic dysfunction associated with hemochromatosis. Ann Intern Med 1985; 103:550–551.

PART VI
Emerging Areas of Neuroendocrinology

29 Neuroendocrine Immunology

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Contents

1. OVERVIEW

The neuroendocrine and immune systems function to preserve organismal homeostasis. The role of the neuroendocrine system is to regulate the effects of various metabolic, osmotic, reproductive, and external stressors on the body, whereas the function of the immune system is to eliminate, or at least control, the presence of foreign organisms and substances. To the casual observer, the functions of the neuroendocrine and immune systems may appear independent. However, abundant data now indicate that the integration of these systems enables survival, through extensive *bidirectional communication* that couples homeostasis and immune balance. One example of the bidirectional communication between the neuroendocrine and immune systems is the regulation and control of systemic infection, and its attendant stress on the body. As the brain is alerted to septic stress by cytokines released from the immune system, neurotransmitters and hormones are secreted from the nervous and neuroendocrine systems. These factors serve to stimulate both the immune and stress response during the infection, and to downregulate both of these responses when the foreign agent is eliminated. As shown in Fig. 1, achieving immune balance is critical for survival, as too much immunostimulation may lead to autoimmune disease, whereas too little may result in immunosuppression, opportunistic infections, or death. Thus, from a teleologic perspective, bidirectional communication between the neuroendocrine and immune systems coordinates the body's responses and provides a distinct survival advantage. The goals of this chapter are to detail the communication pathways that exist between the neuroendocrine and immune systems, to describe how such transmissions are integrated, and to document the effects of neuroendocrine-immune interaction on health and disease.

2. STRESS EFFECTS ON IMMUNITY ARE CLINICALLY RELEVANT AND OCCUR BY MULTIPLE MECHANISMS

There are centuries of anecdotal clinical observations of individuals who became sick after stressful

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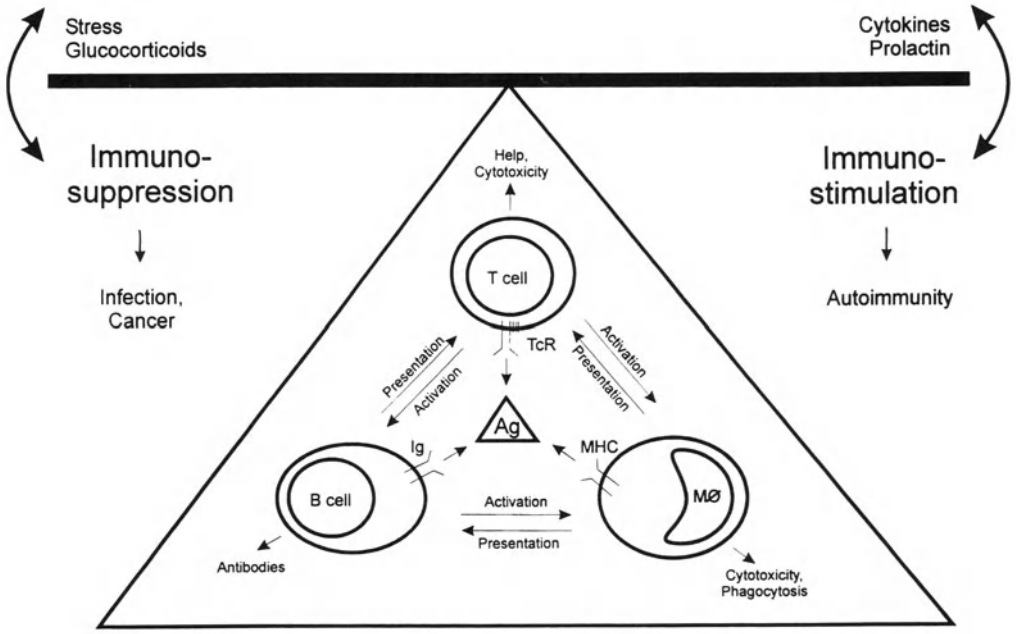

Fig. 1. The neuroendocrine regulation of immunologic homeostasis seeks to achieve a balance between immunosuppressive and immunostimulatory hormones and cytokines. As indicated by the functional inset within the triangle, the interaction of these factors with the immune system influences many aspects of immune function.

situations. Indeed, stress and depression have been increasingly reported to be associated with immunosuppression. For example, clinical studies have shown a correlation between the subjective level of stress in one's life and the probability of contracting a cold. In one study, participants were assessed for the subjective level of stress in their lives and then inoculated with one of several cold viruses. In general, participants with higher levels of stress had an increased incidence of infection that could not be explained by personality, health, or other behavioral factors. Similar studies have found correlations between stressors, such as examinations, bereavement, sleep deprivation, or life changes, with specific changes in immune function, such as cytokine production or immune cell proliferation.

Immune responses can be divided into nonspecific (or innate) and specific (or acquired) immunity. Nonspecific immunity exists from birth and operates in a generalized fashion, whereas specific immunity involves the recognition of a particular foreign substance followed by its destruction and/or removal. Although much of this chapter will focus on specific immunity, it is important to remember when considering the possible interactions between stress and immunity that there are nonimmune factors of host resistance that can be affected by stress. In particular, such factors including peristalsis, mucosal secretions, coughing, and gastric acid provide protection from infection without involvement of immune cells. Each of these functions can be modulated by stress hormones and autonomic nervous system activity. For instance, as a stressor activates the sympathetic nervous system, there will be a reduction in mucus production, which normally contains lysozymes that cleave bacterial cell walls, and a decrease in gastric acid secretions, which normally kill pathogens. Thus, in studies where vulnerability to illness is measured, both specific immunity and nonspecific immunity mechanisms may contribute to the effects of stress.

3. PARALLELS EXIST BETWEEN THE NEUROENDOCRINE AND IMMUNE SYSTEMS

Classically, the principal cellular constituents of the neuroendocrine system consist of a hard-wired network of neurons and neuroepithelium that mediates its principal functions at a distance by the secretion of hormones. In contrast, the immune system is composed of motile *T and B lymphocytes* and *macrophages*, whose initial function is mediated by the migration of these cells to a site of injury, followed by the local elaboration of immune system stimulants, such as *cytokines* and *chemoattractants*, and antimicrobial effectors. A summary of the major compo-

Table 1
Glossary of Immune System Terminology

Tissues	
Generation of cellular components of immunity:	Bone marrow (B-cells, macrophages) Thymus (T-cells)
Sites of antigen presentation:	Lymph organs, spleen, Peyer's patches, skin
Messengers	
Cytokines/Lymphokines (released by immune cells to affect other immune cells or other targets)	
Examples:	Interleukins, Tumor Necrosis Factors, Interferons
Cells	
Lymphocytes:	B-cells (antibody production)
	T-cells (cell-mediated cytotoxicity, B- and T-cell help, cytokine production)
Natural killer cells	(tumor surveillance)
Monocytes/macrophages	(phagocytosis, antigen presentation, cytokine production)
Mast cells	(inflammation)
Neutrophils	(phagocytosis)
Eosinophils	(immunity to parasites)
Basophils	(mast cell precursors)
Cell-Surface Recognition Proteins	
Immunoglobulins	on or secreted by B-cells to mark foreign antigens for clearance
T-cell receptors	on T-cells to detect foreign antigen
MHC complexes	present antigens to B-cells and T-cells

nents of the immune system is listed in Table 1. At first glance, it would appear the neuroendocrine and immune systems have little in common. However, detailed research over the past 40 years has demonstrated a striking degree of parallelism between these systems, when viewed from the perspective of communications networks. As seen in Table 2, both the neuroendocrine and immune systems contain transmitting and receiving effector cells that utilize both feedback and amplification mechanisms to achieve the desired goals. When examined from this perspective, the rigid distinctions between these systems fall apart. Indeed, although hormones derived from the neurohypophysis do function at a distance, they also locally autoregulate their own secretion, in an autocrine/paracrine-like manner, by interacting with specific receptors found within the pituitary and hypothalamus. Similarly, although cytokines and chemoattractants act locally, their effects, as detailed below, can extend throughout the body. Thus, if the neuroendocrine and immune systems need to work in a coordinate manner, it would not be of surprise to find that certain elements of their communication pathways, such as the transmitters and receivers would be shared. An overview of this bidirectional communication between the neuroendocrine and immune systems is presented in Fig. 2. Thus, cytokines released by lymphocytes in the periphery can bind to specific

Table 2
Communication Parallels Between the Immune and Nervous Systems

	Immune System	*Nervous System*
Transmitter:	MHC-Antigen Complex Antibody-Antigen Complex	Neurotransmitter (Ach, DA, NE, EP, Se)
Receiver:	T-cell receptor	Neurotransmitter receptors
Feedback Loops:	Cytokines, chemoattractants	Neurotransmitters, hormones
Signal Amplification:	Clonal Expansion of T-/B-cells	Synaptic potentiation, recruitment
Output:	Response to Antigen	Response to External Stimulus
Long-term effects	Antigenic Memory	Learned Memory

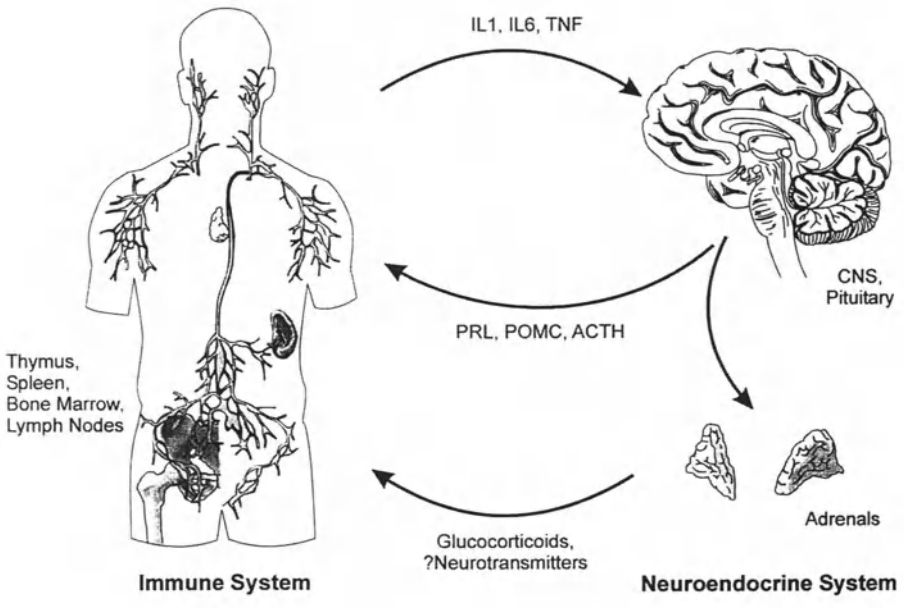

Fig. 2. Communication between the neuroendocrine and immune systems is bidirectional. Cytokines released from the peripheral immune system alter both hypothalamic and pituitary function, in terms of physiologic set points and hormone release. In turn, releasing factors and hormones from the hypothalamus, pituitary, and endocrine glands modulate the immune system function.

receptors in the hypothalamus and pituitary, altering physiologic set points, e.g., temperature, appetite, and hormone secretion. In turn, hormones released by the central nervous system can alter immune function by stimulating or suppressing lymphocyte and macrophage proliferation, cell-mediated cytotoxicity, and cytokine and antibody production. The remainder of this chapter details the manner and effects of such bidirectional communications between the neuroendocrine and immune systems.

4. SYMPATHETIC NERVES AND ADRENAL STEROIDS ARE IMMUNOMODULATORY

Selye was one of the first experimentalists to document that both stress and adrenal extracts cause involution of the thymus gland in experimental animals. Since then, many different stress paradigms, including crowding, isolation, noise, presentation of a predator, restraint stress, uncontrollable shock, and cold stress also have been found to affect adversely various immune parameters or the survival of infected animals. Modulation of immune function not only influences the response to pathogen infection, but also the progression of cancer and autoimmune diseases. Although not all mechanisms have been fully characterized, glucocorticoid and autonomic effects have been well delineated.

The two major pathways by which the central nervous system can communicate with immune system are the *hypothalamic-pituitary-adrenal* (HPA) *axis* and the autonomic nervous system, both of which have demonstrated effects on immune function. The components of the HPA axis include the hypothalamic paraventricular nucleus, which contains neurosecretory cells that release *corticotropin-releasing hormone* (CRH) into the portal vessels that supply the anterior pituitary. In response to CRH, corticotrophs in the anterior pituitary release *adrenocorticotropin hormone* (ACTH) into the general circulation, which is the primary stimulus for synthesis and secretion of *glucocorticoids* from the adrenal cortex. The glucocorticoids are the main effector for the HPA axis, and their receptors are found in virtually all peripheral tissues. In addition, glucocorticoid receptors are found in both the hypothalamic paraventricular nucleus and anterior pituitary, where they exert negative feedback effects on HPA activity (*see* Fig. 3).

In contrast, the output from the autonomic nervous system reaches immune target tissues through direct nerve fibers. The two-neuron chain that comprises the peripheral autonomic nervous system is in turn controlled by various hypothalamic and hindbrain structures. Of particular interest is the hypothalamic paraventricular nucleus, which maintains direct projections to sympathetic preganglionic neurons. Because of its key role in HPA function, the *hypothalamic paraventricular nucleus* may serve to relay stress responses to sympathetic target tissues. In addition to neurosecretory CRH neurons, there are CRH neurons that project within the central nervous system and appear to mediate various stress responses.

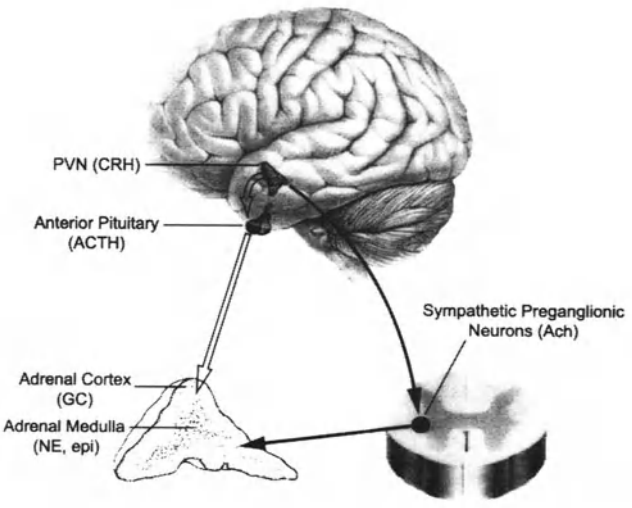

Fig. 3. Neuroendocrine regulation of the immune system by the hypothalamus utilizes both the HPA axis (open arrows) and the autonomic nervous sytem (filled arrows). Abbreviations: Ach, acetylcholine; ACTH, adrenocorticotropic hormone; CRH corticotropin releasing hormone; epi, epinephrine; GC, glucocorticoids; NE, norepinephrine; hypothalamic paraventricular nucleus, paraventricular nucleus.

4.1. There is Direct Neuronal Innervation of the Immune System

It has been known for some time that sympathetic nerves accompany the vasculature supplying by the thymus and spleen. Early speculation that these nerve fibers could influence immune responses required proof that communication could possibly occur at these sites. Formal proof of such communication requires demonstration of the presence of the following: (1) an appropriate transmitter (i.e., a neurotransmitter-containing axonal terminal in juxtaposition with a lymphocyte), (2) receiver (i.e., an appropriate neurotransmitter receptor on such lymphocytes), and (3) demonstration of in vitro and in vivo functional effects when such a communication pathway is stimulated or blocked. Proof of a neural "transmitter" has come by way of electron microscopic examination of nerve fibers within the spleen and thymus. These analyses revealed axonal terminal endings on both vascular walls and T and B lymphocytes found within these organs. In parallel, biochemical analysis of lymphocytes has demonstrated the cell surface expression of β-adrenergic receptors. Taken together, these data make a suggestive case for direct neural-immune communication. A formal demonstration of a functional effect of neural transmitters on lymphoid tissues, however, has remained elusive. In vitro studies using large (i.e., pharmacologic) doses of catecholamines have suggested a suppressive effect of these agents on immune response, as measured by decreased antigen- or mitogen-induced lymphocyte proliferation, cytotoxicity, and cytokine production. Although antagonists can reverse the specificity of this effect, the sizable doses of neurotransmitter required to observe these results raises a reasonable question of biologic significance. The data obtained from animal models also have been tantalizingly suggestive. Interruption of catecholamine signaling in vivo by either surgical or pharmacologic blockade of the thymus or the spleen is immunosuppressive, as measured by antigen-induced antibody production or cell-mediated cytotoxicity. However, many of these studies have neglected to examine whether these experimental approaches could induce the release of other hormones known to mediate stress response and immunosuppression, such as ACTH and glucocorticoids. Furthermore, despite millions of man-years of pharmacologic therapy with β-adrenergic blockers (i.e., propranolol therapy for hypertension), no evidence to date exists in humans that the direct modulation of sympathetic outflow influences immune response. Although these caveats are significant, they should serve to stimulate further investigation of the direct communication between neural networks and the immune system via catecholamines.

4.2. Glucocorticoids are Immunosuppressive (Classic Endocrine Theory)

The effects of glucocorticoids on the immune response were first noted by Thomas Addison in the 18th century in his seminal observations that adrenal hypofunction is associated with an "increase in white corpuscles" in the blood. Hans Selye formalized the connection between stress, the elaboration of steroid hormones, and modulation of the immune response in the 1930s to 1940s in his *general adaptation* or *stress* theory. The general adaptation theory states that in response to a major stress, such as trauma, infection, etc., the body seeks to achieve homeostasis through resistance or adaptation, as mediated in large part by glucocorticoids. If the stress is overwhelming or chronic, decompensation ("stage of exhaustion") can occur. As part of the adaptation syndrome, glucocorticoids were thought to play a major role in the immunosuppression, observed as thymic involution and increased susceptibility to infections, in patients

Table 3
Classic Functions of Glucocorticoids on Immune System

	Numbers in Circulation	*Function*
T-lymphocytes	Decreased	Decreased (↓Mitogenesis, CD4 help)
B-lymphocytes	Decreased	Decreased (↓Mitogenesis, ↓Ab)
Monocytes	Decreased	Decreased (↓Chemotaxis, ↓Ag Presentation)
Neutrophils	Increased	Altered margination
	Quantity	*Effect*
Cytokines	Decreased	Decreased

undergoing chronic stress. The clinical use of pharmacologic doses of glucocorticoids to inhibit autoimmune disease and allograft rejection was interpreted to support this "classic dogma" as glucocorticoids as immunosuppressants.

The classic effects obtained from data gathered from 1950 to 1980 suggest a broad immunomodulatory effect of glucocorticoids, as outlined in Table 3. In the peripheral blood, all major immune system cell types are decreased, with the exception of neutrophils. The relative increase in neutrophils is thought to be secondary to altered cellular adhesion, resulting in neutrophilic demargination. The mechanism of the loss of mononuclear cells within the blood, however, remains uncertain and may result from cellular apoptosis, altered cell adhesion, or combinations of both. Similarly, examination of the thymus under prolonged stress or glucocorticoid administration reveals thymic involution, with a reduction of T-cell progenitors secondary to glucocorticoid-induced apoptosis. Whereas much of this programmed cell death may be directly induced by glucocorticoids, secondary alterations in cytokine release and response also may contribute to the loss of T-lymphocyte progenitors.

Munck first provided an adaptive context for this effect, proposing that adrenal steroids protect the body by preventing a potential overshoot of immune responses, containing or preventing immunologic responses to "self" antigens released during tissue damage. The danger of such a "self-attack" can be seen in autoimmune diseases, where destruction of tissues by monocytes, cytotoxic lymphokines from the T lymphocytes and/or antibodies from B lymphocytes can be chronically debilitating or, in some cases, lethal. Aside from autoimmune diseases, various infections can lead to septic shock, during which the immune response is more detrimental to the host than are the invading microbes. Specifically, cytokine cascades can be lethal because of circulatory collapse, induced by their action at high concentrations. It has long been known that corticosteroids can inhibit the lethal actions of endotoxins in such circumstances. It is now known that endotoxin-induced activation of the HPA axis is mediated by cytokines. In addition, adrenalectomy results in increased sensitivity to the lethal effects of endotoxin. Thus, it appears that as cytokines are induced by pathogens, adrenal steroids are concomitantly evoked to dampen the self-destructive effects of cytokines. Such scenarios provide a basis for understanding the importance of neuroendocrine responses to help buffer and regulate immune function.

It has been suggested that individual differences in stress responses represent a continuum, making some individuals more susceptible to tumor growth (i.e., robust stress responders) and others more vulnerable to autoimmune disease (i.e., weak stress responders). Different strains of rats have provided an opportunity to explore two ends of such a continuum (Fig. 4). The inbred Lewis rats are extraordinarily susceptible to a broad array of experimentally induced autoimmune diseases, including experimental models of rheumatoid arthritis and multiple sclerosis compared with a control strain of rats such as Fischer 344. In addition, the Lewis rats have different physiological and behavioral profiles. In specific, Lewis rats have a profound defect in their stress response system, with very low levels of circulating corticosterone basally and after an acute stressor, whereas the Fischer 344 rats have a robust increase in corticosterone after a stressor. Moreover, Lewis rats are docile, serene, hypoarousable, and nonaggressive, whereas the Fischer 344 rats are easily aroused, nervous, and potentially aggressive. Importantly, physiological replacement of corticosteroids helps protect Lewis rats from the induction of severe autoimmune diseases. The possibility that such relationships apply to humans is supported by the fact that the development of autoimmune disease is rare in patients with

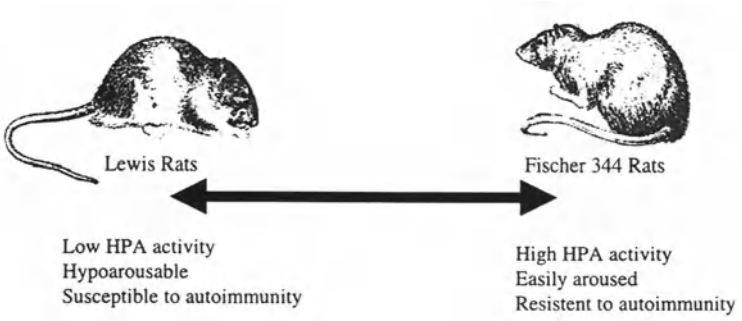

Fig. 4. The proposed spectrum of relationships between the hypothalamic-pituitary-adrenal system, behavior, and susceptibility to autoimmune disease in two different strains of rats.

untreated Cushing's syndrome, a state of hypercortisolemia. Although there are certainly many genetic and environmental factors involved in both cancer and autoimmune disease, support of the idea that neuroendocrine-immune modulation also may influence vulnerability to these diseases has been found by correlating stress responses and tumor and autoimmune resistance in different strains of rats.

Several caveats should be mentioned regarding the "central dogma" of immunomodulation by glucocorticoids. First, many of the above data were obtained using pharmacologic doses of glucocorticoids. Second, many studies focused on rodent models, long known to have a higher sensitivity to glucocorticoids than humans. Third, most prior models of glucocorticoid action had envisioned the effects of glucocorticoid-receptor interaction in a linear, stochastic manner and discounted potential biphasic responses. Fourth, these studies failed to take into account the effects of other cytokines on the immune response. Finally, many studies used pure glucocorticoids capable of interaction with only the glucocorticoid receptor, such as dexamethasone, whereas the principal circulating glucocorticoid, corticosterone, is capable of interaction with both the glucocorticoid and mineralocorticoid receptors. Indeed, most of the receptor-engaged corticosterone in the body is bound to the mineralocorticoid receptor. Thus, although the central dogma of steroid-induced immunosuppression remains largely valid during steroid pharmacotherapy, the physiologic function of steroids within the immune system has been largely unexplored.

4.3. Glucocorticoids Modulate the Immune System through Autocrine/Paracrine and Endocrine Mechanisms

Recent studies from the laboratory of Robert Ashwell have shed new light on the potential physiologic function of glucocorticoids and the immune system. In the course of studying immortalized T-cell hybridomas it was observed the stimulation with either antigen or glucocorticoids alone induced cell cycle arrest and cellular death by apoptosis. These data were consistent with the classic observations of glucocorticoid action on the immune system. It was therefore expected that treatment with both glucocorticoid and antigen would induce an even greater degree of cell death, i.e., the two death signals would summate. Instead, when this experiment was performed with the coadministration of both agents, the T-cell cultures survived! This phenomenon, known as *mutual antagonism* is thought to result from interfering crosstalk between the T-cell and glucocorticoid receptors (more on a potential mechanism for this phenomenon below). If crosstalk between these receptors were to play a meaningful role in the development of the T-cell repertoire, such cross-signaling would need to occur during fetal and adult immune system development. However, very low levels of glucocorticoids are present at such times in the serum. This presented a conundrum—if steroids were critical for promoting T-cell survival or death during fetal thymic development, where were they coming from, if not from the fetal adrenal?

To address this question, another dogma of endocrinology needed to be tackled. Since the inception of endocrinology as a scientific field, in the mid-18th century, the production of hormones has been believed to be the sole domain of the endocrine organs. The discovery in the middle of the 19th century that the placenta could elaborate large quantities of hormones did little to damage this philosophy—instead, the placenta became an endocrine organ. However, with the discovery of the synthesis of pituitary hormones, such as prolactin (PRL) and growth hormone (GH), in tissues such as the breast, prostate, and even lymphocytes, the paradigm began to change. Thus, "pure" endocrine hormones also can be synthesized at a local level and act as *autocrine/paracrine* growth, survival, or differentiation factors.

Given this paradigmatic shift, it was reasoned that fetal thymic tissues might make their own steroid hormones. Indeed, if fetal thymic cultures were fed radioactive cholesterol, labeled glucocorticoids were

subsequently identified in such cultures, proving that T cells were capable of synthesizing such "hormones." If such a locally produced glucocorticoid was functioning to modulate T-cell survival, it would be predicted that inhibition of such synthesis would promote the survival or death of cultures that were respectively unchallenged or stimulated with antigen. Indeed, culture of such fetal thymic cultures with an inhibitor of steroid synthesis (aminoglutethimide) resulted in the enhanced survival of T lymphocytes in unstimulated cultures, whereas antigen-challenged cultures demonstrated marked cell death. These in vitro findings have been extended in vivo by the development and testing of transgenic mice expressing an antisense glucocorticoid receptor gene. These mice demonstrate lower levels of glucocorticoid receptor throughout the body; when stimulated with antigen, fetal thymic lymphocytes demonstrate significant cell death. This again confirms the hypothesis that the unopposed action of either the T-cell or glucocorticoid receptor supplies a death signal, whereas the costimulation of both receptors permits cell survival. Thus, alteration of the relative set-points for T-cell and glucocorticoid receptor crosstalk could enable the survival of autoreactive T lymphocytes, and thus directly contribute to the pathogenesis of autoimmune disease.

4.4. CRH and ACTH can Directly Modulate the Immune System

Until as recently as the mid-1980's, the "classic" endocrine function ascribed to corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) has been to stimulate the release of glucocorticoids from the adrenal cortex. When viewed from an immunologic perspective, this linear cascade of releasing and tropic hormones (CRH → ACTH → Corticosterone) resulted in a suppression of the immune response, as schematized in Fig. 5. This was supported by studies using pharmacologic doses of glucocorticoids, as well as experiments performed on adrenalectomized rodents that demonstrated heightened immunoresponsiveness and/or an increased incidence of autoimmunity in specific animal strains. From a classic perspective, neuroendocrine-immune interaction was essentially mediated by the action of glucocorticoids on lymphocytes. In essence, the signaling between the immune and endocrine systems was thought to be linear and nonoverlapping, as indicated by the heavy lines in Fig. 5.

Recent data, however, has indicated that the supposed dichotomy in neuroendocrine versus immune system signaling may not exist. Data gathered over the past 15 yr has indicated that all of the HPA-associated ligands, i.e., CRH, proopiomelanocortin (POMC), ACTH, and β-endorphin and their corresponding receptors are expressed in lymphocytes. As such, these findings would suggest that these ligands could function both at the endocrine, as well as autocrine/paracrine levels. In the extreme view, which is not shared by all neuroendocrinologists, some proponents have suggested that lymphocytes may serve as local source for HPA hormones, which blurs the distinction between hypothalamic/pituitary and lymphocyte functions. Several in vitro and in vivo models now indicate that CRH, ACTH, and ACTH-derived peptides can modulate lymphocyte responsiveness and gene expression. For instance, both in vitro and in vivo CRH stimulation of human mononuclear cells induces the expression of β-endorphin. The effect on CRH on immunoresponsiveness is site-dependent. Thus, although stimulation of the central nervous system (CNS) with CRH is immunosuppressive in adrenalectomized animals, its peripheral administration in CRH −/− knockout mice is immunostimulatory, regardless of adrenal status. As opposed to the classic "linear" view of the HPA immunoregulatory cascade of hormones, these recent data support the concept that multiple levels of crosstalk exist between the neuroendocrine and immune systems. As a major unresolved issue, the relative importance of these increasingly complex autocrine, paracrine, and endocrine HPA/immune pathways to overall physiologic immunoregulation will remain an active area for continued research.

4.5. The Central Nervous System (CNS) Integrates Immune Modulation

Beyond the issue of glucocorticoid- versus catecholamine-mediated effects of stress on immune function is the CNS integration of stress-induced immune regulation. Brain lesions in rodents in areas known to control either the HPA axis or the autonomic nervous system have been shown to modulate immune function, including various hypothalamic and limbic nuclei. Opioid peptides have been shown to mediate stress-induced suppression of natural killer cell activity by influencing opiate receptors in the brain. In addition, centrally acting CRH appears to mediate stress effects on natural killer cell activity. In sum, the function of direct connections between neural networks and the immune system has not been fully investigated, nor has the brain circuitry and neurochemistry that controls such autonomic nervous sys-

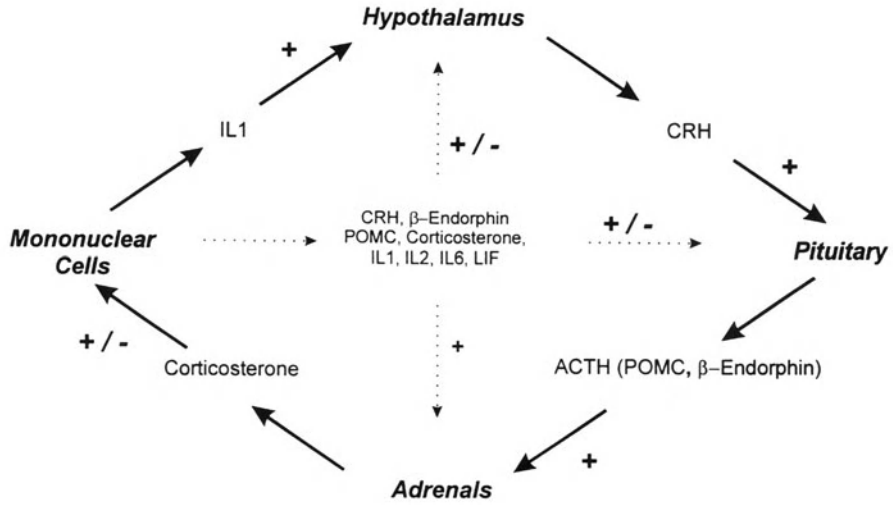

Fig. 5. Bidirectional communications within the HPA-axis utilizes both classic and alternative pathways. The classic pathway (as indicated by the heavy arrows) is largely linear, i.e., hypothalamus - CRH → pituitary - ACTH → adrenals - glucocorticoids → mononuclear cell - cytokines. The identification of the synthesis/secretion of a variety of hormones and cytokines, and their corresponding receptors, at nonclassical sites (i.e., synthesis of cytokines/cytokine receptors in the pituitary, glucocorticoids in lymphocytes) has lead to recognition of the role of alternative pathways (as indicated by the dashed arrows) in neuroendocrine-immune communication.

tem outflow, and this field remains ripe for future investigation.

In addition to immediate and direct effects of neuroendocrine systems on immune function, it also has been established that immunomodulation can be learned in certain cases. Specifically, in experiments by Ader and Cohen, Pavlovian conditioning could be observed in immune responses. In this learning paradigm, a neutral substance, saccharin, served as the conditioned stimulus. This flavor was paired with a noxious immunosuppressive agent, cyclophosphamide, which served as the unconditioned stimulus. When the animals were reexposed to the saccharin flavor they displayed a suppressed antibody response to an antigen challenge. Thus, under the appropriate circumstances, aspects of the immune response may be conditioned in a manner similar to that of endocrine and autonomic reflexes.

5. GONADAL STEROIDS MODULATE IMMUNE FUNCTION

5.1. Sexual Dimorphisms Exist in the Immune Response and Autoimmunity

Physiological, experimental, and clinical data have substantiated the presence of *sexual dimorphisms* within the immune system and its associated responses. In general, women have a more vigorous immune response, a more developed thymus, higher immunoglobulin concentrations, stronger primary and secondary responses, more resistance to the induction of tolerance, and a greater ability to reject tumors and allografts.

The majority of patients suffering from various types of autoimmune disease, however, are also female. For example, a higher female/male susceptibility ratio is seen in systemic lupus erythematosus (SLE), rheumatoid arthritis (rheumatoid arthritis), myasthenia gravis, and multiple sclerosis. As an extreme, autoimmune thyroid disease is 50-fold more common in women than men. Nevertheless, not all human autoimmunity demonstrates a female predominance the female preponderance; juvenile-onset insulin-dependent diabetes mellitus is one such example.

Several lines of evidence suggest a role for gonadal steroids in the development of SLE, where the female to male ratio is approximately 10:1. First, female SLE patients have an increased aromatase activity in a number of tissues, leading to a decreased androgen–estrogen ratio. Second, administration of estrogen-containing contraceptives can aggravate the disease symptomatology. Third, a therapeutic effect has been observed with treatments that increase the androgen/estrogen ratio. Fourth, estrogen also exacerbates the symptoms in experimental models of lupus. The nature of the interaction between sex steroids and the immune system to cause this autoimmune disease remains an area of active investigation.

Unlike SLE, rheumatoid arthritis is ameliorated by estrogens, and oral contraceptives tend to mitigate

the development of the disease. In general, pregnancy reduces the clinical and biological activity of rheumatoid arthritis and a variety of other autoimmune diseases, including Graves' disease, Hashimoto's disease, and myasthenia gravis. The mean age of onset of rheumatoid arthritis in women is usually at approximately 40–59 years of age, which also corresponds to passage through menopause. Although such a correlation might appear to be an attractive etiologic mechanism, estrogen levels in rheumatoid arthritis patients are similar to controls.

Like humans, differential gender susceptibility is seen also in many animal models of autoimmune diseases and transplant rejection. In general, nonpregnant female animals more rapidly reject allografted transplants. In several rodent models, the female of the species demonstrates a more robust and prolonged immune response both at the level of antibody production (i.e., B-cell related immunity) and cell mediated immunity (i.e., T-cell related). This includes the both the NZB/NZW and *lpr/lpr* mouse models of SLE, the Lewis rat model of rheumatoid arthritis, and the nonobese diabetic (NOD) mouse model of type I diabetes. Administration of testosterone to female NZB/NZW mice largely prevents the onset of autoimmune disease, whereas castrated males demonstrate disease acceleration. Thymectomy ablates the beneficial effects of testosterone; hence T-lymphocytes likely contribute to this phenomenon.

5.2. Mechanisms of Gonadal Steroid Function with the Immune System

The examination of immune system cells for the expression and function of sex steroid receptors is incomplete, but both androgen and estrogen binding sites have been reported in thymic, bone marrow, and peripheral immune system tissues. In organs involved in the maturation of T and B cells, sex steroids can have profound effects. Gonadectomy in males and females induces hyperplasia of the thymus, whereas the administration of exogenous androgens and synthetic estrogens can cause thymic involution. Receptors for both androgen and estrogen have been found in progenitor T lymphocytes, and the effects these hormones have on the immune response are most likely secondary to the modulation of specific gene transcription by the sex steroid/receptor complex. Like the thymus, estrogens and progesterones exert a negative effect on B-cell lymphopoiesis within the bone marrow. B cells and stromal support cells have been shown to express the estrogen receptor. The role and expression of androgen receptors within the bone marrow and B cells, however, remains incompletely characterized. Sex steroids also modulate the function of support cells in the immune system. Physiologic concentrations of these hormones stimulate both antigen presentation and cytokine production from macrophages and upregulate the expression of adhesion factors (i.e., integrins) on endothelial cells.

Both in vivo and in vitro studies have shown that sex steroids have a number of effects on the peripheral immune system, influencing the function of mature T and B cells. Estrogen may concomitantly increase the activity of CD4+ ("helper") T cells, and decrease the activity of CD8+ ("cytolytic") T cells. Estrogen treatment has been shown to increase the number of autoreactive immunoglobulin-secreting B cells. Convincing effects of androgens on peripheral T- and B-cells, as well as the expression of androgen receptor on these cells, remain to be demonstrated. Sex steroids also influence the cytokines elaborated from mature circulating lymphocytes. Females are known to produce higher levels of IFN-γ than males. In vitro studies have shown that estrogen enhances mitogen-induced IFN-γ mRNA and protein expression probably through an estrogen response element on the gene for IFN-γ. This cytokine is known to serve a proinflammatory role, so the effects of estrogen on this gene may contribute to the tendency towards autoimmune disease in females. Taken together these data would indicate that alteration of the relative ratio of estrogen–androgen might enhance the overall pathologic immune response to a targeted organ and/or antigen, leading to disease progression.

Reminiscent of the dual endocrine-immune functions of CRH and ACTH, gonadotropin-releasing hormone (GnRH) may also assist in the coordination of the neuroendocrine and immune systems. In addition to its synthesis within the hypothalamus, GnRH RNA has also been detected in both the spleen and thymus. In addition, GnRH receptors have been detected on T cells. Recent data have suggested that GnRH may also be involved in the maturation and senescence of cells within the immune system.

5.3. A Dichotomy Exists Between Pregnancy and Autoimmune Disease

It has been known for sometime that the effects of estrogen and progesterone on the immune response are dichotomous. Specifically, females have a marked predisposition to develop autoimmune diseases (i.e., a tendency for immune system hyperactivity) and yet are incapable of mounting a significant immune response to the sizable foreign antigenic load pre-

sented by the fetus. In other words, the mother demonstrates *immunologic tolerance* of her fetus, despite ready passage of foreign antigens from the paternal genetic contribution from the fetus into the expectant mother.

During pregnancy a dramatic change in the overall responsiveness of the female immune system occurs. In animal models, measures of cell-mediated immunity are suppressed and humoral immunity is preserved or enhanced during pregnancy. During pregnancy, levels of a variety of cytokines decline, including INF-γ, which is a powerful abortifacient. These measures generally correspond with the increasing levels of estrogen, progesterone, and their metabolites, which on average increase 20–30-fold above normal cycling levels. In addition, pregnancy is associated with elevated levels of glucocorticoids, which are potent inhibitors of INF-γ. Thus, in the pregnant female, an inverse correlation exists between the levels of gonadal steroids and immunoresponsiveness. Obviously, factors other than the relative serum levels of estrogen and progesterone must contribute to the lack of an immune response to the fetus. Several hypothesized factors may contribute to this phenomenon including the markedly increased death of T- and B-cell progenitors, the partial exclusion of maternal lymphocytes from the placental barrier secondary to alterations in lymphocyte adhesion, the "tolerizing" presentation of fetal antigens to the maternal lymphocytes, and the elaboration of unidentified immunosuppressive factors.

These changes in immune function may explain fluctuations in autoimmune symptoms during pregnancy. Rheumatoid arthritis, which is dependent on cell-mediated immunity, is generally suppressed during pregnancy, whereas SLE symptoms, which are dependent on humoral immunity processes, can be aggravated during pregnancy.

6. SOMATOLACTOGENIC HORMONES ARE IMMUNOMODULATORY

Of the many functions attributed to PRL, its immunomodulatory role was discovered relatively late. First identified in the 1930s, PRL has been classically regarded as a hormone regulating lactation in mammals, and reproductive functions in nonmammalian species. Although an immunomodulatory role for this hormone was implicated as early as the 1970s in avian species, another decade elapsed before the first definitive studies in rodents were performed. Initial studies at this time had indicated that hypophysectomy produced an immunosuppressed stated in rats. Berczi and Nagy subsequently expanded these findings by hormonal reconstitution of these hypophysectomized animals with pituitary hormones, as summarized in Fig. 6. In terms of immunoresponsiveness, as measured by antibody production and T-cell proliferation, PRL alone reconstituted 70 to 80% of basal immunoresponsiveness. The remaining immunostimulatory activity within purified pituitary hormones was found to reside in growth hormone; none of the other hormones found within the pituitary significantly contributed to the reconstitution of the immune response. The association between PRL and the immune response was further confirmed by the treatment of rats with bromocryptine, a dopamine agonist that serves as a specific inhibitor of PRL release from the pituitary. Rats treated in such fashion demonstrated markedly reduced serum PRL levels and profound immunosuppression, but otherwise normal levels of pituitary hormones. Subsequent studies have also found that reduction of circulating PRL levels via bromocryptine therapy also results in reduced mitogen-induced T-cell proliferation, macrophage phagocytosis, and decreased survival following infection with intracellular bacteria or parasites (i.e., Listeria, Toxoplasma). Parallel decreases in serum PRL and T-cell responsiveness in humans have also been documented in patients in intensive care units receiving pressor support through intravenous dopamine. Whereas recent studies using either PRL or PRL receptor knockout mice have found no intrinsic defect in cellular numbers in the homozygous immune systems, no data have yet been presented on the survival of these animals to infectious stress. In contrast to these studies, disruption of a major PRL signaling pathway, namely Stat5, has provided support for an immunostimulatory role for signals emanating from the somatolactogenic receptors. Indeed, analyses of the Stat 5a-/-/Stat5b-/- double knockout mice have revealed a profound inability of mature, peripheral T lymphocytes to proliferate in response to appropriate stimuli.

6.1. Prolactin Immunomodulation Involves Intracellular Signal Integration

If PRL is an immunomodulatory hormone, its actions should be reproducible and dissectable in vitro. Analysis of isolated peripheral blood lymphocytes, cloned T lymphocytes, and T-cell lines have found that PRL serves as both a necessary comitogen and survival factor for T cells in vitro. As a comitogen, PRL is necessary but not sufficient for proliferation, acting

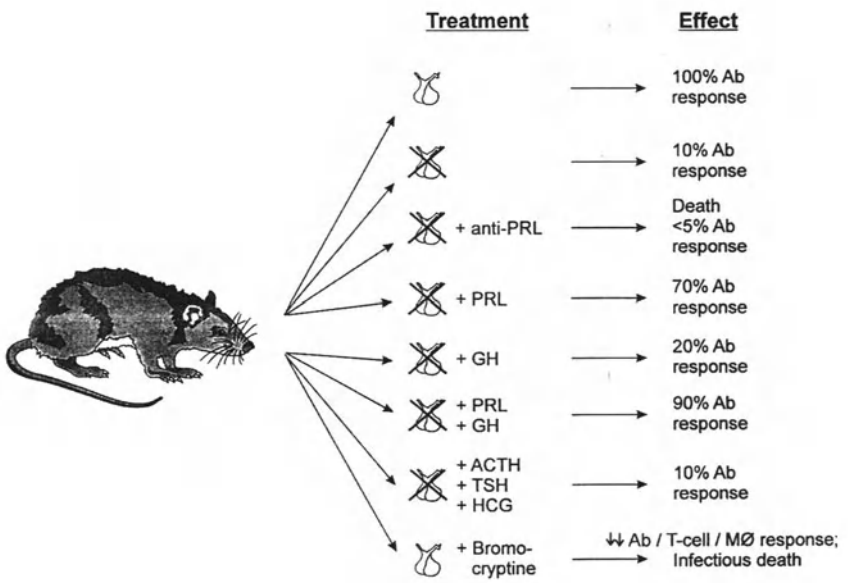

Fig. 6. Effect of pituitary hormone ablation and reconstitution in adult rodents on the immune response. Such studies have convincingly demonstrated that PRL, and to a lesser extent GH, significantly contribute to immunologic homeostasis in adult animal models.

in concert with antigen and IL2 to stimulate cell cycle progression, as schematized in Fig. 7. IL2-stimulated cloned T cells that lack PRL do not enter the S-phase of the cell cycle and do not undergo active DNA replication. Northern blot analysis of mRNA of such cells has revealed that the expression of transcripts of gene products necessary for entry into S-phase, such as the cyclins and histones, fails to occur in the late G1 phase. PRL-induced gene expression is initiated by several PRL receptor-associated signaling pathways, as outlined in Fig. 8. Many of these pathways involve cascades of either tyrosine or serine/threonine kinases, or proteins that allosterically or enzymatically alter the activity of such kinases.

Two protein kinase cascades, namely, the JAK/Stat and the Raf/mitogen activated protein kinase (MAPK) pathways, bear some brief mention. As the PRL receptor itself lacks intrinsic kinase activity, PRL-induced signaling results from the activation of these kinase cascades as a consequence of ligand-induced PRL receptor dimerization. Activation of the JAK2 tyrosine kinase associated with the PRL receptor enables phosphorylation of the transcription factors Stat 5a

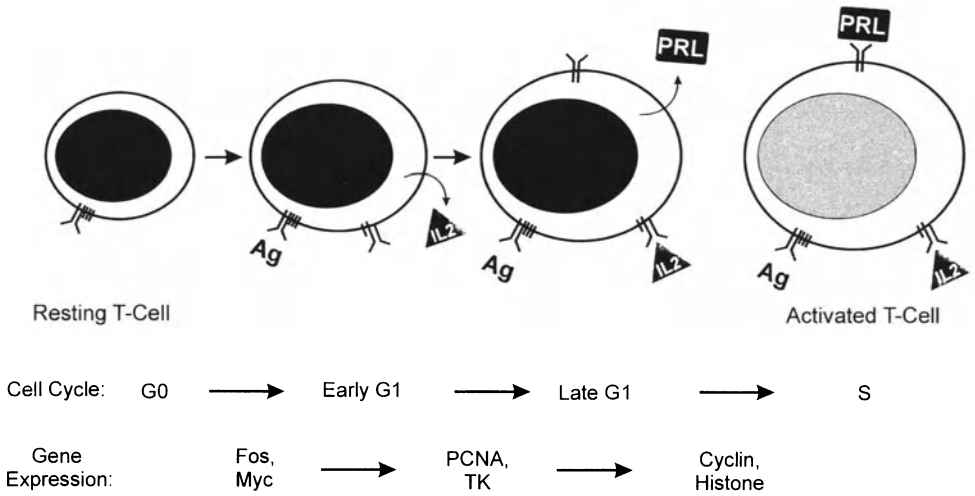

Fig. 7. Function of PRL during an immune response. During antigen- or cytokine-driven immune cell proliferation ("clonal expansion") PRL acts as necessary comitogen. Thus, antigen stimulation of the T-cell receptor induces the expression of immediate early genes, G0 → G1 cell cycle progression, and the generation of both IL-2 and its corresponding receptor. The interaction of IL-2 with its receptor, in turn stimulates the expression of early gene products, early G1(late G1 progression, and the synthesis of both PRL and the PRL receptor. The PRL/PRLr complex in turn enables the T cell to synthesize late gene products necessary for entry into S-phase.

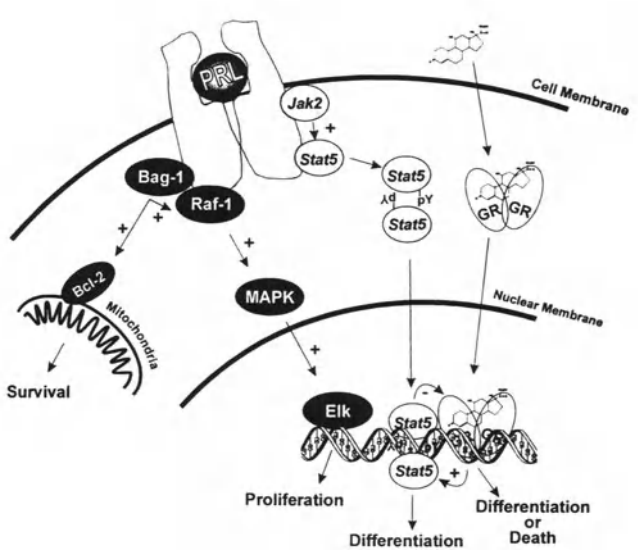

Fig. 8. Modulation of the immune response by PRL involves the integration of multiple signaling pathways. Engagement of PRL with the PRLr induces the formation of a signaling complex that activates mitogenic (i.e., Raf-1/MAPK), anti-apoptotic (Bag-1), and differentiation (JAK2/Stat5) cascades. These cascades in turn can interact with other signaling systems (i.e., glucocorticoid/Stat 5 interaction) leading to the integration of multiple transduced signals.

and 5b. Tyrosine phosphorylation of Stat 5 induces the dimerization of this protein through SH2-phosphotyrosine interaction. This autodimerization is requisite for the entry of Stat 5 into the nucleus, where it specifically interacts with specific gene promoter sequences and stimulates the transcription of PRL-induced gene expression. Whereas the activation of Stat5 appears necessary for PRL-induced differentiation, recent data have indicated that the Stats are unnecessary for ligand-induced proliferation. In contrast, PRL-receptor activation of the polykinase cascade of Raf-1/MAPK ultimately induces the activation of transcription factors such as elk and fos that do contribute to PRL-induced proliferation.

6.2. Prolactin and Glucocorticoids Signaling Pathways are Convergent

How are the divergent signals of the PRL receptor (that triggers survival, proliferation, and differentiation in vitro) and glucocorticoid receptor (that triggers survival and differentiation at physiologic levels and death at pharmacologic levels) integrated within cells of the immune system? Recent research has discovered that extensive crosstalk mechanisms may exist between these apparently distinct pathways. As seen in Fig. 8, such crosstalk converts these biochemically "linear" signaling cascades into highly integrated transduction networks. Thus, although the serine/threonine kinase MAPK phosphorylates transcription factors such as Elk and fos that are associated with the induction of cell proliferation, the serine phosphorylation of Stat5 by MAPK is necessary for this transcription factors full activation as a transactivator of cell differentiation. One level of crosstalk between the proliferation and survival signals occurs at the level of Bag-1. Bag-1 levels are upregulated by the PRLr; in turn, Bag-1 acts as an allosteric activator of the Raf-1 kinase (stimulating proliferation) and the mitochondrial ion channel Bcl-2 (stimulating cell survival). Cross-talk can also occur even at the level of transcription factors, resulting in altered patterns of cellular differentiation or cell death. One such example is the interaction that occurs between the dimerized Stat 5 complex and the glucocorticoid–glucocorticoid receptor complex. While the interaction with the ligand bound glucocorticoid receptor complex upregulates the transcriptional activity of Stat5 from PRL-inducible promoters, this same interaction downregulates the activity of glucocorticoid responsive promoter. The utility of these crosstalk mechanisms in regulating orchestrated patterns of gene expression is readily evident; it would serve the organism little use if an infection-triggered clonal expansion of lymphocytes also resulted in lymphocyte death or inappropriate differentiation.

7. CYTOKINES REGULATE NEUROENDOCRINE FUNCTION AND BEHAVIOR

A variety of behavioral, physiological, endocrine and neural changes are produced by infection, which as a group have been termed the *illness response* because they are thought to work in concert to promote survival. These responses include fever, increased slow wave sleep, decreased activity and social interaction, decreased ingestion and digestion, the formation of taste aversions to novel foods, and hyperalgesia. Fever, immune cell proliferation, and tissue repair are energy intensive, and therefore many of the other illness responses serve to conserve energy. Simultaneously, the release of glucocorticoids enables energy mobilization.

These illness responses are in part produced by IL-1, TNF, and IL-6 either released during infection or after systemic administration. In addition, treating animals with receptor antagonists to these cytokines can prevent illness responses. The CNS is required to mediate the illness responses, as cytokines cannot

act directly on brown adipose tissue to produce fever or alter activity levels. Systemic administration of cytokines produces activation of various brain regions, as shown by molecular markers of neuronal activation, such as the Fos protein, and electrophysiological studies. In fact, brain lesions can block the effects of infection or cytokine administration on decreased ingestion, decreased social interaction, increased slow wave sleep, and HPA activation.

Although it is clear that systemic cytokines affect brain function, several possibilities exist regarding the initial site of action of these factors. First, there are the circumventricular organs, which have a weak blood-brain barrier, and could permit cytokine entry. Second, there is an endothelial-associated transport system that allows low levels of circulating IL-1 to enter the brain. Given such low levels of transport, however, such a mechanism is unlikely to explain the neural effects of IL1. Third, cytokines can infiltrate the CNS after brain damage, but brain damage does not accompany most infections. Fourth, sensory fibers on the subdiaphragmatic vagus nerve appear to mediate at least some of the behavioral and physiological responses to systemic cytokines, including fever, decreased food intake, learned taste aversions, and decreased social behavior.

There also appears to be an autocrine/paracrine utilization of cytokines within the CNS, as evidenced by the synthesis of both IL-1 and its receptor in the brain. Immunoreactive IL-1 has been observed in a variety of brain regions, including the hypothalamus, which suggests a central contribution of IL-1 to neuroendocrine function. IL-1 receptor binding activity and IL-1 mRNA also have been detected in the hypothalamus, hippocampus, and dorsal raphe nuclei. Although a number of other cytokines are synthesized in the brain by neurons and microglia, it is unclear how neural IL-1 release might be stimulated by systemic infection.

7.1. Cytokines Have Global Behavioral Effects

7.1.1. Cytokines Promote Sleep

In humans, IL-1 blood levels are related to sleep and increase during sleep deprivation. Human patients receiving IL-1 therapy have reported somnolence. In cats, IL-1 levels in the cerebrospinal fluid are elevated during sleep. Exogenous IL-1 can increase sleep by either systemic or central administration in rodents and rabbits. Conversely, antibodies directed against IL-1 or an IL-1 receptor antagonist blunt the sleep rebound seen after sleep deprivation in experimental animals, but only when administered centrally. At present, the endogenous source of sleep-promoting IL-1, whether neural, glial, or blood-borne, and the responsive brain regions that are relevant to sleep are unknown. However, growth hormone-releasing hormone is thought to mediate the effects of IL-1 on sleep.

7.1.2. Cytokines Mediate Appetite Suppression

A major cause of morbidity and mortality in the cancer patient is a generalized body wasting known as *cachexia*, which combines the rapid expenditure of caloric stores with food aversion. There are many possible explanations for the tissue wasting of cachexia including mechanical obstruction or ulcerations of the gastrointestinal tract, malabsorption of nutrients, emetic properties of chemotherapeutic agents, suppressed food intake because of the emotional reaction to the illness, and infection. However, in many instances, weight loss is the first manifestation of occult cancer leading to medical advice. Although depletion of host tissues can result from one of several mechanisms of inappropriate energy utilization, inadequate nutrient intake is a major factor. Indeed, implantation of small tumors into laboratory animals can yield a rapid decrease in food intake and body weight, which are reversed by the surgical removal of the tumor. These observations lend credence to the hypothesis that some tumors secrete anorexigenic humoral factors. An alternative hypothesis is that the immune system, in response to the tumor, secretes cytokines with anorexic properties.

Centrally administered IL-1β causes a dose-dependent decrease in food intake and body weight that does not show tolerance after 7 d. At pathophysiological doses, the effects of centrally administered IL-1 on caloric intake and body weight are pronounced. Peripheral IL-1β also suppresses food intake, and although the mechanism is not known, it does not appear to involve vagal afferents. Central administration of IL-1 upregulates the expression of IL-1, its receptor, and *tumor necrosis factor* (TNF), indicating that during illness the brain may become sensitized to the effects of cytokines.

A role for TNF in anorexia and cachexia was originally proposed based on reports that TNF promotes weight loss and lowers food intake. The actual role of TNF in the anorectic effects of tumors is unclear for several reasons. First, rapid tolerance develops to the anorexic effect of exogenously administered TNF. Second, there is no correlation between circulating

levels of TNF and anorexia symptoms in tumor bearing animals. Third, anti-TNF antiserum has only a limited effect at reducing tumor-induced anorexia. At the same time, however, there are several similarities between tumor- and TNF-induced anorexia. First, both treatments induce learned taste aversions to a novel diet, which is mediated by the area postrema, a circumventricular organ in the brain stem. Second, the effects of both tumors and TNF on food intake are mediated by afferent fibers in the vagus nerve. Given the mixed evidence, it has been proposed that TNF acts in concert with other cytokines, such as IL-1, to mediate cancer anorexia.

7.1.3. Peripheral Cytokines Trigger Fever and Hyperalgesia

Fever is considered to be a key element of the illness response. Fever represents an increase thermostatic set-point of the anterior hypothalamus, resulting in an increase in core body temperature. Increased body temperature is thought to enhance survival during illness because it slows replication of pathogens and accelerates immune responses. Fever can be induced by either *exogenous* or *endogenous pyrogens*. Exogenous pyrogens are derived from the offensive microorganisms and include both endo- and enterotoxins from gram-negative and -positive bacteria, respectively. These pyrogens are highly potent and can produce a fever response in the ng/kg range. Not all febrile responses, however, are because of the elaboration of exogenous pyrogens. Indeed, in response to several kinds of injuries lymphocytes and macrophages can elaborate endogenous pyrogens, also known as *pyrogenic cytokines*. The two most potent of these are *interleukin-1* (IL-1) and *tumor necrosis factor alpha* (TNF). In humans, doses as low as 10 ng/kg body mass can produce increases in body temperature of 1.0–1.5°C within 30 min. Other cytokines, such as the *interferons* and *interleukin-6* (IL-6) may also contribute to the generation of fever. Indeed increases in circulating levels of IL-1 can induce the secondary release of IL-6, which provides additional positive feedback during the onset of fever. IL-6 also induces many aspects of the *acute phase response*, an alteration of the proteins elaborated by the liver into the serum. These proteins include antitrypsin, fibrinogen, and complement factors; their alteration within the serum is believed to enhance the bodies' defenses to invading microorganisms.

The mechanism through which fever is induced by the pyrogenic cytokines is incompletely understood. Doses of cytokines that are threshold for producing a fever activate very few brain regions that include the hypothalamic paraventricular nucleus (organ vasculosum lamina terminalis) and the ventrolateral preoptic area. These hypothalamic areas are critical in the generation of fever; their ablation in animal models dramatically reduces the febrile response to pyrogenic stimuli. In response to pyrogenic cytokines, marked increases in the levels of *prostaglandin* E_2 (PGE_2) have been noted in the hypothalamic thermoregulatory centers. Indeed, injection of PGE_2 into the ventrolateral preoptic area mimics the effect of pyrogenic cytokines and induces a febrile response. Immunocytochemistry for Fos, a marker of neuronal activation, has been used for functional neuroanatomical mapping of brain regions involved in cytokine-mediated febrile responses. When small amounts of prostaglandins are infused into the ventrolateral preoptic area, Fos expression is induced in the hypothalamic paraventricular nucleus. It is currently believed that projections from the ventrolateral preoptic area to the hypothalamic paraventricular nucleus mediate the thermogenic response during infection. How circulating pyrogenic cytokines gain access and/or signal the hypothalamic thermoregulatory centers to elaborate PGE_2 is not clearly characterized, although mediation by the intervening endothelium is thought to play a critical role. The inhibition of such synthesis within the hypothalamus is of immense importance to the pharmaceutical industry in that modulation of PGE_2 levels serves as the basis antipyretic therapy by aspirin and acetaminophen. Indeed, many agents that reduce the hypothalamic activity of cyclooxygenase, a key enzyme in the synthesis of prostaglandins, effectively reduce fever. Like cyclooxygenase inhibitors, corticosteroids can also reduce fever by inhibiting cyclooxygenase; in addition, glucocorticoids also inhibit the transcription and synthesis of pyrogenic cytokines.

The CNS incorporates circuitry to both facilitate and inhibit transmission of pain. It is thought that during illness, hyperalgesia may function to decrease energy use, and therefore promote recuperation. The neural circuitry involved in this immune-neural interaction has been well characterized. Infectious agents lead to hyperalgesia by inducing IL-1 and TNF systemically, which activate sensory fibers in the hepatic vagus nerve. These fibers activate the nucleus of the solitary tract in the medulla, which in turn projects to the nucleus raphe magnus. These neurons then send substance P-, cholecystokinin-, and glutamate-containing projections to the spinal cord, which enhances pain neurotransmission in the spinal cord. The purpose of hyperalgesia during infection may be

Table 4
Expression and Function of Cytokines and Cytokine Receptors in the Pituitary

Cytokine	*In:*	*Receptors In:*	*Function:*
IL1	Thyrotrophs, ?others	Somatotrophs	↓ TRH, ?ACTH in vivo; ↑ACTH, LH, GH, TSH in vitro
IL2	Corticotroph	Pituicytes, ?cell type	↑POMC, ACTH
IL6	Folliculostellate Cell	Pituicytes, ?cell type	↑ACTH in vivo; PRL, GH, FSH, LH in vitro
LIF	Pituicytes, ?cell type	Pituicytes	↑ACTH

to promote social isolation, inactivity, and care for any sites of injury.

7.2. Cytokines Stimulate the HPA Axis

In addition to their above-described effects on the CNS, TNF, IL-1, and IL-6 significantly contribute to the activation of the HPA axis activation and the elaboration of a stress response Each cytokine stimulates the HPA axis independently, and their combined effects are synergistic. For example, IL-6 causes a greater cortisol secretion in humans than can be achieved by maximal doses of CRH. This is caused by the IL-6-induced release of arginine vasopressin (AVP) that acts synergistically with CRH as an ACTH secretagogue. IL-6 may also contribute to the inappropriate secretion of AVP, and concomitant hyponatremia, that occurs with a variety of inflammatory diseases.

As mentioned above, IL-1 function has been localized to the hypothalamic paraventricular nucleus (paraventricular nucleus) using immunocytochemistry and receptor autoradiography. In addition, the endogenous IL-1 receptor antagonist also is found in the brain, and its mRNA has been localized in the paraventricular nucleus. Central administration of IL-1 triggers a cascade of CRH, ACTH and adrenal steroid release. In vitro studies have shown that IL-1 also directly stimulates ACTH synthesis and release that suggests IL-1 may act at multiple sites to promote HPA activity (discussed later).

Mental and behavioral components of the stress response include enhancement of central nervous system processes that control arousal, alertness, mood, vigilance, focused attention, and cognition. Hypothalamic CRH appears to play a central role in setting the level of arousal, controlling mood, and modulating attention. Physiological consequences of HPA activation during the stress response serve a critical role in redirecting nutrients and energy utilization. Here again, central CRH directly activates the sympathetic nervous system, and as a consequence blood glucose, heart rate, and blood pressure are increased. At higher doses, centrally administered CRH inhibits appetite and reproductive functions. Therefore, the ability of central cytokines to activate central CRH activity provides a mechanism for global effects on behavior and physiology that are adaptive during illness.

Cytokine-induced activation of the HPA axis also serves as a negative feedback mechanism for immune responses, as studies have shown that brain IL-1 causes immunosuppression mediated by central CRH. In particular, the effect of central IL-1 to suppress cellular immune responses, such as natural killer cell activity and IL-2 production, was blocked by immunoneutralization of central CRH. The suppressive effect of centrally administered IL-1 also was attenuated by peripheral blockade of autonomic ganglia, which suggests that the suppressive effect of central CRH is mediated in part by the sympathetic nerves. Of course, neurosecretion of CRH into the median eminence also modulates the immune system by triggering glucocorticoid release.

Classic endocrine theories have taught that the elaboration of hormones from the pituitary is stimulated by hypothalamic releasing factors and downregulated by hormonal feedback. A growing body of evidence, however, suggests that cytokines may function within the pituitary to regulate the synthesis and secretion of hormones. Although these regulatory cytokines may arrive in the pituitary from the portal circulation, recent data indicate that the expression of some of these cytokines and their receptors occurs directly within the pituitary, as indicated in Table 4. The primary effect of these cytokines, whether lymphocyte- or pituitary-derived, is to modulate the level of hormonal secretion from hormone-secreting cells. Depending on the cytokine, several different effects on hormone secretion have been observed, nevertheless as noted in Table 4, a consistent effect of all cytokines within the pituitary is to increase the level of released ACTH. This is significant in that the ACTH-induced increase in glucocorticoid levels dampens both the direct immunostimulatory effect of these cytokines on lymphocytes, and also served to mitigate some of the "toxic" effects induced by high levels of circulating cytokines (i.e., vascular shock, etc.).

There is also evidence for another feedback mechanisms in HPA-immune interactions. In particular, various cytokines have been shown to decrease expression and affinity of the glucocorticoid receptor. This effect potentially could reduce the immunosuppressive effect of glucocorticoids. Whether cytokines mediate the apparent hypofunction of the glucocorticoid receptor seen in depressed patients is a current area of investigation.

7.3. Does the Immune Response to Brain Injury Cause Brain Damage?

The brain had previously been considered inaccessible to circulating lymphocytes because (1) there is no lymphatic drainage, (2) the presence of the blood-brain barrier, and (3) the minimal expression of major histocompatability antigens in the CNS. More recent experiments, however, have indicated that the CNS undergoes routine surveillance by activated T-lymphocytes, which have the ability to abrogate the blood-brain barrier. Subsequent inflammation only occurs if a second wave of infiltration occurs because of the presence of antigen recognition. Astrocytes can be induced by T-cell mediators to express major histocompatibility complex Ia, which then enhances the astrocyte ability to present antigen.

Evidence for cytokine involvement in neurodegeneration derives from experimental and clinical observations in a wide range of brain insults. It is important to distinguish between the very early acute phase of cell death after brain insults and the subsequent period of delayed neurodegeneration. Studies on cerebral ischemia in the rat indicate that IL-1 contributes to acute neuronal death. At present, the mechanisms of action of cytokines in neurodegeneration are only partially characterized, but include direct neuronal effects on excitatory amino acid release, alterations in glial function, and invasion of peripheral immune cells.

8. SUMMARY AND CONCLUSIONS

This chapter has given an overview of the many levels of crosstalk between the neural, endocrine and immune systems. As clinical research has found clear evidence for immune impairment associated with stress, basic research has found numerous channels of communication that allow immunomodulation by neuroendocrine systems, as well as behavioral modulation by the immune system, especially by cytokines that mediate inflammation. There are many examples of the pathology that ensues when immune responses are unchecked, including septic shock and autoimmune diseases, which indicates the importance of the immunosuppression evoked by cytokine activation of the brain. Conversely, chronic stress can yield inadequate immune responses, leading to susceptibility to infections and possibly tumors. Thus, wellness requires walking a tightrope between these extremes, which explains the redundancy in communication and feedback mechanisms between the brain and the immune system.

SELECTED READINGS

Ashwell JD, King LB, Vacchio MS. Cross-talk between the T cell antigen receptor and the glucocorticoid receptor regulates thymocyte development. Stem Cells 1996; 14:490–500.

Besedovsky HO, Del Rey A. Immune-Neuro-Endocrine interactions: facts and hypotheses. Endocrine Rev 1996; 17:64–102.

Berczi I. The role of the growth and lactogenic hormone family in immune function. Neuroimmunomodulation 1994; 1:201–216.

Black PH. Central nervous system-immune system interactions: Psychoneuroendocrinology of stress and its immune consequences. Antimicrob Agent Chemother 1994; 38:1–6.

Chrousos GP. The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. New Eng J Med 1995; 332: 1351–1362.

Dinarello CA. Thermoregulation and the pathogenesis of fever. Infect Dis Clin N Amer 1996; 10:433–449.

Freidman EM, Irwin MR. Modulation of immune cell function by the autonomic nervous system. Pharmacol Ther 1997; 74:27–38.

McEwen BS, Biron CA, Brunson KW, Bulloch K, Chambers WH, Dhabhar FS, Goldfarb RH, Kitson RP, Miller AH, Spencer RL, Weiss JM. The role of adrenocorticoids as modulators of immune function in health and disease: neural, endocrine, and immune interactions. Brain Res Rev 1997; 23:79–133.

Olsen NJ, Kovacs WJ. Gonadal steroids and immunity. Endocrine Rev 1996; 17:369–384.

Ray D, Melmed S. Pituitary cytokine and growth factor expression and action. Endocrine Rev 1997; 18:206–228.

Selye H. Stress and disease. Science 1955; 122:625–631.

Weigent DA. Immunoregulatory properties of growth hormone and prolactin. Pharmacol Ther 1996; 69:237–257.

Wilder RL. Neuroendocrine-immune system interactions and autoimmunity. Annu Rev Immunol 1995; 13:307–338.

30 Electrophysiology of Hypothalamic Neurons

F. Edward Dudek, PhD, Bret N. Smith, PhD, Kelly J. Suter, PhD and Jean-Pierre Wuarin, MD

CONTENTS

1. INTRODUCTION: WHY ARE ELECTROPHYSIOLOGICAL MECHANISMS CRITICAL TO HORMONE SECRETION?

Just as the neuroendocrine cell is universally considered to be the "final common pathway" for hormone secretion in the field of neuroendocrinology, the action potential and its firing rate and pattern are the "final common mechanism" for release of transmitters, modulators, and hormones. Although graded changes in membrane potential control the secretion of these substances in some situations, nearly every excitable cell in both the endocrine system and the nervous system secretes its transmitter, modulator, or hormone through the generation of action potentials. In traditional terms, action potentials are initiated at the axon hillock near the soma and propagated in an all-or-none fashion to the terminals. This concept is generally true for both conventional neurons and the neuroendocrine cells of the hypothalamus. Thus, if our primary concern is how much transmitter or hormone is released from a terminal of a hypothalamic neuron or neuroendocrine cell, then we must understand how action potentials are generated and what determines their firing frequency and pattern.

The axon and its terminals are the output of hypothalamic neurons and neuroendocrine cells, and the cell body (or soma) and its associated dendrites represent the region specialized for synaptic inputs and their integration. Synaptic connections from other parts of the nervous system are critical in controling the generation of action potentials. Although many neurotransmitters and neuromodulators undoubtedly contribute to the regulation of the firing rate of hypothalamic neurons and neuroendocrine cells, electro-

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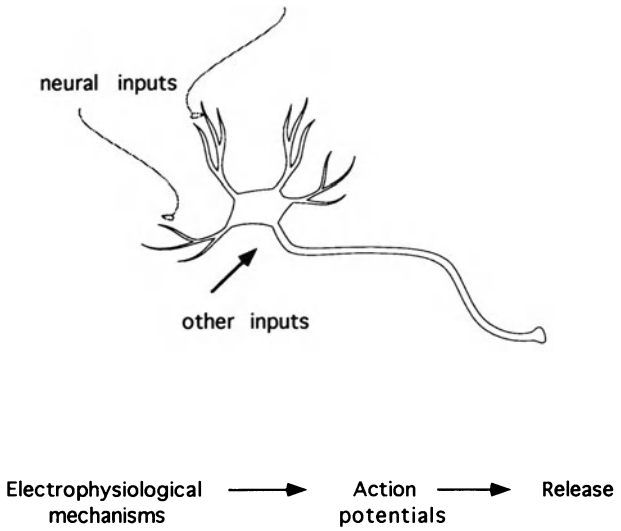

Fig. 1. Neuroendocrine cells integrate neural inputs in the form of excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs), which lead to the generation of action potentials. The action potentials that are conducted along the neurosecretory axon to its terminal are the final common pathway for secretion of neurohormones.

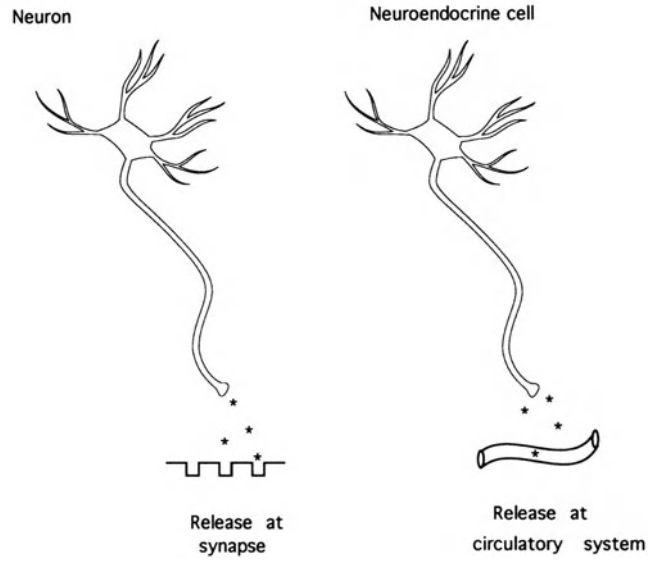

Fig. 2. Conventional neurons and neuroendocrine cells are very similar, except that neuroendocrine cells release hormones into the circulatory system.

physiological studies over the last decade have provided strong evidence that—like neurons throughout the nervous system—the amino acid transmitters glutamate and γ-amino butyric acid (GABA) generate, respectively, the excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs) of hypothalamic neurons. The available evidence indicates that virtually all of the fast synaptic potentials recorded in hypothalamic neurons are the result of these two amino acid transmitters. Other neural inputs apparently do not generate fast synaptic potentials, but are responsible for slow changes in membrane conductance that may "modulate" membrane potential and affect the probability that a cell will generate action potentials. These neuromodulators are generally amines and peptides, and they include, but are not limited to, acetylcholine, norepinephrine, histamine, oxytocin, and vasopressin. A wide range of other biologically active chemicals, such as steroid hormones, act on the somadendritic region of hypothalamic neurons and neuroendocrine cells to regulate the integrative functions of these cells (Fig. 1). These substances may act on membrane and/or nuclear receptors, but to have an impact on hormone secretion, they must alter the likelihood that a hypothalamic neuron or neuroendocrine cell will generate action potentials. Thus, these inputs to hypothalamic neurons and neuroendocrine cells determine their action potential firing behavior, which is the final element that regulates the release of transmitter, modulator, or hormone in the hypothalamus. An understanding of the fundamental electrophysiological mechanisms responsible for this integration of electrical signals is the goal of this chapter.

2. NEUROENDOCRINE CELLS ARE SIMILAR TO CONVENTIONAL NEURONS: A HISTORY OF COMPARISON

For more than a quarter of a century, a fundamental question in the combined fields of neurophysiology and neuroendocrinology has been, "How are neuroendocrine cells and traditional neurons the same, and how are they different?" Classic histological studies in both invertebrates and vertebrates have revealed that neuroendocrine cells possess large neurosecretory granules at the release sites, whereas conventional neurons have synaptic vesicles. Furthermore, neurons, including hypothalamic neurons, form synapses, whereas the axons of neuroendocrine cells terminate at specialized neurohemal organs that link the nervous system to the circulatory system (Fig. 2). Numerous studies in the 1970s and 1980s using invertebrate preparations described the distinctive appearance of neuroendocrine cells, even at the level of the light microscope. For example, neuroendocrine cells in gastropods tend to have a white coloration that gives them an appearance distinctive from conventional neurons. Early electrophysiological recordings often showed that these putative neuroendocrine cells

may also have unique electrophysiological characteristics, such as action potentials of particularly long duration and a greater tendency to fire action potentials in the pattern of burst discharges. These differences in the overall cell biology of neuroendocrine cells vs conventional neurons implied that they could be fundamentally different, but the degree to which this was true remained a question. To the contrary, numerous studies on the electrophysiological properties of neurons and neuroendocrine cells in the hypothalamus, particularly in mammalian preparations, have emphasized the similarities rather than the differences in these two types of cells.

The long history of comparing conventional neurons and neuroendocrine cells began with invertebrates and lower vertebrates, but has continued with a variety of mammalian hypothalamic systems, with particular focus on the magnocellular neuroendocrine cells of the supraoptic and paraventricular nuclei. Two structural characteristics have long been attributed to neuroendocrine cells: large synaptic terminals and simple dendrites. However, these features can by no means be considered unique for neuroendocrine cells, because other neurons are known to have large characteristic synaptic terminals (e.g., motor neurons and the mossy fibers of the dentate granule cells). Furthermore, a host of neurons throughout the nervous system have dendrites as simple (e.g., few branches and spines) as those characteristic of hypothalamic neurons and neuroendocrine cells. Early studies also suggested that neuroendocrine cells might have unusually long action potentials and that their integrative mechanisms might be more likely than neurons to incorporate the use of neuromodulators and peptides. However, both of these concepts are also probably erroneous. Although large terminals, simple dendrites, long-duration action potentials, and extensive use of neuromodulators and peptides might characterize some neuroendocrine cells, these features are common throughout the nervous system and are hardly unique to hypothalamic neurons or neuroendocrine cells. Thus, the underlying thesis of this chapter is that neurons in the hypothalamus, and even hypothalamic neuroendocrine cells, are characterized more by their similarity to other neurons than their differences.

3. ACTION POTENTIALS AND THEIR FIRING PATTERNS MEDIATE HORMONE RELEASE

The rate and pattern of action potentials is the final determinant of the quantity of hormone released from the pituitary. These two characteristics of action potential discharge are critical throughout the nervous system and are the basis of all integrative mechanisms. Thus, if an individual cell fires an action potential, a quantitative description of how repetitive action potentials are generated is essential to defining the overall state of activation of that neuron or neuroendocrine cell. Obviously one definitional state of activity is a "silent cell" that is not generating any action potentials in that particular condition or situation. Action potential generation can be either slow or fast, and the pattern can either be regular, irregular, or bursting (Fig. 3). An example of a slow regular pattern would be the generation of one action potential once every several seconds (i.e., the interspike interval is several seconds). If the action potential frequency increased to many spikes every second (e.g., 5/s, 5 Hz), then the firing rate and pattern would be considered to be fast regular. Many cells in the nervous system do not have a regular pattern, however; interspike interval can be quite variable and would thus be classified fast irregular. The characteristic firing pattern of neuroendocrine cells, particularly during

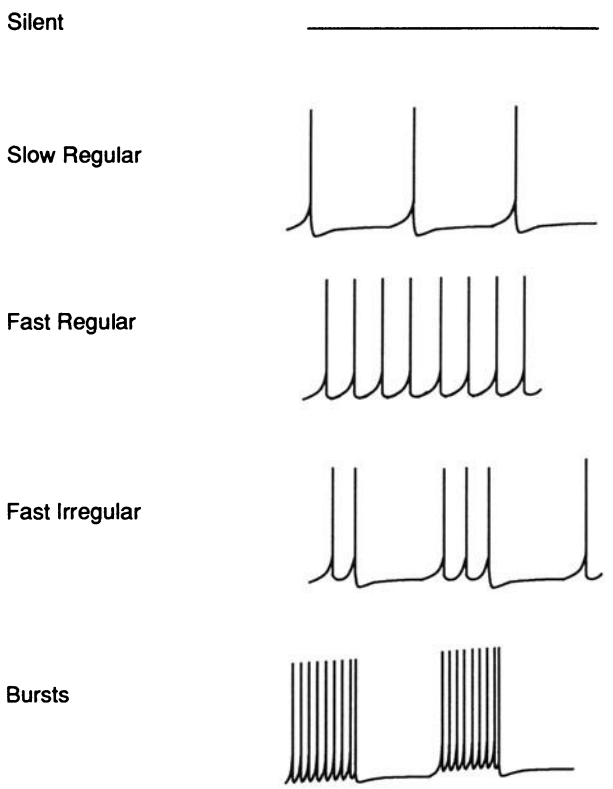

Fig. 3. Neuroendocrine cells have the typical range of firing patterns. These include silent, slow regular, fast regular, and burst discharges. Hormone release is thought to be maximal during burst firing.

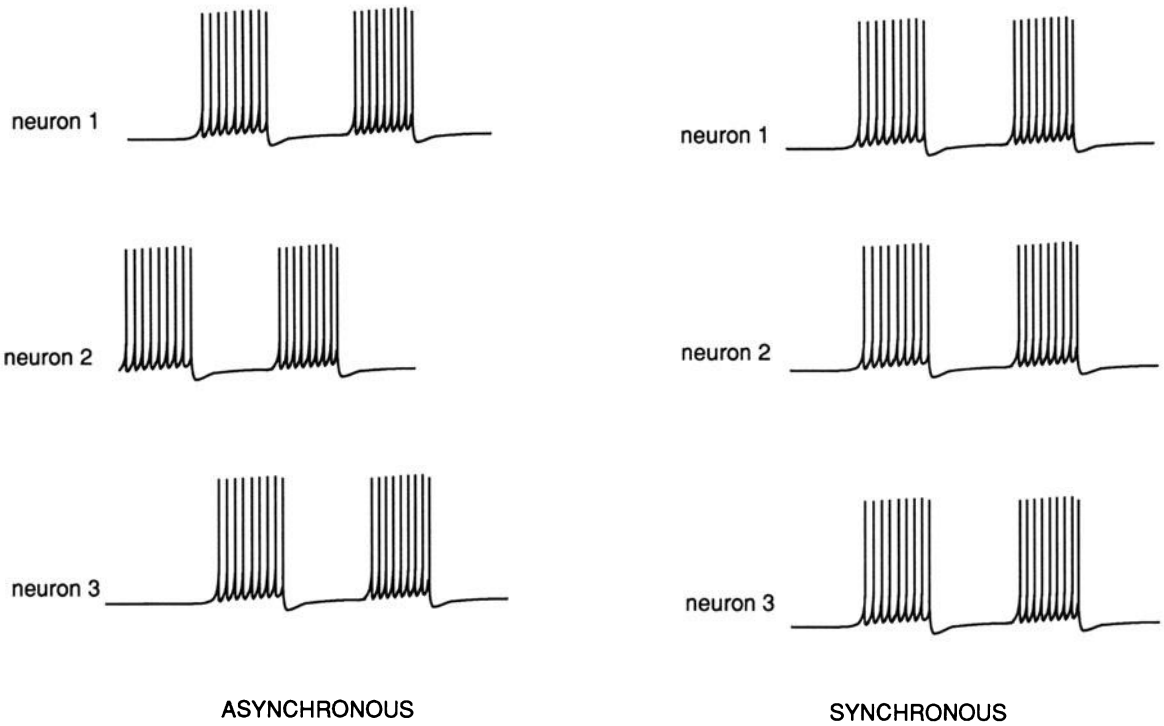

Fig. 4. Bursts of electrical activity in neuroendocrine cells can be asynchronous or synchronous in the population. When the bursts are asynchronous in the population, as in the vasopressin system, neuroendocrine control is often graded. When the bursts are synchronous throughout the network, as in the oxytocin system, hormone secretion tends to be pulsatile.

intense activation, appears to be a burst discharge pattern. Again, the bursts can be either regular or irregular. An example of regular bursting would be one where the interspike interval within any burst is short and constant, and the interval between bursts is long and also relatively constant (e.g., interspike interval of 10 ms during a 1-s-duration burst, yielding a transient spike frequency of 100 Hz, with a constant interburst interval of 10 s). A more realistic situation, however, is that both the bursts and the interburst intervals are variable. Thus, an understanding of how individual neurons or neuroendocrine cells contribute to the output of any hypothalamic system involves the definition of how that neuron is firing. Is it silent, slow, or fast-firing? And if it is firing, are its action potentials regular, irregular, or in a burst-generating mode?

If individual neurons can have a wide range of firing rates and patterns, then a typical population of neurons or neuroendocrine cells in the hypothalamus can obviously have a much more complicated overall discharge rate and pattern. Probably the most elementary distinction when considering a population or network of hypothalamic neurons is whether the activity is asynchronous or synchronous (Fig. 4). This distinction can be applied to individual action potentials, but is probably more important when considering bursts of action potentials. Secretion of hormones can obviously be either graded or pulsatile in nature, and it is widely believed that these characteristics and the magnitude of hormone release depend on whether bursts are either asynchronous or synchronous. Thus, to optimize graded release of hormone, one mechanism is to have the action potentials be in a pattern of asynchronous bursts. An increase in hormone release would then be the result of an increase in the number of neuroendocrine cells firing burst discharges, and of the overall frequency and characteristics of the action potentials and bursts. It is well known that many neuroendocrine populations have a pulsatile pattern of hormone release; such a pattern presumably derives from synchronization of the bursts of action potentials across the population. As will be discussed later, the mechanisms responsible for generating bursts of action potentials are both intrinsic to the individual cell and the consequence of network interactions mediated by several possible mechanisms. Both asynchronous and synchronous bursts can involve intrinsic mechanisms, but obviously the synchronous bursts also depend upon some form of inter-

action or communication between neurons or neuroendocrine cells in order for them to generate and maintain a synchronous pattern of discharge.

4. WHICH ASPECTS OF CELLULAR NEUROPHYSIOLOGY ARE FUNDAMENTAL DETERMINANTS OF FIRING RATE AND PATTERN?

Hypothalamic systems share many neurophysiological characteristics with other neuron systems, whether it be under normal conditions such as sensory or motor integration or pathophysiological conditions such as seizures and epilepsy. The most basic characteristic of any neuron is the resting membrane potential. It is not fully appreciated, however, that resting potential is quite variable across neurons and neuroendocrine cells, and this feature can be regulated by extrinsic sources. Obviously, as discussed above, action potentials are the fundamental characteristic that is the final outcome of synaptic and nonsynaptic integration, which ultimately leads to communication of signals along axons and secretion of transmitter, modulator, or hormone from terminals. Many investigators do not appreciate, however, the extraordinary importance of the membrane potential fluctuations known as afterpotentials that follow each action potential or bursts of action potentials. These afterpotentials play a critical role in generation of the pattern of action potentials (e.g., burst generation).

Another fundamental characteristic of any neuronal system is the excitatory and inhibitory synaptic input. Like other neurons, hypothalamic neurons and neuroendocrine cells use glutamate and GABA as the primary transmitters mediating fast communication. The receptor subtypes for these two main transmitter systems are similar in the hypothalamus to other areas of the nervous system. After fast transmission, one must consider the slower neuromodulatory mechanisms that play an important role in controlling firing rate and pattern. Neuromodulatory mechanisms can be either presynaptic or postsynaptic; some neuromodulators use both systems and use them simultaneously. The degree of synchronization, and the cellular mechanisms that mediate synchronization of activity in hypothalamic systems, ultimately determines such critical factors as whether hormone secretion will be graded or pulsatile. It is the complex combination of these fundamental neurophysiological features and mechanisms that determine how an hypothalamic system integrates information and how neurohormones are secreted into the circulatory system.

4.1. Resting Potential and Other Intrinsic Membrane Properties

Most cells have a resting potential determined primarily by a high permeability or conductance to potassium, plus a small, but distinct, sodium permeability. Resting potential is not always constant in any given neuron and is not the same across all neurons. Many studies over the last quarter of a century on a range of neurons throughout the nervous system, including hypothalamic neurons and neuroendocrine cells, have shown that resting membrane potential varies across cell types. Although there is significant potential for error in measurements of resting potential, a large body of data strongly suggest that resting potential can vary from less than −50 mV to more than −85 mV. It must always be appreciated, however, that the actual process of recording intracellularly from any cell requires penetration or disruption of the membrane; if this is associated with any injury to the cell, the resting potential is likely to be artificially low (i.e., less negative than if the membrane were not penetrated or disrupted). As might be expected, the rate of spontaneous activity (i.e., the rate of action potential discharge) and the apparent amplitude of excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs) are a function of resting potential (Fig. 5). Just as resting potential can be variable across different types of neurons, so can action potential threshold.

Nonetheless, if all other factors are equal, the more depolarized a neuron is at any one time, the more likely it is to generate action potentials at a higher frequency. Thus, if a neuron has a relatively depolarized resting membrane potential, such as −50 to −60 mV, then it is more likely to generate action potentials spontaneously (Fig. 5A). If a neuron or neuroendocrine cell has a resting potential of −60 to −70 mV, then it is more than likely to fire slower (Fig. 5B). As one might expect, those cells with more negative resting potentials, such as −80 to −90 mV, are most likely to be silent and lacking any action potentials under resting conditions (Fig. 5C). Therefore, all other factors being equal, the resting potential determines the firing rate.

Resting membrane potential affects—if not determines—the apparent amplitude of EPSPs and IPSPs. The basis for this relation is founded in the concept that the ions generating EPSPs have an equilibrium

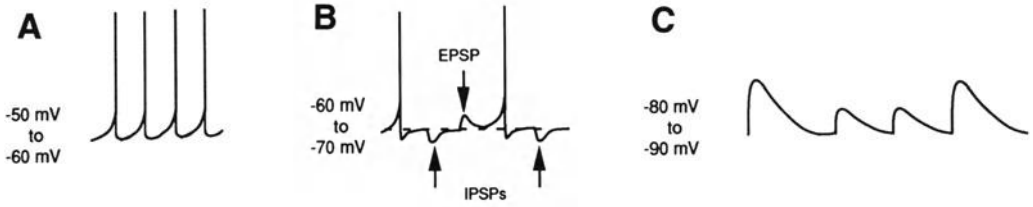

Fig. 5. In both conventional neurons and neuroendocrine cells, resting potential is not a "constant;" it differs depending on the type and state of the cell. **(A)** Relatively low resting potentials (e.g., −50 to −60 mV) are generally associated with spontaneous firing and EPSPs appear small, if they are even observed at all. **(B)** Slowly firing neurons often have resting potentials of −60 to −70 mV, and EPSPs and IPSPs are usually discernable. **(C)** Silent neurons generally have very negative resting potentials (−80 to −90 mV); EPSPs are often large and IPSPs may be reversed in polarity.

potential near 0 mV (the combined equilibrium potential for sodium and potassium), and the ions generating IPSPs have an equilibrium potential at −75 mV or more negative (i.e., equilibrium potentials for chloride and potassium ions). Thus, when a cell has a relatively depolarized resting potential of −50 to −60 mV, the action potentials will tend to obscure the EPSPs (Fig. 5A), yet IPSPs may be apparent. At resting potentials of −70 mV, for example, the slower firing rate allows one to more easily discern synaptic events, and close examination of the baseline of an intracellular recording will reveal the presence of both EPSPs and IPSPs (Fig. 5B). Similarly, when neurons are silent with large negative resting potentials of −80 mV or greater, the lack of action potential generation and the large driving force between the resting potential and the equilibrium potential for the ions generating the EPSPs leads to the observation of large-amplitude EPSPs in the baseline (Fig. 5C). Under these conditions, IPSPs will be small. This fundamental characteristic of intracellular recordings applies not only to hypothalamic cells, but to all neurons throughout the nervous system. Thus, an understanding of the factors that cause changes in membrane potential, no matter how slow they may be, is critical to understanding synaptic integration and generation of action potentials in the hypothalamus.

4.2. Action Potentials and Their Afterpotentials

The traditional explanation of action potential generation is that if depolarization of the membrane exceeds a threshold potential, voltage-dependent sodium channels open in a positive-feedback manner to generate an action potential whose upslope is determined by sodium current and whose downslope is determined by potassium current (Fig. 6A). Less widely understood is the important role of calcium ions and voltage-dependent calcium channels in generating and modulating the waveform of action potentials. Numerous studies in conventional neurons and neuroendocrine cells have shown that the primary charge carrier for most action potentials, particularly in axons, is sodium. A slow but important contribution of calcium, however leads to a small "hump" on the downslope of an action potential, particularly when examined carefully at a fast time-scale (Fig. 6B). Thus, the calcium component of an action potential tends to prolong its duration, and it also is the mechanism by which calcium enters the cell during action potentials, particularly at the soma and the axon terminal. When sodium-mediated action potential generation is blocked with the selective sodium channel blocker tetrodotoxin, depolarization in many cells can lead to the generation of a calcium-mediated action potential with no sodium contribution whatsoever (Fig. 6C). These action potentials tend to have unconventional characteristics; they are often graded in amplitude without a discrete threshold, and their duration can be much longer than the traditional sodium-mediated action potential. Therefore, when one considers action potentials in neurons and neuroendocrine cells of the hypothalamus, one must always consider the relative role of sodium and calcium in the generation of these events.

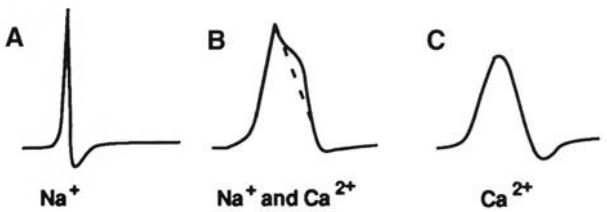

Fig. 6. Action potentials have both sodium and calcium components. The sodium-mediated action potentials are fast, whereas calcium-mediated action potentials are slower.

Any analysis of the ionic basis of the action potential must take into account which part of the cell is under consideration (Fig. 7). The axon, for example,

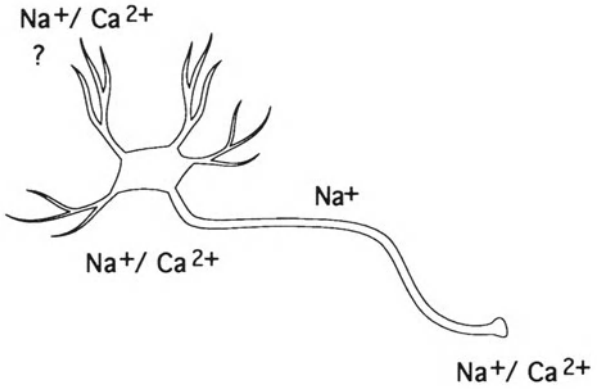

Fig. 7. Action potentials can occur in axons, somata, dendrites, and terminals. Some dendrites have the capability for action potentials, but others do not. Both sodium and calcium action potentials can conceivably occur in the dendrite, both occur in the somata and terminals. It is generally accepted that axons conduct sodium-mediated action potentials exclusively under normal conditions.

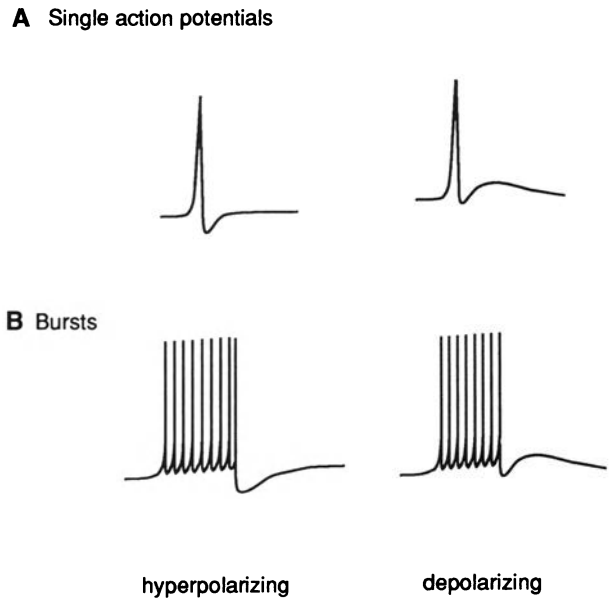

Fig. 8. Action potentials are followed by afterpotentials. A single action potential traditionally is followed by a hyperpolarizing afterpotential, although depolarizing afterpotentials are also observed. Following bursts of action potentials, afterhyperpolarizations and afterdepolarizations can also occur, and these are thought to generate and modulate the bursts.

is the part of a neuron or neuroendocrine cell most likely to have an action potential dependent exclusively on sodium influx and relatively independent of calcium influx. Thus, exposure of a preparation (e.g., a brain slice) to tetrodotoxin blocks action potentials in axons, but does not necessarily block all action potentials in the soma. Tetrodotoxin can be used to block most communication between nerve cells; electrical events are unlikely to spread passively from the soma along the axon to a terminal, because cable properties prevent passive spread of current over long distances.

Virtually all terminals and somata have calcium channels, thus the sodium-mediated action potential in these structures depolarizes the cell to a level so that voltage-dependent calcium channels allow entry of calcium into the cell. At the terminal, it is the influx of calcium that is ultimately believed to be responsible for secretion of transmitter, modulator, or hormone. At the soma, calcium entry probably has a wide range of effects, not the least of which is to modulate other ion channels (e.g., calcium-activated potassium conductance) and influence a variety of second messenger systems and signal transduction pathways. Finally, the most contentious area of neural integration with regard to action potentials is the dendrite. Most of the controversy concerning action potentials and dendrites has centered around other cell types, such as cerebellar Purkinje cells and cortical pyramidal cells. The role of voltage-dependent sodium and calcium channels in action potential generation (or even modulation of synaptic events) is less well understood in hypothalamic neurons. It is likely, however, that sodium and calcium channels play an important role in the integration of synaptic input in hypothalamic neurons and neuroendocrine cells.

As all students of introductory neurophysiology appreciate, the action potential is usually followed by a hyperpolarizing afterpotential. One can view the electrophysiological signature of a neuron as including not only the mechanism of action potential generation, but the succeeding changes in membrane potential that are associated with the afterpotentials (Fig. 8A). It is widely considered that the hyperpolarizing afterpotential is caused by the persistence of a high potassium conductance immediately after an action potential, so that the membrane potential shifts even closer to the potassium equilibrium potential than is normal during the resting state. The hyperpolarizing afterpotential therefore regulates firing rate. Although these hyperpolarizing afterpotentials are as important in the hypothalamus as other areas of the nervous system, less well understood is the role of depolarizing afterpotentials, which are also observed following action potentials (Fig. 8A). The important role of the depolarizing afterpotential, as described in many other neurons (e.g., the cortical pyramidal cell), is its contribution to generation of burst discharges. When a depo-

larizing afterpotential is generated, the depolarization allows generation of a second action potential. Because the second action potential can also cause a depolarizing afterpotential, it is possible to generate repetitive action potentials or burst discharges through this intrinsic mechanism. Using this mechanism, there is evidence that some neuroendocrine cells, such as those comprising the magnocellular neuroendocrine system of the supraoptic and paraventricular nuclei, generate depolarizing afterpotentials that may contribute to the burst discharges characteristic of these neurons. Likewise, just as an individual action potential can have a hyperpolarizing or depolarizing afterpotential, burst discharges can be followed by longer-lasting afterhyperpolarizations and/or afterdepolarizations (Fig. 8B). It is likely that the individual action potentials generate these postburst potential changes through some type of summation during the bursts, most likely involving Ca^{2+} accumulation intracellularly, but other mechanisms are likely to be important also. These postburst afterhyperpolarizations and afterdepolarizations almost certainly contribute to regulation of interburst interval. Thus, whereas not unique to neurons or neuroendocrine cells in the hypothalamus, the slow potential changes that follow both individual action potentials and also bursts of action potentials are important mechanisms of cellular integration. This type of intrinsically generated burst mechanism ultimately contributes to determining the firing pattern of neurons and neuroendocrine cells in the hypothalamus.

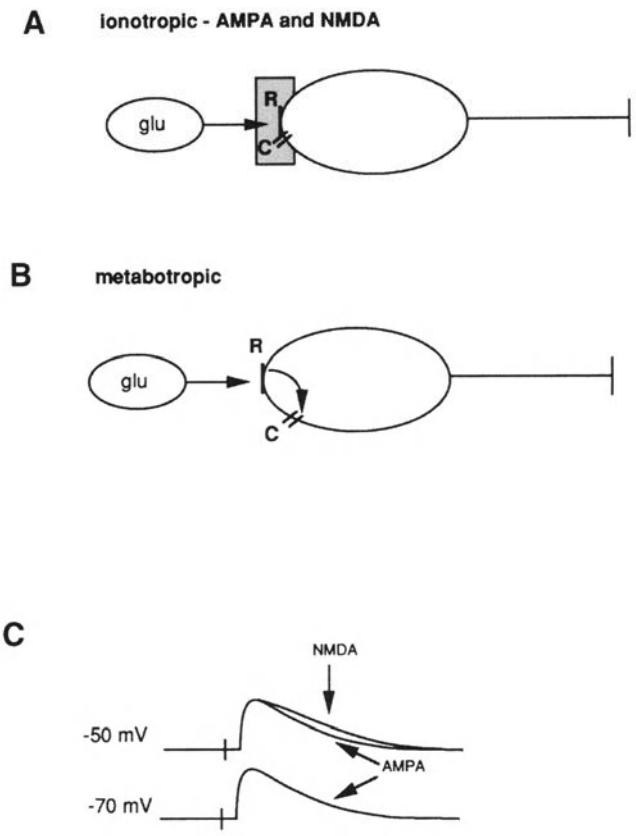

Fig. 9. Fast EPSPs are generated by ionotropic **(A)** and metabotropic **(B)** glutamate receptors, and AMPA and NMDA receptors generate the fast EPSPs **(C)**. At depolarized membrane potentials, there is an increased NMDA component.

4.3. Excitatory and Inhibitory Postsynaptic Potentials (EPSPs and IPSPs): The Importance of Glutamate and GABA

Although the properties of resting potentials, action potentials, and afterpotentials are critical for determining the output or activity of hypothalamic neurons, the first line of integration of input is the fast excitatory and inhibitory postsynaptic potentials resulting from activation of glutamate and GABA receptors. Although many neuroactive substances control the rate and pattern of action potentials, it is undoubtedly glutamate and GABA that generate most fast EPSPs and IPSPs in the nervous system, including hypothalamic neurons and neuroendocrine cells. There has been a long-standing concept that the hypothalamus preferentially integrates information based on neuromodulators and neuropeptides. This principle(s) is almost certainly exaggerated and probably wrong; glutamate and GABA appear to be as important in regulating electrical activity in the hypothalamus as they are in the rest of the brain, just as neuromodulators and peptides are probably as important in the rest of the brain as in the hypothalamus. Thus, a full understanding of how hypothalamic systems integrate information, particularly on the millisecond-to-second time scale, necessarily involves the amino acid neurotransmitters glutamate and GABA.

Acetylcholine is widely appreciated to generate fast synaptic excitation at the neuromuscular junction, but its possible role in synaptic excitation in the hypothalamus appears to be nonexistent, at least as a fast synaptic mechanism. Numerous studies, particularly in slice preparations over the last decade, have shown that probably all EPSPs result from the excitatory transmitter glutamate. Glutamate receptors can be separated into ionotropic (receptor directly linked to channel, Fig. 9A) and metabotropic types (receptor modulates channel through second messenger systems, Fig. 9B). The ionotropic receptors can be further divided into AMPA and NMDA types. At resting

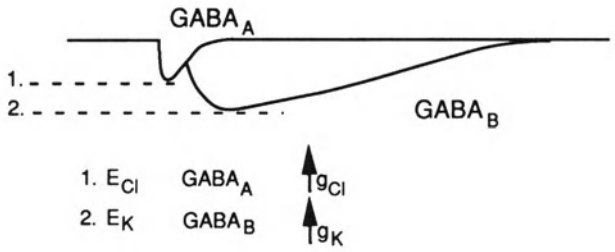

Fig. 10. IPSPs are generated by ionotropic and metabotropic GABA receptors. $GABA_A$ receptors generate fast IPSPs through an increase in conductance to chloride, whereas $GABA_B$ receptors generate slow IPSPs because of an increase in conductance to potassium.

potentials more negative than –60 mV, the transmitter glutamate primarily causes current to flow through AMPA-receptor-activated channels (Fig. 9C). When the cells are depolarized, however, an important contribution from NMDA receptors is realized. The fundamental mechanism by which glutamate activates NMDA receptors is founded on the concept that magnesium ions block the channel of NMDA receptors when cells are at negative membrane potentials, but as they become depolarized, the magnesium ion is shifted out of the channel. This allows sodium and calcium ions to cross the membrane when glutamate binds to the NMDA receptor at depolarized potentials. The synaptic component from NMDA receptors is generally slower than the AMPA-receptor-mediated component. One area of confusion in the field of neuroendocrinology is the lack of appreciation that AMPA vs NMDA receptor involvement depends primarily upon the background level of depolarization at the time of glutamate receptor binding. Activation of ionotropic AMPA and NMDA receptors is the fundamental mechanism of generation of fast synaptic potentials, and yet these mechanisms can also have long-term effects on neuronal activity. Finally, glutamate receptor activation is more complex, because the metabotropic glutamate receptors have long-term regulatory influences acting through second messengers.

GABA is the predominant transmitter mediating fast synaptic inhibition. Both ionotropic and metabotropic receptors are involved in GABAergic inhibition. $GABA_A$ receptors mediate fast postsynaptic inhibition through activation of chloride channels, and $GABA_B$ receptor activation leads to slow IPSPs through activation of potassium channels (Fig. 10). In other neuronal systems besides the hypothalamus, synaptic activation with electrical stimulation ultimately leads to fast $GABA_A$-mediated IPSPs and slow $GABA_B$-mediated IPSPs, although $GABA_B$-mediated IPSPs have not been so clearly detected in the hypothalamus. In neurons throughout the nervous system, including the hypothalamus, spontaneous inhibitory synaptic input is primarily if not exclusively the result of $GABA_A$ receptor activation of chloride channels. The $GABA_B$ receptor mechanisms, although likely present on hypothalamic neurons, appear to depend upon more intense synaptic activation; these slower mechanisms are metabotropic in nature and appear to depend on second messenger systems. It is likely that in the hypothalamus and elsewhere in the nervous system, presynaptic modulation by GABA is mediated by $GABA_B$ receptors on presynaptic terminals, which tend to regulate the amount of hormone or transmitter released from presynaptic terminals. Thus, as with other types of neurons, $GABA_A$ receptor activation on postsynaptic membranes leads to the characteristic IPSPs of hypothalamic neurons. These IPSPs are presumably a critical determinant of firing rate and pattern.

4.4. Neuromodulation: Presynaptic Versus Postsynaptic Mechanisms

Our present understanding of the nervous system suggests strongly that substances other than glutamate and GABA modulate neuronal activity through mechanisms that are primarily slow and secondary to the fast actions of amino acid transmitters. Neurons that project to the hypothalamus release neuromodulators (such as acetylcholine, serotonin, and histamine) and peptides (such as oxytocin, vasopressin, angiotensin I, neuropeptide Y, and opioid peptides) on dendrites, somata, and other terminals. Those terminals that connect to the dendrites and/or somata of hypothalamic neurons, by definition, have postsynaptic actions. Compared to fast IPSPs and EPSPs, the postsynaptic effects of neuromodulators are much slower, lasting from hundreds of milliseconds to many minutes. In one sense, these slow inhibitory and excitatory inputs change the resting potential of the hypothalamic neuron. The primary mode of action, however, is on an ion channel through some second messenger-mediated signal-transduction pathway; because ion channels are ultimately affected, the resistance or conductance of the neuron is changed. If the resting potential is close to the equilibrium potential for the ions involved in the neuromodulatory mechanism, there may be no change in membrane potential, but changes in membrane resistance may be altered, thus having an impact on neuronal activity. An analysis of the mechanism of action of many neuromodulators has

revealed that slow changes in membrane conductance with little or no change in membrane potential can still influence not only action potential generation, but also the amplitude of fast synaptic events. Thus, slow postsynaptic actions may be relatively undetectable with traditional extracellular and intracellular recordings unless care is taken to examine long-term changes in firing behavior.

Powerful mechanisms of presynaptic neuromodulation exist in the hypothalamus and elsewhere in the nervous system. Because of the difficulty in the mammalian nervous system of recording from presynaptic terminals, our understanding of presynaptic modulation is limited at best. We know that substances like GABA acting on presynaptic $GABA_B$ receptors or serotonin acting on presynaptic $5HT_{1B}$ receptors can have particularly powerful effects because of our ability to examine how these pharmacological agents modulate postsynaptic potentials and currents. Presynaptic inhibition leads to the attenuation of EPSPs and/or IPSPs through a mechanism that influences the membrane potential and/or the waveform of the action potential in the presynaptic terminal. It is widely believed that presynaptic inhibition involves shortening the duration of the action potential (presumably through a reduction in the calcium component) at the presynaptic terminal. On the other hand, presynaptic excitation, which is manifest as an enhancement of EPSPs and IPSPs, is thought to broaden the presynaptic action potential by increasing the calcium component. Our understanding of how presynaptic modulation occurs in the hypothalamus is based on studies primarily conducted in other parts of the nervous system, which themselves are relatively indirect because of our inability to directly examine the physiological events in presynaptic terminals.

4.5. Mechanisms of Neuronal Synchronization

A fundamental characteristic of many hypothalamic neuroendocrine systems, and even nonneuroendocrine systems (e.g., the circadian timing system of the suprachiasmatic nucleus), is the presence of some degree of synchronization of electrical activity between neurons. For example, it is well established that the pulsatile release of oxytocin during the milk ejections associated with lactation are owing to synchronized electrical activity across populations of oxytocinergic neuroendocrine cells. Regardless of the possible postsynaptic mechanisms, it is likely that presynaptic mechanisms are also involved in this particular system and presumably several others. Most or all oxytocinergic neuroendocrine cells in the paired supraoptic and paraventricular nuclei fire synchronously to generate the bursts of activity required for each pulse of oxytocin. The neurons that project to the oxytocinergic system are presumably synchronized, because it is difficult to understand how local synaptic circuits could synchronize neuroendocrine cells across four distinct and widely separated nuclei. In addition to these presynaptic mechanisms, however, postsynaptic mechanisms such as ephaptic and gap junctional coupling (i.e., nonsynaptic mechanisms) and synaptic mechanisms such as recurrent excitation and recurrent inhibition are likely involved in the synchronization of hypothalamic neurons, similar to neurons in areas such as the hippocampus and neocortex.

Both ephaptic transmission through electrical field effects and electrotonic transmission through gap junctions have been postulated as important mechanisms of synchronization in hypothalamic neurons and neuroendocrine cells. Although not usually considered as a specific mechanism of neuronal communication, intense activation of any population of neurons has the potential to cause increases in the concentration of extracellular potassium and decreases in the concentration of extracellular calcium. Although these mechanisms may be important in some pathological situations (e.g., during epileptic seizures), it seems less likely that they would play a critical role in synchronizing hypothalamic neurons and neuroendocrine cells during normal activation of these networks. The mechanism of ephaptic transmission (i.e., electrical field effects) has been studied extensively in the hippocampus and is likely to contribute importantly to synchronization during seizures and possibly also during certain "sharp waves" known to occur in cortical structures. The close apposition of neuronal membranes with little or no intervening glia and the parallel arrangement of neuronal structures (particularly dendrites) could lead to communication of electrical events through the extracellular space. In the hippocampus, where this has been studied most extensively, this mechanism appears to depend on the parallel orientation and alignment of the various neuronal elements, which is much less likely to occur in hypothalamic nuclei. These two mechanisms of synchronization through the extracellular space (i.e., ionic and electrical), have been proposed to be important in synchronization of hypothalamic networks, but additional studies will be required

to provide more compelling evidence that these mechanisms are important in the regulation of hormonal secretion.

Particular interest has focused on the possible role of electrotonic coupling through neuronal gap junctions in the synchronization of hypothalamic neurons, particularly neuroendocrine cells. Although many researchers would consider gap junctional communication to be nonsynaptic, the presence of the specialized structure of intercellular channels associated with the gap junction provides the basis for an argument that electrotonic coupling through gap junctions represents electrical synaptic transmission. Numerous reports have suggested the presence of gap junctions among neuroendocrine cells, although this result and the field in general remain highly controversial. The presence of electrotonic coupling through gap junctions has been suggested by intracellular injections of low-molecular-weight tracer molecules, such as Lucifer Yellow and biocytin during intracellular recording from a single neuron. The subsequent determination that more than one neuron or neuroendocrine cell has been stained by such injections has led to the proposal that these cells are "dye coupled," presumably through gap junctions. Although careful controls have been undertaken, this line of investigation alone is unlikely to provide compelling evidence for electrotonic coupling through gap junctions. Gap junctions are expected to transmit in an attenuated fashion the electrical currents associated with action potentials. Partial spikes (or "spikelets") have been considered as the possible representation of interneuronal gap junctions and electrotonic synapses. A variety of electrophysiological experiments involving single and even dual intracellular recordings have suggested the presence of electrotonic coupling through gap junctions in the hypothalamus, particularly in such areas as the suprachiasmatic, supraoptic, and paraventricular nuclei. Again, further experimentation is required before we can be certain that gap junctions and electrotonic synapses are critical in the synchronization of neuronal activity in neuroendocrine systems.

The primary form of neuronal communication in local circuits is chemical synaptic transmission, involving recurrent and feedforward excitation and inhibition. The difficulty with invoking recurrent mechanisms in the neuroendocrine hypothalamus is that one must assume either neuroendocrine cells secrete a fast transmitter (such as glutamate or GABA) in addition to their peptide hormone, or that the synchronization is limited temporally by the speed of action of the neuropeptide on other neuroendocrine cells. Because the likely time-scale by which neuropeptides influence electrical activity in the hypothalamus and elsewhere is relatively long (i.e., seconds to minutes), how these mechanisms could operate to synchronize activity in the neuroendocrine hypothalamus is unclear. On the other hand, conventional neurons in the hypothalamus, even those that secrete neuromodulators and peptides, may also secrete glutamate and GABA, thus allowing the recurrent excitatory and inhibitory synaptic mechanisms well known to occur in hippocampus and neocortex to be important in the hypothalamus. Glutamatergic recurrent excitatory pathways are considered to be critical for synchronization of pyramidal cells in cortical structures during seizures, and recurrent inhibitory mechanisms with "rebound depolarization" also are thought to synchronize cortical networks that may be important in the hypothalamus. Our present understanding of the organization of local synaptic circuits—and in particular the role of fast transmitters, neuromodulators and peptides in these local circuits—is sorely lacking in the hypothalamus compared to higher levels of the brain. Additional study in this particular area will be required before we will have a complete understanding of how neurons and neuroendocrine cells in the hypothalamus are synchronized. Local circuits, however, cannot explain synchronization between cell populations located in different nuclei, only within one nucleus.

5. WHAT ARE THE MODEL SYSTEMS AND THE TECHNICAL DIFFICULTIES IN RELATING ELECTROPHYSIOLOGY TO HORMONE SECRETION?

Like all areas of scientific endeavor, an understanding of fundamental principles relating to how neurophysiological mechanisms regulate hormone secretion in neuroendocrine systems requires one or more model systems. In the mammalian hypothalamus, the magnocellular neuroendocrine system responsible for the secretion of oxytocin and vasopressin has served as a model system for understanding neuroendocrine regulation, and, in particular, how electrophysiological mechanisms generate the firing pattern associated with physiological mechanisms of hormone secretion. The oxytocinergic and vasopressinergic neuroendocrine cells are located in the supraoptic and paraventricular nuclei and they project their axon terminals

to the posterior pituitary (i.e., neural lobe of the hypophysis). One fundamental difficulty in understanding how changes in firing frequency and pattern regulate hormone secretion is to know under which physiological conditions the particular hormones are secreted. Our understanding of how hormones are released under different physiological conditions is probably better established for oxytocin and vasopressin than for other neuroendocrine systems. Similarly, because the supraoptic and paraventricular nuclei contain almost exclusively oxytocinergic and vasopressinergic neurons and neuroendocrine cells, it has been possible to evaluate, at least indirectly, how these two populations of neuroendocrine cells behave under a variety of physiological conditions known to lead to secretion of these hormones. Experiments using extracellular, intracellular, and whole-cell patch-clamp techniques in brain-slice preparations and acutely dissociated cells have led to a good understanding of hypothetical mechanisms responsible for generating the distinctive firing patterns of the oxytocinergic and vasopressinergic neuroendocrine cells.

The physiological conditions under which the oxytocinergic and vasopressinergic neuroendocrine systems have been most extensively studied are lactation on the one hand, and dehydration and hemorrhage on the other hand. Extracellular recordings from anesthetized animals have shown that a brief synchronous burst of activity in approximately half of the cells in the supraoptic and paraventricular nuclei precedes a pulse of oxytocin, which then leads to milk ejection. Thus, brief high-frequency bursts of action potentials occur synchronously in most if not all of the oxytocinergic neurons before each pulsatile release of oxytocin. The vasopressinergic system appears to operate differently, and graded secretion of vasopressin during hemorrhage or dehydration is thought to involve phasic burst discharges, but not particularly in a synchronized mode. Thus, during hemorrhage or dehydration the number of putative vasopressinergic neuroendocrine cells that fire in a phasic pattern increases with increased hormone demand. The phasic bursting pattern is more effective for releasing vasopressin than is continuous neuronal activity. The phasic burst is a much longer burst than the typical oxytocin burst, often lasting tens of seconds. Simultaneous extracellular recordings have revealed that although physiological conditions that lead to vasopressin secretion tend to progressively recruit the electrical activity of vasopressinergic neuroendocrine cells, neither the action potentials nor the bursts themselves are thought to be synchronized. The mechanism of graded secretion of vasopressin is thought to involve both an increase in the intensity of the burst discharge of individual vasopressinergic neuroendocrine cells, and also an increase in the number of vasopressin neurons firing burst discharges. The magnocellular neuroendocrine system therefore represents a model for different mechanisms by which action potential generation leads to optimization of hormone secretion, with the oxytocin system generating brief high-frequency synchronized bursts of action potentials to cause pulsatile release of oxytocin, and the slow progressive recruitment of vasopressinergic neurons into a phasic firing pattern leads to graded secretion of this hormone. Complete understanding of the cellular mechanisms responsible for these electrophysiological events will require a range of experimental approaches, some of them quite traditional and some state-of-the-art methods currently under development.

6. WHAT ARE THE ESTABLISHED APPROACHES AND NEW TECHNIQUES FOR STUDYING ELECTROPHYSIOLOGICAL MECHANISMS OF HORMONE SECRETION?

Many questions could be answered directly if the neuroendocrine electrophysiologist could record action potentials, synaptic events and the properties of single channels in awake, freely behaving animals secreting hormone under physiologically controlled conditions without anesthesia and without in vitro preparations. This is technically impossible now, and will almost certainly remain so for the foreseeable future. Our strategy for understanding the electrophysiological mechanisms of the different neuroendocrine systems will require several in vivo and in vitro approaches and a wide range of electrophysiological techniques. The in vivo approaches will continue to require chronic, freely behaving animals and anesthetized preparations. It is to be expected that traditional extracellular recordings from single neurons and populations of neurons will remain a fruitful avenue of investigation, particularly as we attempt to relate behavioral and anatomical issues to the action potential discharge of neurons and neuroendocrine cells in the hypothalamus. Further use of slice preparations and patch clamp techniques, combined with intracellular staining and subsequent immunohistochemical identification of the recorded neurons and neuroendocrine cells, should provide new insights into synaptic mechanisms and their relation to the different firing patterns characteristic of particular neuroendocrine

systems. New slice preparations involving direct visualization of neurons with differential interference contrast optics and infrared illumination have tremendous potential in the future to allow important new advances in our understanding of the electrophysiology of hypothalamic neurons and neuroendocrine cells. Methods involving acutely dissociated neurons and cell cultures will continue to be useful for direct electrophysiological studies, although they will be of little or no value in analyzing local synaptic circuits. Our understanding of the electrophysiological mechanisms of hypothalamic neurons will depend upon combining results obtained with in vivo and in vitro methods with a wide range of electrophysiological techniques. However, methods are available now to greatly advance our understanding of the hypothalamus and its electrophysiological mechanisms over the next decade.

SELECTED READINGS

Armstrong WE. Morphological and electrophysiological classification of hypothalamic supraoptic neurons. Prog Neurobiol 1995; 47:291.

Dudek FE, Tasker JG, Wuarin JP. Intrinsic and synaptic mechanisms of hypothalamic neurons studied with slice and explant preparations. J Neurosci Meth 1989; 28:59.

Hatton GI. Emerging concepts of structure-function dynamics in adult brain: the hypothalamo-neurophysial system. Prog Neurobiol 1990; 34:437.

Renaud LP. Magnocellular neuroendocrine neurons: update on intrinsic properties, synaptic inputs and neuropharmacology. TINS 1987; 10:498.

Renaud LP, Allen AM, Cunningham JT, Jarvis CR, Johnston SA, Nissen R, Sullivan MJ, Van Vulpen E, Yang CR. Synaptic and neurotransmitter regulation of activity in mammalian hypothalamic magnocellular neurosecretory cells. In: Joose J, Buijs Rm, Tilders JH, eds. Progress in Brain Research, vol 92. New York: Elsevier Science, 1992:277.

Renaud LP, Bourque CW. Neurophysiology and neuropharmacology of hypothalamic magnocellular neurons secreting vasopressin and oxytocin. Prog Neurobiol 1991; 36:131.

Smith BN, Dudek FE. Intracellular recording from hypothalamic cells that regulate neuroendocrine function. In: Van de Kar LD, ed. Methods in Neuroendocrinology. Boca Raton, FL: CRC, 1998:95.

van den Pol AN, Wuarin J.-P, Dudek FE. Glutamate neurotransmission in the neuroendocrine hypothalamus. In Mahesh VB, Brann DW, eds. Excitatory Amino Acids: Their Role in Neuroendocrine Function. Boca Raton, FL: CRC, 1995:1.

Yagi K, Iwasaki S. Electrophysiology of the neurosecretory cell. Int Rev Cytol 1977; 48:141.

31 Anatomical Markers of Activity in Hypothalamic Systems

Gloria E. Hoffman, PHD and Anne Z. Murphy, PHD

CONTENTS

1. INTRODUCTION

1.1. Assessing Hypothalamic Activity Generates a Need for Anatomical Markers

Attempts to study the activity of individual hypothalamic neurons directly have been fraught with problems. The location of some hypothalamic nuclei close to the base of the brain or ventricles makes access difficult. Moreover, with the exception of the magnocellular neurons that contain vasopressin (VP) and oxytocin (OT), the remaining hypothalamic cells are small and rarely concentrated within cytoarchitectonically defined nuclear boundaries. Even within some of the distinct nuclei of the hypothalamus, functionally diverse neuronal populations are present. For example, in the paraventricular nucleus (PVN), whereas the VP cells that regulate water balance are clustered into large magnocellular groups in the lateral subnucleus, the OT neurons of the same nucleus do not form homogeneous groups. Moreover, OT neurons that regulate posterior pituitary function are interspersed among OT neurons that do not innervate the posterior pituitary, but rather, project to the brain stem and spinal cord autonomic regions. In the more medial regions of the PVN, OT neurons intermix with parvocellular neurons (not containing OT) that participate in a number of diverse functions, such as regulation of temperature, blood pressure, and gastric motility and secretion. This heterogeneity is widespread within the hypothalamus and points to the necessity of being able to identify, *a priori*, which populations of neurons participate in a specific function.

Previous approaches to studying hypothalamic neuronal activity such as ^{3}H-*2-deoxyglucose* or assessment of *cytochrome oxidase* activity have been employed with some success, but these techniques lack the cellular resolution required to distinguish individual active neurons from their inactive neighbors. For anterior pituitary regulatory systems, use of sensitive *radioimmunoassay* techniques for measurement of pituitary hormone secretion has been used as an indicator of hypothalamic releasing hormone activity. However, this approach is hampered by the fact that some pituitary systems are regulated by more than one neuroendocrine system (e.g., growth hor-

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mone regulatory systems). Furthermore, even when only one neuroendocrine system controls pituitary function, feedback loops of pituitary or peripheral target hormones can greatly alter the anterior pituitary's sensitivity to the releasing factor. Yet even when used successfully, detection of changes in hormone levels reveals relatively little about the *dynamics* of individual hypothalamic neurons functioning within a population.

As emphasized in Chapter 6 of Section 3, single unit recordings provide valuable information regarding the physiological characteristics of individual hypothalamic cell types. However, this technique shares in common the disadvantage of the aforementioned techniques in that it does not allow for identification of neuronal populations, which as a whole, are responsive to a particular type of stimulus.

Until recently, addressing the issue of activity in heterogeneous diffuse neuronal systems appeared irresolvable largely due to the absence of experimental techniques designed to measure responses of identifiable *individual* neurons participating within a population. In the late 1980's, a novel approach for examining neuronal activity emerged that capitalized on the ability of certain neurons to express immediate early genes upon depolarization.

In 1988, a group of scientists reported that electrical stimulation applied to the cerebral cortex rapidly (and transiently) evoked expression of a gene, *c-fos*, in neurons of the brain. The gene encoded for a protein, cFos, which was detected in the stimulated cells' nucleus within 1 h following stimulus onset and persisted for approximately 3–5 h thereafter. Because these neurons did not express cFos under basal conditions, the use of antibodies directed against cFos provided a powerful means of identifying individual cortical neurons that were depolarized following electrical stimulation. Figure 1 shows an example of cortical neurons stained for cFos from a control animal (left panel) and one which had been administered a potent stimulant (right panel). Note the marked intense staining of neuronal nuclei in the stimulated animal.

1.2. What is Fos?

Fos is one product of a family of *transcription factors* that in neurons are rapidly induced when the cells activity is stimulated. In cells that can proliferate, Fos proteins may regulate mitogenesis. The *c-fos* gene was cloned from mouse liver DNA in studies of homologs to the **F**BR **o**steogenic **s**arcoma virus, *v-fos*. It encodes a 55 kDa product that is posttranslationally modified to yield a product which migrates as 62 kDa. The Fos protein has a leucine zipper motif that promotes *dimerization* with other gene products, most commonly with members of the Jun family (c-Jun, Jun B, and Jun D). Binding of the dimers occurs at a specific site on DNA, the *AP-1 binding domain*. Hence, immunocytochemical localization of Fos or Jun proteins yields staining exclusively in the cell nucleus.

The *c-fos* gene is considered one of the *immediate early genes* because its expression occurs very rapidly and does not require prior protein synthesis. For this reason, the expression of immediate early genes and the immunocytochemical detection of their protein products have provided valuable tools for the identification of stimulated neurons. Yet, their functions are also of great interest. The immediate early gene products serve as transcriptional factors that modify the expression of other genes within the neuron (Fig. 2). The binding of Fos–Jun heterodimers at the AP-1 site can either promote or inhibit the transcription of specific target genes bearing a similar AP-1 binding domain in their promoter region. Whereas all the target genes influenced by Fos translation have not been firmly established, many genes encoding for neuropeptides (e.g., enkephalin, dynorphin, galanin, LHRH), steroid receptors (e.g., the estrogen receptor), or certain transmitter precursor enzymes have AP-1 sequence in their promoter region.

There are other chemically similar proteins in addition to Fos. These *Fos-related antigens*, or FRAs, are formed by different genes, but share in common the leucine zipper motif, the ability to form heterodimers with Jun proteins, and binding to AP-1 recognition sites. Three FRAs have now been characterized: Fos-B, FRA-1, and FRA-2. Antibodies generated against the cFos protein can sometimes detect both Fos and FRAs. As might be predicted, Fos antibodies generated against the portion of Fos that includes the leucine zipper (essential for the formation of dimers) are most likely able to detect both Fos and FRAs equally well. By contrast, antisera generated against the N-terminal of Fos are specific for Fos.

As was mentioned earlier, under basal conditions, most neurons express little or no Fos. However, following membrane depolarization and calcium entry into the cell, *c-fos* gene expression is rapidly induced with mRNA peaking within 15–30 min, and the protein product reaching maximum expression 60–90 minutes after stimulation. Detectable levels of Fos persist for 2–5 h poststimulation. Despite lack of precise knowledge about what Fos does in specific

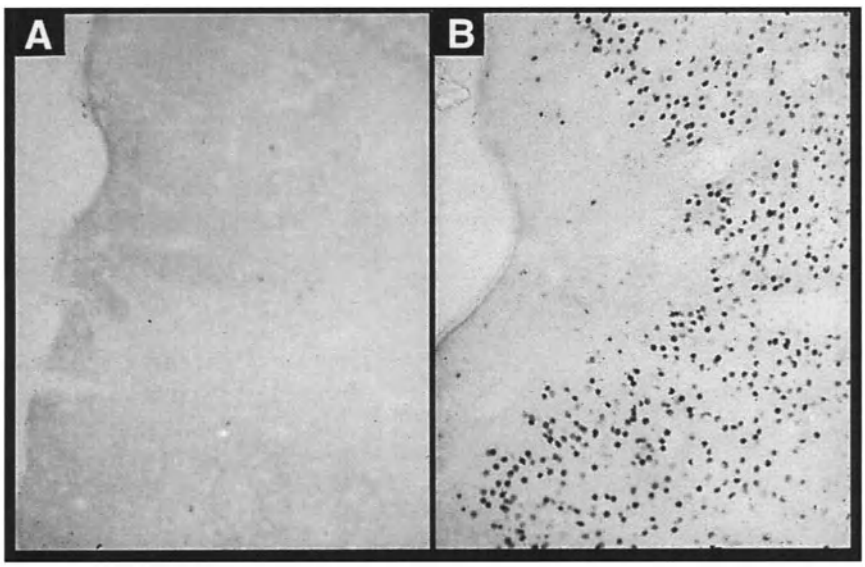

Fig. 1. cFos protein induction of in cortical neurons. The micrograph on the left **(A)** shows a section of the cerebral cortex from a control animal that received saline injection. On the right **(B)**, a section from the same region was obtained from an animal that received stimulation with the glutamate agonist, *N-methyl-D,L-aspartic acid*, (NMA) one hour prior to sacrifice. Note the intense nuclear staining for cFos after stimulation.

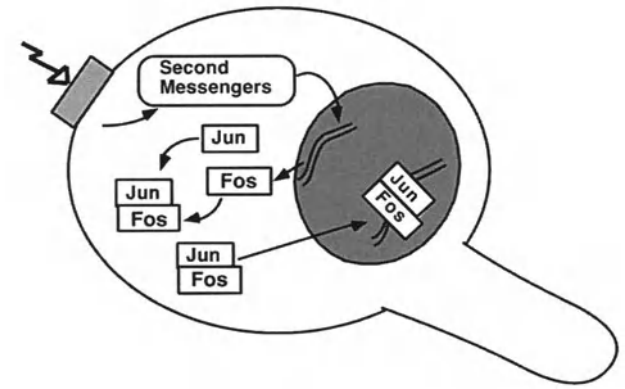

Fig. 2. Cartoon depicting nuclear Fos induction in a stimulated cell. Transmitter stimulation evokes second messenger systems within this cell, which in turn results in cFos induction. cFos and Jun form a heterodimer in the cytoplasm of the cell, which subsequently translocates to the nucleus and binds to the AP-1 site on the promoter region of the cell's DNA and influences the transcription of target genes.

systems, for neurobiological studies, use of Fos localization as a marker for neuronal activation provides a powerful tool by which populations of neurons may be examined under varying physiological conditions.

1.3. What are the Advantages of Using Immunocytochemical Localization of the Fos Protein over In Situ Hybridization for Fos mRNA?

Studies of changes in *c-fos* expression can be accomplished by examining either the messenger RNA for the *c-fos* gene (generally with use of radiolabeled probes and autoradiographic development of emulsion-dipped slides) or by localizing the cFos protein immunocytochemically. Both techniques are potentially useful for assessing neuronal activation, but immunocytochemical localization of the protein offers several advantages.

1.3.1. Fos Immunocytochemistry Offers Superior Cellular Resolution

In several hypothalamic nuclei (e.g., the MPN), the majority of neurons are small and tightly compacted. Therefore, the ability to distinguish between a stimulated neuron and its unstimulated neighbor requires a high level of cellular resolution that immunocytochemical localization of the protein provides. Figure 3 shows micrographs from two sections of the preoptic area of animals stimulated with a glutamate agonist and killed either at the time of peak Fos mRNA production (top) or at the time of peak Fos protein expression (bottom). As is immediately obvious, the number of cells stimulated is more easily discerned when the protein is localized versus when *in situ* hybridization with ^{35}S-labeled riboprobes is used. The latter problem can be solved by use of non-radioactive methods for *in situ* hybridization.

1.3.2. Having a Nuclear Rather than a Cytoplasmic Signal Enables Use of Double Labeling Methods for Cytoplasmic Markers

Whereas Fos protein expression is confined to the nucleus of the cell, Fos mRNA is localized in the cytoplasm of the activated cells. The confinement of Fos protein to the cell's nucleus permits easy detection of Fos along with cytoplasmic markers using standard double labeling techniques (Fig. 4). For studies of

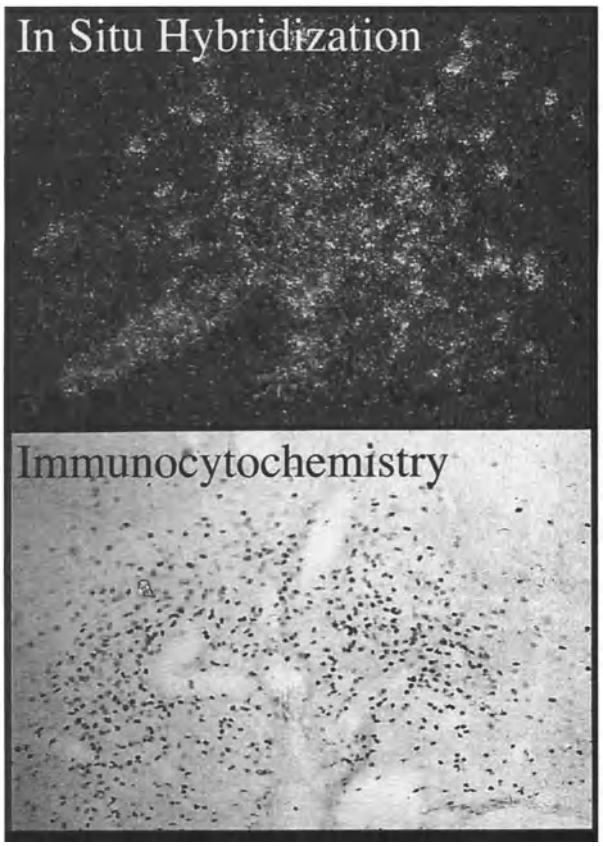

Fig. 3. Comparison of the localization of cFos protein **(A)** and *c-fos* mRNA **(B)** in animals treated with a glutamate agonist. The animal prepared for localization of cFos protein was killed 1 h after stimulus delivery; the animal prepared for *in situ* hybridization of *c-fos* mRNA was killed 15 min after stimulation.

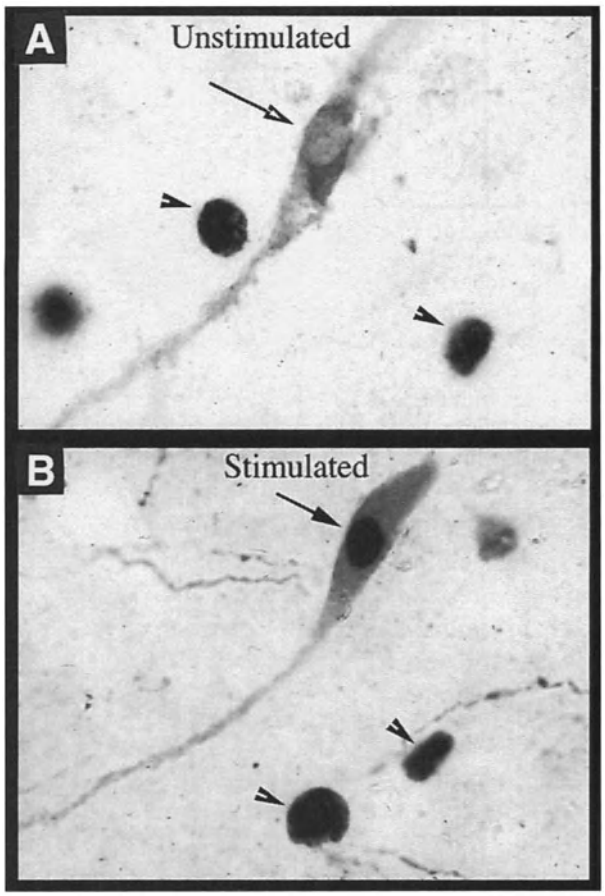

Fig. 4. Micrographs showing the localization of a cytoplasmic marker (in this instance, LHRH) and Fos in an unstimulated **(A)** and stimulated **(B)** condition. In each instance, Fos-positive cells that are unstained for the cytoplasmic marker are present (arrowheads).

hypothalamic function, this is particularly important since often the cells under study are intermingled among functionally different populations, necessitating use of a second marker to distinguish the cells under investigation. While double labeling of one mRNA and an immunocytochemically identified marker is technically feasible, generally there is compromise in the quality of either the mRNA label, the immunocytochemical marker, or both. In contrast, the staining of a cell's nucleus combined with double labeling of the cytoplasm is quite easy and straightforward (*see* Fig. 4).

3.3.3. Fos Protein Synthesis takes Approximately 30–45 min after Stimulus Delivery

A third major advantage of localizing the Fos protein over the mRNA is that its appearance is delayed by at least 30–45 min after the stimulus is delivered; by contrast, *c-fos* mRNA increases within 5–10 min following stimulation. This delay in protein expression provides an interval in which the investigator can move or prepare the animal for euthanasia without concern that such action will result in visualized immediate early gene expression from the handling of the animal in addition to that induced by the desired stimulus. For example, handling and moving an animal in preparation for euthanasia can induce Fos expression in various hypothalamic regions. If even a few minutes elapse before the animal is killed, Fos mRNA can be detected. The same handling will not have sufficient time to result in detectable Fos protein synthesis, and thus will not appear in the tissue studied. Apart from handling issues, a change in setting can also pose problems, as is well illustrated for studies of light activation of the suprachiasmatic nucleus. To study c-fos mRNA expression during the dark phase of the light–dark cycle, animals must be sacri-

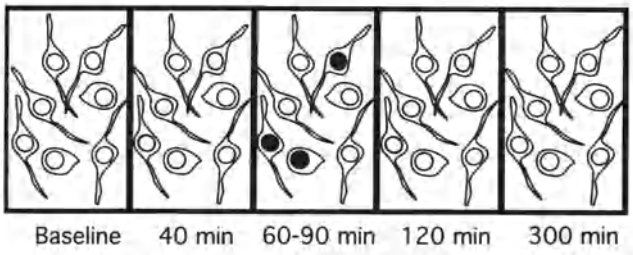

Fig. 5. Cartoon depicting the time course of Fos protein induction and decline. At baseline, no Fos is detectable. Forty minutes following stimulation, Fos is detectable in low levels in some cells; 60–90 min after stimulation, Fos protein expression peaks with the majority of cells Fos positive. Two hours after stimulation, Fos protein is beginning to degrade, and is back to baseline 5 h poststimulation.

ficed under safelight conditions close to the site where the animals are housed. In contrast, the delay in cFos protein expression (detected immunocytochemically) allows an animal to be anesthetized and brought into the light for perfusion without generating spurious cFos positive nuclei.

1.3.4. Fos Proteins Persist for a Longer Time than Does its mRNA

A fourth advantage in localizing the Fos protein over the mRNA is the longer duration of expression. As mentioned previously, *c-fos* mRNA expression is brief, with a half-life of 15–30 min. This makes mRNA analyses for hypothalamic cell activation risky, particularly when the timing of stimulation cannot be predicted accurately (as often is the case with naturally occurring stimuli). For example, in studying the hypothalamic mechanisms involved in the preovulatory gonadotropin surge, the timing of onset of the surge cannot be precisely determined. Thus, euthanizing the animal at the time of the expected surge may miss the key event, necessitating the use of many animals. Selecting later times may then miss those animals whose surge began early. By contrast, the Fos protein has a much longer half-life than the mRNA, with detectable levels generally lasting hours rather than minutes (Fig. 5). Thus, using animals at later times will optimize detection of neurons that were activated even if that activation had begun 2–3 h earlier.

1.3.5. Some Antibodies Against Fos also Recognize Fos Related Antigens

In using antibodies to localize proteins, one is always concerned with the possibility that the antibody will recognize proteins related to, but different from, the antigen. Comparisons of staining patterns with different anti-c-Fos sera indicate that the brain contains more than one class of Fos family proteins and scientists have begun to identify each of these proteins. However, in the majority of studies, the purpose of using Fos is to detect neuronal "activation." Thus, an antibody that cross-reacts with FRAs may still be a useful probe for identifying neuronal activation, and may even prove more sensitive at labeling these populations than a very specific Fos antibody. Thus, in using broadly reactive Fos antisera, a potential disadvantage of antibody cross reactivity is transformed into a benefit.

2. HOW IS IMMEDIATE EARLY GENE INDUCTION USED TO STUDY HYPOTHALAMIC FUNCTION?

2.1. Fos Identifies Neurons Activated by Specific Treatments and Conditions

The first step toward acceptance of Fos expression as a method of identifying activated neurons required verification that known stimuli evoked Fos expression. Initially, such studies included stressful stimuli and examination of the hypothalamic neuroendocrine neurons regulating the stress axis. The neurohypophyseal system also represented an ideal model for evaluating whether neuronal Fos induction bore a reliable relation to neurosecretion from the neural lobe. These initial studies reported Fos expression in magnocellular neurons in the supraoptic (SON) and paraventricular (PVN) nuclei of dehydrated rats. Subsequent studies have confirmed in many hypothalamic systems that Fos accurately tracks stimulated neurons.

Whereas verifying that stimulated systems express Fos was important, it was equally important to determine that the Fos induction was selective. An example of the specificity of Fos expression for identifying activated magnocellular neurons is provided from the studies in which sulfated cholecystokinin octapeptide (CCK) was administered systemically to rats. CCK induces oxytocin (OT) secretion without an associated (measurable) increase in vasopressin (VP) release. Similarly, CCK selectively depolarizes OT, but not VP, neurons. If Fos induction may be interpreted as a marker for neural activation, then CCK administration should only induce Fos expression in OT neurons. By contrast, hyperosmolarity and hypovolemia stimulate both OT and VP neurons and induce Fos in both AVP and OT neurons. Figure 6 shows that this is indeed

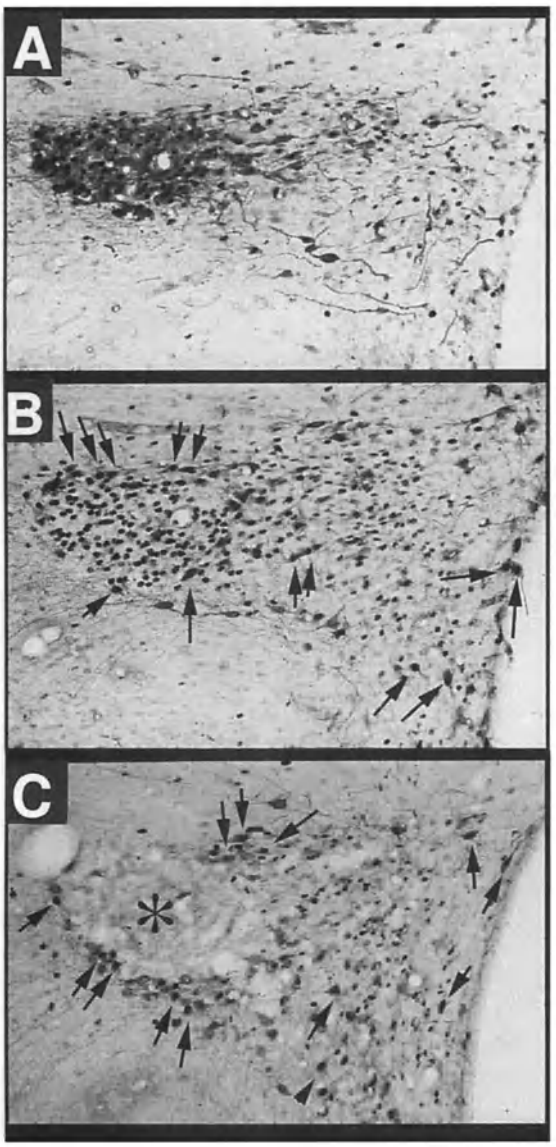

Fig. 6. Comparison of the stimulation of VP (**A**) and OT (**B** and **C**) neurons of the paraventricular nucleus by hypertonic saline (A and B) or CCK (C) treatment. Acute increases in osmolarity induced Fos in both VP (A) and OT (B) neurons, whereas CCK administration induced Fos only in the OT neurons (C). In B and C, some of the Fos-positive OT neurons are indicated by arrows. The * in C indicates the region occupied by VP magnocellular neurons.

the case. The top two panels show the PVN of a rat made hyperosmolar with an acute injection of 2M NaCl (i.p.). The bottom panel shows the PVN of a rat that received CCK (100 μg/kg, ip). The section on the top was double labeled with Fos and VP; the bottom figures were double labeled with Fos and OT. As shown, hyperosmolarity induces Fos in both OT and VP neurons of the PVN. By contrast, CCK-induced Fos was limited to OT cells with no expression present in VP containing cells. The specificity of Fos expression as a marker for neural activity has been confirmed in a wide range of hypothalamic systems including those controlling neuroendocrine secretions, and those regulating circadian rhythms, sex behavior and autonomic functions.

2.2. Staining of Fos can Determine the Magnitude of Stimulation

The studies presented above establish that Fos expression may be interpreted as a marker for neural activation. Ideally, the sensitivity of such a marker should correspond to the *magnitude* of neuronal stimulation. Indeed, that is the case, both in terms of the number of neurons expressing Fos, and the magnitude (intensity) of the staining in each cell. For example, the amount of OT release induced by CCK administration is directly proportional to the dose of CCK administered. The threshold for detecting an increase in OT plasma levels is 1 μg/kg; at this dose a few OT cells express Fos whereas no Fos is present following administration of lower doses. By contrast, a 100 μg/kg dose of CCK produces both maximal OT secretion and Fos induction in PVN magnocellular OT neurons.

Many of the other hypothalamic functions respond similarly and indicate that the magnitude of Fos expression is closely correlated with the extent of hypothalamic neuronal stimulation. For example, during sexual behavior, the number of Fos positive cells in the preoptic area directly reflects the number of ejaculations emitted by the male, or the number of intromissions received by the female.

Equally important to verifying that Fos induction represents neuronal activation of hypothalamic neurons are studies that demonstrate the absence or attenuation of Fos expression at times when responses are blocked or reduced. Indeed, several studies report that Fos expression is suppressed when neuronal activity is inhibited or reduced. The changes observed are manifest by a reduction in Fos intensity, a reduction in the number of Fos positive cells, or both.

2.3. Changes in the Pattern of Fos Activation can Suggest a Mechanism whereby the Magnitude of a Stimulus Increases

In some instances, the pattern of change in Fos expression in a particular neuronal population can suggest a mechanism of stimulus processing. In analyzing the role that LHRH neurons play in initiating an LH surge, scientists were faced with the question

of how the relatively slow rise in plasma LH on proestrus was generated. It was thought that either all LHRH neurons, as a population, were active at the beginning of the LH surge and slowly increased their firing rate, or alternatively, that greater numbers of LHRH neurons were being recruited over time into an active state as the surge progressed. As LHRH neurons are small in size and not localized within a discrete nuclear region, extracellular recordings of these cells would be difficult and painstakingly slow. In addition, to answer the above question would require chronic long term recordings. However, studies using Fos immunocytochemistry (ICC) are ideally suited for these types of questions. By monitoring the pattern of Fos expression in LHRH neurons during the rising phase of the LH surge, investigators noted that increasing numbers of LHRH neurons expressed Fos as the LH surge progressed. This result suggested recruitment of active neurons. In addition, examination of Fos induction at different times prior to the surge pinpointed the time of initiation of the increase in neural activity. Presumably, as more and more LHRH neurons were recruited into the active state, LHRH secretion also increased, leading to the generation of the ascending phase of the LH surge. Analysis of this type of data also dispelled a previous theory which postulated that LH surges are the result of synchronization of LHRH cell firing. If this theory were true, some neurons should be firing and expressing Fos at all times during the estrous cycle. However, the presence of Fos in LHRH neurons *only* at the time of the LH surge suggests that increased afferent neuronal stimulation of LHRH neurons, and not synchronization of ongoing activity, initiated the LH surge.

2.4. Fos can Reveal Functional Differences in Subpopulations Activated by Specific Stimuli

As mentioned previously, a major advantage in the use of Fos staining for study of hypothalamic activation is the ability to separate subpopulations of neurons participating in an event from functionally distinct neighboring neurons. For the hypothalamic PVN, this feature is essential. Anatomical studies have defined three magnocellular and five parvocellular subdivisions of the PVN, each of which contains admixtures of multiple cell types and none of which has clearly defined, unequivocal borders. Consequently, attempts to analyze the physiological functions of this nucleus by traditional techniques have frustrated investigators because of sheer complexity. What is clearly required to study the functional characteristics of the PVN subnuclei is the ability to examine neuronal activity using techniques that allow resolution at a cellular level; these requirements are well met by Fos immunocytochemistry.

As discussed above, CCK administration to rats induces OT release and Fos expression in PVN and SON magnocellular oxytocin neurons. However, unlike the global *magnocellular* oxytocin activation produced by CCK administration, activation of *parvocellular* oxytocinergic neurons is much more selective in that not all parvocellular subdivisions containing OT neurons respond to CCK. The absence of Fos expression in neurons within the dorsal parvocellular subdivision of the PVN following CCK administration offers a particularly striking example, and clearly indicates *functional heterogeneity* of parvocellular oxytocinergic neurons in the PVN.

In addition to OT neurons, corticotropin releasing hormone (CRH) neurons in the parvocellular subdivision of the PVN are also excited by CCK. In contrast to the selective OT neuronal excitation, PVN CRH neurons appear to be more globally activated. This finding was not surprising, as CCK administration is associated with elevated levels of plasma corticosterone and ACTH in rats. However, earlier studies were not able to address directly whether the observed ACTH secretion was secondary to activation of central CRH neurons or a direct effect of CCK on pituitary corticotrophs to stimulate ACTH release. The results of studies using Fos ICC indicated that the former is more likely to be the case, and that systemically administered CCK potently activates central secretion of CRH as well as OT. Studies in control animals verified that this response was specific for CCK administration rather than a result of nonspecific stress from handling the animals or the injection procedures. Consequently, the use of techniques such as Fos ICC now provides us with a much more sensitive tool to ascertain what systems are activated by specific treatments, and consequently, to understand better the effects produced by such treatments.

2.5. Fos Enables Further Characterization of Neuronal Populations Participating in the Processing of Selected Stimuli

Earlier sections of this chapter have demonstrated that hypothalamic cells activated by specific stimuli can be identified using Fos immunocytochemistry, and that the degree of Fos expression is proportional

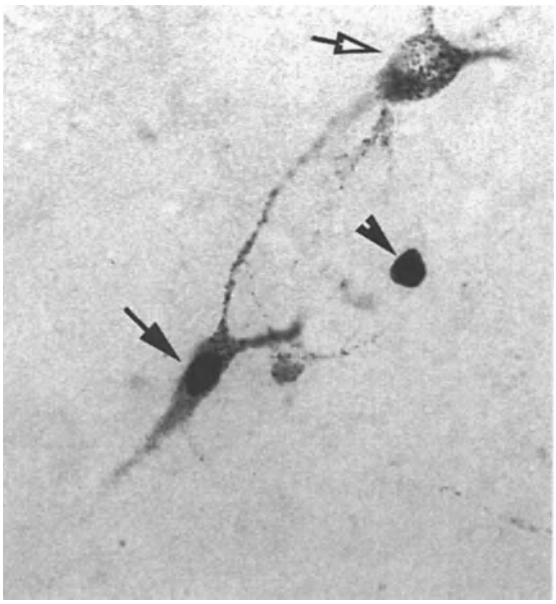

Fig. 7. Example of the determination of pathways of activation by combining Fos staining with retrograde tracer localization. In the field are neurons that are retrogradely labeled (open arrow), Fos positive (arrowhead) or double labeled (filled arrow).

to the stimulus intensity. Also, mapping studies using Fos have identified brain regions that are coactivated with hypothalamic neuroendocrine cells. Perhaps the most exciting use of Fos is the ability to use neuroanatomical tract tracing techniques to establish that a given *neural circuit* becomes engaged following application of a specific stimulus. An example of this is shown in Fig. 7. This approach has the potential of elucidating the *functional* connectivity of a given pathway.

A simple but apt example is provided by the hypothalamohypophyseal portal system. Multiple peptidergic neurons project to the median eminence and secrete releasing or inhibiting factors into hypophyseal portal blood. However, this observation only becomes physiologically significant when it can be ascertained under what conditions each of the many peptides are actually released into the portal circulation and thereby gain access to the pituitary. Fos staining, in combination with neuroanatomical tract tracing techniques, addresses such questions.

In order to accomplish meaningful (interpretable) functional neuroanatomical mapping, a number of criteria must be met. First, potential projection neurons must be sufficiently distant from their targets to allow local injections of *retrograde tracers* without direct diffusion into the site where the cell bodies are located. Second, the retrograde tracer itself must not induce Fos expression in neurons taking up the label. To date, incorporation of most tracers into the cell body does not produce sufficient depolarization or injury to induce Fos expression *per se*; viral tracers are the exception to this rule. Third, a cell that has taken up the retrograde tracer must still be capable of expressing Fos upon stimulation.

Fos staining in combination with neuroanatomical tract tracing has been applied to delineate a number of hypothalamic circuits. These include the delineation of the pathways to or from the hypothalamus activated by anorexigenic stimuli, hypotension, osmotic stimulation, stress, sleep, maternal behavior, and mating.

2.6. FRAs can be Used as Markers in the Study of Decreased Activity

The previous discussions have focused on applications of immediate early gene localization as a method of identifying an increase in neuronal activity. However, for some systems, *reducing* tonic activity is a prominent means of control. For example, it is well established that the pudendal motoneurons of the lumbosacral spinal cord are under tonic descending inhibition from the ventrolateral medulla. It is thought that activation of the medial preoptic area during male sex behavior functions, in part, to remove this tonic descending inhibition. Unfortunately, as disinhibition does not involve voltage gated Ca^{+2} entry in the cell or increases in other second messengers, localization of Fos is not likely to prove valuable for investigating this type of circuit. Rather, what is needed is a cellular marker present when neurons are firing normally but which decreases when an inhibitory signal is imposed (Fig. 8).

Some years ago, scientists observed that staining with antisera generated against the region of cFos close to the DNA binding domain of Fos and Jun labeled a large population of neurons in brains of unstimulated control animals. N-terminally directed cFos antisera (more specific for cFos) showed little or no baseline expression in the same neurons (Fig. 9). Because Western blots of brain tissue using the DNA binding domain antisera suggested the presence of multiple proteins in brain, it was reasoned that some FRAs, different from c-Fos, are likely expressed under baseline conditions. Consequently, scientists sought to determine if changes in basal FRA expression could be used to examine *decreases* in hypothalamic activity.

The tuberoinfundibular dopamine (TIDA) neurons served as the initial model system. TIDA neurons,

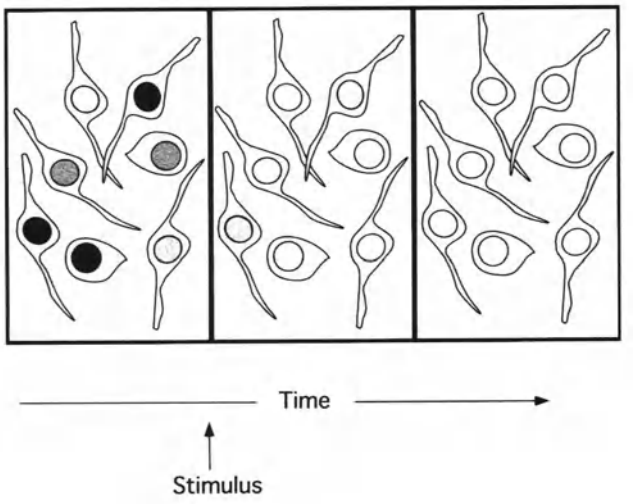

Fig. 8. Cartoon illustrating use of activity-driven transcription factors that can be used to study decreases in activity produced by a stimulus. Certain FRAs appear to operate in this way.

located within the arcuate nucleus of the hypothalamus, tonically inhibit prolactin release. These cells normally express no cFos but have staining for FRA proteins. Following the onset of suckling, prolactin is released, due in part to an inhibition of dopaminergic tone. When lactating rats (postpartum day 12) with continually suckling pups were compared to pregnant or diestrous rats for the presence or absence of FRA proteins within TIDA neurons, approximately half of the TIDA neurons from diestrous and pregnant rats expressed prominent FRA immunoreactivity. In contrast, no FRA immunoreactivity was present in TIDA arcuate neurons from the lactating rats suckling their pups. After pups were removed, FRA staining was again very prominent. These data suggest that FRA immunoreactivity reflects the status of tonic inhibitory drive present in these neurons. Thus immunocytochemical localization of FRA can be used to reveal reductions in tonic activity within a neuronal population. Since that time, further studies in the TIDA system have indicated that daily shifts in dopamine release are reflected by changes in FRA expression, substantiating the usefulness of this approach.

3. WHAT ARE THE LIMITATIONS AND PITFALLS IN THE USE OF FOS?

No method is without limitations. Thus, while quite useful for a number of studies, questions of hypothalamic function cannot all be answered by employing Fos staining. This section will discuss some of the limitations and pitfalls in using Fos to study hypothalamic activity.

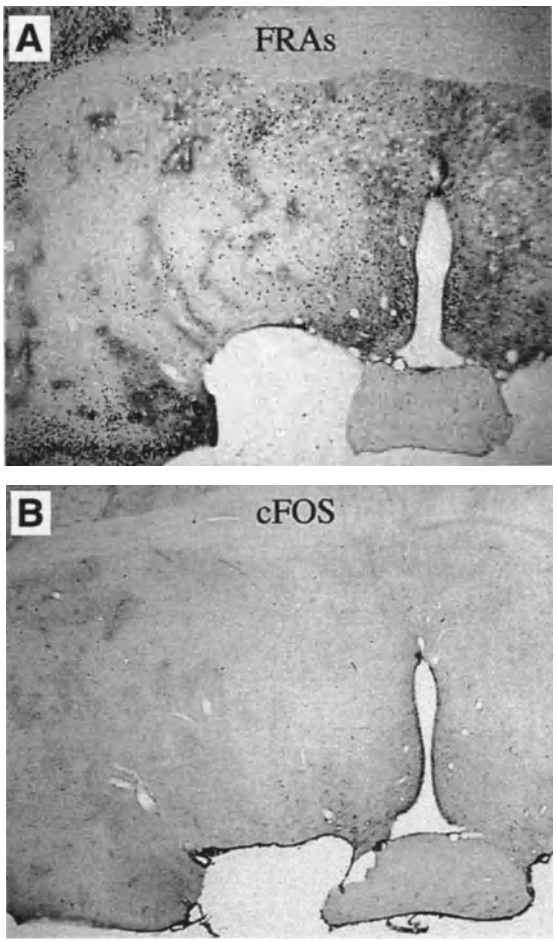

Fig. 9. Staining of adjacent brain sections for FRAs (**A**) and cFos (**B**) show marked differences in baseline expression.

3.1. "The Absence of Proof is Not the Proof of Absence" (Walle Nauta)

A premier neuroanatomist, Dr. Walle Nauta, in discussing neuroanatomical staining cautioned that for many techniques, the absence of proof cannot be equated with the proof of absence. For Fos staining, the same is true. While the presence of Fos following a stimulus is relatively straightforward to decipher, interpreting the absence of Fos is more complicated. Thresholds for Fos/FRA induction may differ across neuronal populations. Moreover, the ability of a stimulus to effectively induce Fos/FRA depends not only the ability to release neurotransmitter, but on the ability of the stimulus to alter second messengers. Neurons can differ in their second messenger responses. Recent studies show quite clearly that antidromic activation which bypasses conventional 2nd messenger induction fails to evoke Fos expression in hypothalamic neurons, whereas orthodromic stimulation does. Even administration of transmitter agonists can result

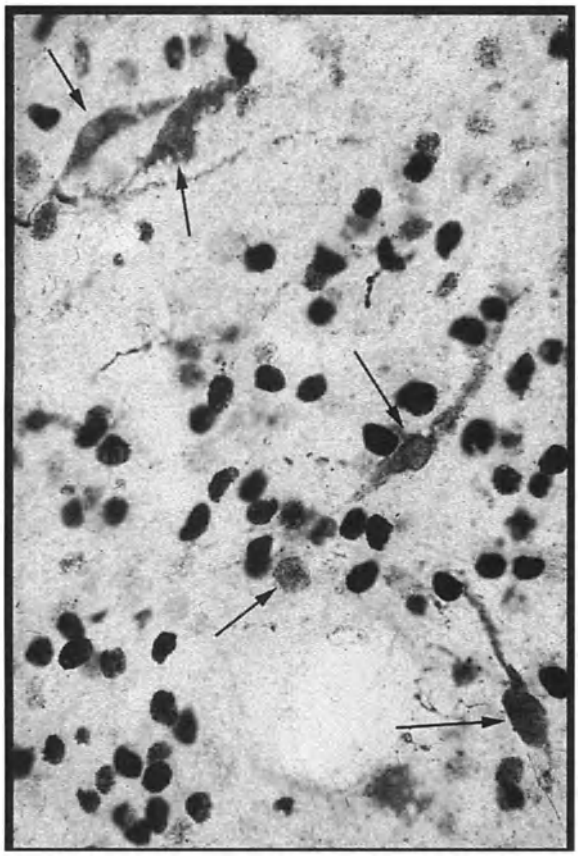

Fig. 10. Staining of the preoptic area of a rat treated with an NMDA agonist NMA shows strong Fos staining in many neurons, but the LHRH neurons in the regions (arrows) show no Fos induction despite the fact that the drug caused marked elevations of plasma LH.

in the dissociation of second messenger increases (and hence, Fos induction) and transmitter release or firing. An example of this is the observation that administration of the NMDA receptor agonist, N-methyl-D,L-aspartate (NMA), fails to induce Fos in LHRH neurons despite this agent's ability to produce a substantial increase in LHRH or LH secretion and marked Fos expression in surrounding neurons (Fig. 10). LHRH neurons are able to produce recognizable levels of Fos during natural stimulation and after electrochemical stimulation. Thus, one possibility is that the signal at the cell body may have been insufficient in strength to induce Fos expression (although cumulatively there is enough LHRH released to produce a marked change in LH secretion). Alternatively, NMA's action on LHRH neurons, unlike natural stimuli, may fail to include other transmitter signals (normally co-released) that enhance second messengers in the LHRH cells.

3.2. The Induction of Fos Does Not Always Indicate that a Neuron's Firing Rate has Increased

There are other aspects of using pharmacologic agents to examine stimulus processing that complicate interpretation of Fos staining. In particular is the ability of a stimulus to induce Fos without substantially increasing neuron firing. In such cases, certain excitatory pharmacological agents may disproportionately raise second messenger levels without concomitantly changing transmitter release, thus dissociating the link between Fos expression and neuronal secretory activity. Growth factors have such a capacity, as do certain peptide neuromodulators. Thus, if administered directly, such factors may induce Fos expression by 2nd messenger induction without concurrently increasing the firing rate of the cell.

Because the intracellular events responsible for releasing transmitter can be separated from the second messenger cascade, one can also envision instances where release is blocked, but second messenger changes persist. The latter is illustrated in the accompanying data from a study of treatment of rats with phenoxybenzamine, an alpha adrenergic antagonist designed to block the hypothalamic pituitary gonadal axis. Administration of phenoxybenzamine, at a dose of 2 mg/kg, blocked LH secretion (Fig. 11). However, luteinizing hormone releasing hormone (LHRH) neurons still displayed marked Fos induction despite the apparent failure of the cells to release LHRH. Thus, without assessment of transmitter (or in this instance hormone) release in addition to Fos staining, erroneous conclusions could be reached.

3.3. Fos is Not a Good Marker for Baseline or Persistent Stimuli

As was mentioned in the initial discussion of cFos, neurons do not normally express cFos. That feature is somewhat perplexing in that many neurons exhibit substantial baseline activity. This raises the question of how neurons respond when firing is persistent. Recall that Fos protein expression lasts for only 3–5 h after stimulus delivery and that time course is essentially unaltered when the stimulus persists. The decline in Fos despite persistent stimulation is thought to involve an inhibitory feedback of the Fos protein on mechanisms of *c-fos* mRNA induction. Yet in some parts of the nervous system (e.g., the midline thalamus) neurons appear to express Fos constitutively. In addition, in the suprachiasmatic nucleus,

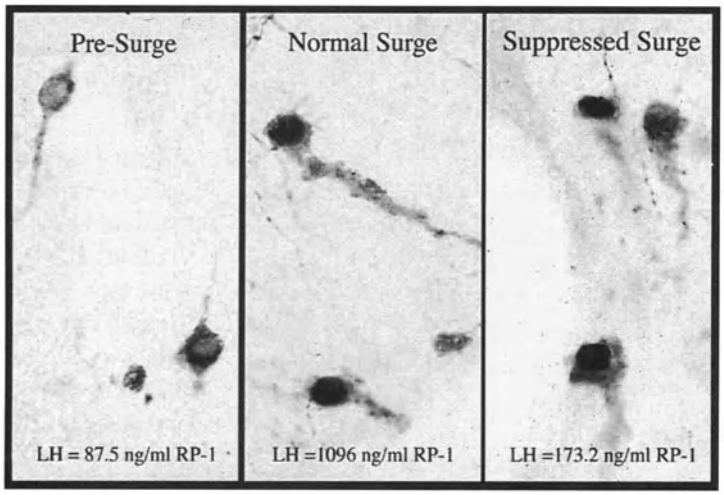

Fig. 11. Micrographs of LHRH cells that were **(A)** unstimulated before the LH surge, **(B)** activated to express Fos during an LH surge, or **(C)** activated but had LH secretion blocked by phenoxybenzamine (2 mg/kg, ip) administered 4 h prior to the expected preovulatory LH surge. Note that the induction of Fos in LHRH neurons remained unchanged even though phenoxybenzamine suppressed LHRH release. The LH values from these animals are indicated at the bottom of each micrograph (expressed as ng/ml of RP-1 LH standard).

continued exposure to light causes very long-term Fos expression. The reasons for Fos' persistence in these situations are not clearly understood and indicate that caution should be used in interpretation of data when stimuli are persistent.

3.4. Fos Expression Does Not Indicate When a Stimulus Ends

Because of the lack of persistent Fos expression in most hypothalamic neurons after stimulation, one loses the ability to derive information concerning stimuli termination. The longevity of Fos protein depends mainly on the half-life of the protein in a particular cell rather than the duration of the stimulus. Detection of Fos' decline will also depend on the sensitivity of the staining method used as well as the quality of the antiserum. Thus, it may be difficult to derive conclusions across studies from different laboratories in which different antibodies against Fos were used.

3.5. Too Much of a Good Thing is Not Ideal: Problems with Use of FRAs to Mark Stimulated Neurons

As was mentioned in the early portions of this chapter, some broadly reacting Fos antisera recognized FRAs in addition to cFos. That capacity, in some instances, increased the sensitivity of detecting activity changes in hypothalamic systems. In other systems, the baseline FRAs appear to be present in greater amounts than cFos. For example, in the magnocellular neurons, FRAs are present in relatively high amounts at times when little or no cFos is present. Thus, in those instances, it is exceedingly difficult to detect increased neuronal activity since the FRAs mask any changes in cFos that are present after stimulation. This problem is easily solved by using antisera specific for cFos.

4. OTHER IMMEDIATE EARLY GENE PRODUCTS CAN BE USED AS MARKERS OF STIMULATED NEURONAL ACTIVITY

Implicit in the analyses presented up to this point is that Fos should be present if the neurons under examination are sufficiently excited. Although true in many instances, it is also likely that neuronal activation alters the expression of a number of different early genes, and depending on the nature of the stimulatory input or the timing of the presented stimulus, the same cell may express different constellations of immediate early genes. In the PVN, for example, there have been rigorous examinations of a number of transcription factors. Following stress, the PVN expresses not only cFos and Jun, but also nerve growth factor-IB (NGFIB). Time courses vary among the factors expressed as does the degree to which the proteins are expressed in the basal condition. Relying on the expression of only protein at a single time after stimulation can thus limit the knowledge obtained. For example, after a stimulus to the magnocellular system, cFos, Jun, and NGFIB are all increased. However, the Jun expression increases more slowly than does cFos. Had Jun been the only protein examined and determinations made relatively soon after stimulation, one might conclude that the system was relatively insensitive to induction. Thus, in considering use of other transcriptional regulators for marking activity changes, knowing the dynamics of each protein induction is critical.

5. FUTURE DIRECTIONS

There are many exciting potential uses of Fos or other immediate early gene proteins in understanding hypothalamic function. Previous sections of this chapter have already described the ways in which Fos has been used to determine when, where, and how stimuli to hypothalamic neurons are processed. However, a challenge for the future will be an assessment of exactly how immediate early gene products affect neuronal function following their expression. Expression of immediate early genes undoubtedly alters transcription of other gene products following neuronal depolarization. However, yet to be determined for the neuroendocrine systems, are exactly what target genes are affected. Understanding the consequences of Fos expression is complicated by the fact that this factor has actions beyond the simple formation of heterodimers with Jun. Fos can affect the functions of other transcriptional regulators, and other transcriptional regulators can influence the ability of AP-1 proteins to regulate gene expression as well. Thus, functional changes based on the presence or absence of only one regulator may be unpredictable. Yet this knowledge is critical.

As scientists move into the 21st century, novel approaches for addressing these problems will need to be devised. Some have already begun to be tested. The use of transgenic mice with reporter proteins linked to the *fos* or *jun* promoters, or transgenic mice which lack the ability to express the immediate early genes offer some means of determining how immediate early gene factors participate in hypothalamic function. As such approaches become more sophisticated (controlled knockouts, for example), we will be better able to understand the complexities of stimulus transcription regulation in hypothalamic systems.

SELECTED READINGS

Cahill MA, Janknecht R, Nordheim A. Signalling pathways: jack of all cascades. Curr Biol 1996; 6:16.

Chaudhuri A. Neural activity mapping with inducible transcription factors. Neuroreport 1997; 8:V.

Herrera DG, Robertson HA. Activation of c-fos in the brain. Prog Neurobiol 1996; 50:83.

Hoffman GE, Verbalis JG, Smith MS. c-Fos and related immediate early gene projects as markers of activity in neuroendocrine systems. Frontiers Neuroendocrinol 1993; 14:173.

Karin M, Liu Zg, Zandi E. AP-1 function and regulation. Curr Opin Cell Biol 1997; 9:240.

Pennypacker KR, Hong JS, McMillian MK. Implications of prolonged expression of Fos-related antigens. Trends Pharmacol Sci 1995; 16:317.

Sagar SM, Sharp FR. Early response genes as markers of neuronal activity and growth factor action. Adv Neurol 1993; 59:273.

Sharp FR, Sagar SM, Swanson RA. Metabolic mapping with cellular resolution: c-fos vs. 2-Deoxyglucose. Crit Rev Neurobiol 1993; 7:205.

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