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THE EFFECT OF CONSTANT ILLUMINATION ON THE HYPOTHALAMO-HYPOPHYSIAL-GONADAL AXIS OF THE FEMALE RAT

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THE EFFECT OF CONSTANT ILLUMINATION ON THE HYPOTHALAMO-HYPOPHYSIAL-GONADAL AXIS OF THE FEMALE RAT

By

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ABSTRACT

The Effect of Constant Illumination on the Hypothalamo-Hypophysial-Gonadal Axis of the Female Rat

By

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1. Mature Sprague-Dawley female rats were exposed to a 14:10 h light:dark regimen (LD) or constant light (LL) for at least 5 weeks. LL rats showing at least 10 consecutive days of vaginal estrus, or regular 4-day cycling LD rats selected on proestrus were used in these studies. Morning (0900-1000 h) and afternoon (1600-1700 h) serum samples were obtained by decapitation from all animals. Basal serum levels of follicle stimulating hormone (FSH) and prolactin (Prl), but not luteinizing hormone (LH), were elevated in LL rats. In addition, an afternoon serum FSH rise, but no preovulatory LH or Prl surge, was seen in LL rats. These results suggest that constant illumination may differentially block preovulatory LH and Prl surges while maintaining a daily afternoon FSH release mechanism.

2. The pituitary response to single or multiple doses of synthetic gonadotropin releasing hormone (GnRH) was compared in LL and proestrous, estrous and diestrous LD rats *in vivo*. LL rat pituitary LH and FSH release was not different from that of LD proestrous (PM) rats, which showed the greatest pituitary sensitivity among those groups of LD rats tested. It was concluded that constant light results in a sensitization of the pituitary to GnRH.

3. Luteinizing hormone releasing hormone (LHRH) content was measured in the anterior hypothalamic area (AHA) and medial basal hypothalamus (MBH) of LL rats and LD proestrous rats in the morning and afternoon. LHRH content was greater in LL than LD rats in both hypothalamic areas and at both times. AHA LHRH content showed a decrease in the afternoon of both LL and LD rats. However, whereas LD rat MBH LHRH content decreased slightly in the afternoon, no change in MBH LHRH content was seen in the afternoon in LL rats. These results suggest that in the LL rat LHRH is synthesized and released from its site of synthesis (AHA), but does not appear to be released from the MBH storage site.

4. Hypothalamic sensitivity to both positive and negative feedback by gonadal steroids was examined in ovariectomized LL or LD rats primed with estradiol benzoate (EB) or progesterone (P). LL rat pituitary LH, but not FSH, response to both negative and positive estrogen feedback was less than in LD rats. In contrast, FSH, but not LH, response to progesterone positive feedback was less in LL than LD rats. These results suggest that LL rats may have a decreased sensitivity to both positive and negative feedback by gonadal steroids on hypothalamic control of gonadotropin release.

5. Plasma estradiol, progesterone and testosterone levels were measured in LL and LD proestrous rats in the morning and afternoon. Morning levels of serum estradiol were not different between LL and LD rats; but unlike LD rats, LL rats showed no afternoon rise in plasma estradiol. Plasma progesterone levels were higher in LD than LL rats at both time periods. Plasma testosterone levels were low in the morning as compared to the afternoon in LD rats, but the opposite pattern

in testosterone release was seen in LL rats. It was concluded that LL rats have altered levels and patterns of plasma gonadal steroid release which may contribute to an insufficient positive feedback effect on hypothalamic gonadotropin release.

6. Biogenic amine turnover index (TI) in LL rats was compared with LD proestrous rats. Morning and afternoon biogenic amine TI was measured in the preoptic-anterior hypothalamic area (AHA) and medial basal hypothalamus (MBH) of all animals. Constant illumination resulted in: a) a depression in norepinephrine (NE) TI and an increase in serotonin (5-HT) in the AHA (AM), and b) a depression in the morning NE and afternoon 5-HT TI in the MBH. It was concluded that constant light may induce changes in hypothalamic biogenic amine metabolism associated with a failure of LH release and increased Prl release.

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

LIST OF FIGURES

LIST OF FIGURES (continued)

INTRODUCTION

Light indicates time of day and time of year, and many species of mammals appear to depend on its diurnal and annual variations for synchronizing their reproductive activities (Hoffmann, 1973). The many examples of continuation of reproductive function in the absence of external cues suggest that endogenous rhythms are merely entrained to one or more environmental signals, of which light seems to be the principle one, but animals are not totally dependent on the external environment. The result is a reproductive cycle that provides the best chance of survival for the species.

The system upon which light acts involves ultimately the gonads, which produce the gametes and also the gonadal steroids. The gonads are stimulated to grow and secrete steroid hormones by gonadotrophic hormones released from the anterior pituitary gland. The pituitary synthesizes and releases its gonadotrophic hormones under the influence of neurohormones produced in the hypothalamus and released into the hypothalamo-hypophysial portal system in the region of the median eminence. The neurohormones may stimulate or inhibit synthesis or release of the gonadotropins from the pituitary. Although there is some evidence for a direct effect of certain agents on the gonads, for the most part the influences on the reproductive system act on the pituitary itself or on the hypothalamic control centers.

The questions addressed by the experiments in this study relate to the nature of changes that occur in several components of the hypothalamo-hypophysial-gonadal axis in response to the absence of photoperiodic change produced by constant illumination (LL) and resulting in persistent estrus. Gonadotropin release is reported to be altered after constant light exposure (Fink, 1975). There appear to be elevated levels of serum follicle stimulating hormones (FSH) and prolactin (Prl), but no pre-ovulatory luteinizing hormone (LH) surge in light induced persistent estrous rats. The latter may well be the major cause by which gonadal function is altered. There may be other reasons for a change in the "normal" pattern of gonadotropin release, not the least of which could be an interruption of the neurochemical signals for gonadotropin release, i.e., the gonadotropin releasing hormones (GnRH) of the hypothalamus. There is a question of whether or not GnRH is synthesized and/or released from the hypothalamus into the portal blood system to stimulate the pituitary in light induced persistent estrus. Therefore, hypothalamic GnRH content of LL rats was measured in these experiments. Another possible cause for failure of ovulation could be reduced LH release due to decreased sensitivity of the pituitary to GnRH. The proestrous stage of the estrous cycle is characterized by an increase in pituitary sensitivity to GnRH (Aiyer et al., 1973). If the pituitary of the LL rat is not sensitive to the GnRH present, adequate LH release would not be expected to occur. The pituitary sensitivity of LL rats to synthetic GnRH was examined in vivo in these experiments.

Since the gonadal steroids exert positive and negative feedback effects on gonadotropin release (Everett, 1969; Brown-Grant, 1974),

constant light exposure may influence gonadotropin release indirectly through changes in the amounts or ratios of serium estradiol, progesterone or perhaps even testosterone levels. Therefore, morning and afternoon serum levels of these steroid hormones were measured in LL rats.

The feedback effects which gonadal steroids have on gonadotropin release is exerted both on the pituitary and the hypothalamus (Chowers and McCann, 1967; Bogdanove, 1963). A change in sensitivity of the hypothalamus to positive and negative feedback of gonadal steroids may determine their effectiveness in inducing gonadotropin release or inhibiting it at the proper time and in a cyclic pattern. Hypothalamic sensitivity to estrogen and progesterone priming was examined in ovariectomized rats under constant light (LL) or regular light:dark (LD) conditions.

Finally, there have been numerous reports implicating hypothalamic monoaminergic neuronal control of gonadotropin release (Fuxe and Hökfelt, 1969). The activity of hypothalamic monoaminergic neurons is in turn influenced by steroidal feedback. Steroid feedback of gonadotropins may occur through mechanisms involving hypothalamic monoaminergic neurons (Kalra et al., 1972). Constant light induced changes in hypothalamic monoaminergic neuronal activity could eventually result in a persistent estrous condition. Therefore, the hypothalamic turnover index (TI) of norepinephrine (NE), dopamine (DA) and serotonin (5-HT) was determined in LL rats.

Functional changes in the above components of the hypothalamo-hypophysial-gonadal axis were examined in light induced persistent

estrous rats, with the hope that this may contribute to a further understanding of the mechanisms by which light influences reproductive functions.

LITERATURE REVIEW

I. Hypothalamic Control of Anterior Pituitary Secretion

A. Early Observations

Early in the 20th century, clinicians recognized that pituitary insufficiency might be related to damage in the region of the hypothalamus, but were unable to resolve whether the effects were those of direct damage to the adjacent pituitary gland (Anderson and Haymaker, 1974; Evans, 1935). On the basis of careful pathologic study, Erdheim (1909) concluded that these changes could be caused by hypothalamic damage alone, and Aschner (1912) demonstrated that gonadal insufficiency (now recognized as being due to gonadotropin failure) could be produced in dogs by hypothalamic lesions which spared the pituitary. Over the following four decades, many workers studied the effects of "denervation" of the pituitary by surgical section of the pituitary stalk, but the results were ambiguous and controversial. In a series of classic experiments, Harris and Jacobsohn (1952) demonstrated the crucial role of the blood vessels of the stalk in this regulation. Stalk section in the rat caused loss of sexual function, which returned when the hypophysial-portal vessels regenerated. When a paper plate was inserted into the stalk section so as to prevent regeneration of the vessels, sexual function failed to return. In all animals, stalk section caused permanent destruction of neural connection. They also repeated and extended the earlier studies of Greep, who had shown that pituitaries transplanted

to the pituitary fossa function normally, whereas pituitary transplants to other sites apparently were devoid of activity (Anderson and Haymaker, 1974). These observations showed that the pituitary fossa was a privileged site for the growth and function of the pituitary, and indicated that the crucial factor was the special blood supply from the hypothalamus. Some criticized the results of the Harris-Jacobsohn experiments as being due to damage to the pituitary caused by transplantation. This reservation was resolved convincingly in the double transplantation experiments of Nikitovitch-Winer and Everett (1958), who demonstrated in rats that the pituitary failure resulting from transplantation of the same pituitary to the region beneath the basal hypothalamus was not seen if anatomic reconnection of the blood vessels occurred. Reconstitution of pituitary function did not occur in control experiments in which the pituitary was re-transplanted to the temporal lobe.

The local trophic function of the hypothalamus was also demonstrated in the early 1960's by transplantation of pituitary fragments directly into the hypothalamus. As reported independently by Halasz et al. (1962) and by Knigge (1962), there is a region of the basal hypothalamus that contains trophic substances capable of maintaining the cellular structure and secretory function of the implants. The term "hypophysiotropic area" was applied to this region.

The hypophysial vessels themselves, now known to be the conduit of the hypothalamic hypophysiotrophic hormones, were first described in 1930 by a Roumanian medical student, Popa (1930), following the lead given by his teacher, Ranier, in Bucharest. The vessels were characterized by a peculiar group of coiled capillaries at the inferior extent

of the hypothalamus that left the brain and joined to form long vessels that traversed the pituitary stalk. The blood in these vessels was incorrectly asserted by Popa, and his subsequent coworker Fielding (popa and Fielding, 1930), to flow from the pituitary upwards to the base of the brain. In 1936, Wislocki and King described similar vessels in the monkey and suggested on the basis of anatomic features that blood probably flowed from the hypothalamus to the pituitary. In 1947, Green and Harris confirmed this suggestion by direct observation in the rat that blood in the hypothalamic portal vessels did indeed flow to the anterior pituitary, an observation which had been made independently and previously in the toad by Houssay et al. (1935). Green and Harris proposed that the hypothalamus secretes into the portal capillaries of the median eminence specific pituitary-regulatory substances which are transported to the adenohypophysis by the portal vessels. A similar proposal was also made at about the same time by Friedgood (1936) in a then unpublished lecture, and the earliest germs of this idea may be found in the writings of Hinsey and Markee a decade before (Anderson and Haymaker, 1974). However, more recent evidence by Hungarian (Palkovits, 1978) and American workers (Oliver et al., 1977; Bergland and Page, 1978) indicates that there is some retrograde flow of blood from the pituitary to the hypothalamus. The significance of this retrograde flow remains to be established.

Vesalius, in De Humani Corporis Fabrica (1543) described the drainage of cerebrospinal fluid through the floor of the third ventricle (named "infundibulum" because of its resemblance to a funnel) into the pituitary and thence into the nose to form mucous (pituita) from which our modern term pituitary is derived. More recent studies indicating

that certain hypophysiotrophic hormones may find their way to the anterior pituitary by way of the cerebrospinal fluid through the floor of the third ventricle and median eminence indicate how leisurely, at times, is the progress of scientific discovery.

Although the anterior pituitary gland lacks a direct nerve supply, the secretion of each of its hormones is under the control of the central nervous system. The pituitary and, in turn, its target glands respond to changes in the external and internal environments through specialized secretory neurons localized in the ventral hypothalamus. In addition, the neurohumoral connections of the anterior pituitary are important in the feedback regulation of a number of hormones, such as cortisol, the gonadal steroids, and thyroxine, and serve as part of the integrated mechanism by which behavior and metabolism adapt to the external environment.

B. Landmarks in Hypothalamic Anatomy

1. General Anatomy:

By definition, the hypothalamic region, though somewhat ill defined, lies below the level of the thalamus. In effect, it comprises the lateral walls of the lower part of the third ventricle, below the level of the hypothalamic sulcus, which, in the adult human, is a shallow and not very conspicuous groove running almost horizontally along the lateral walls of the third ventricle. The rostral boundary of the hypothalamus is indefinite, but may be taken as a plane lying slightly rostral to the optic chiasm (the preoptic area). Its caudal boundary may be taken as a coronal plane immediately posterior to the mamallary bodies. The lateral boundaries of the hypothalamus are the most difficult of all to define. At various coronal levels the

following structures may be found situated laterally; the very lowermost part of the thalamus, the internal capsule, the globus pallidus, the ansa lenticularis, and the optic tract. Below, the lowermost parts of the gray matter making the walls of the third ventricle form, in man, a funnel-shaped cavity, the infundibulum or tuber cinereum, a part of the hypothalamus, which is clearly visible as a protuberance at the base of the brain and which is prolonged downward as the pituitary stalk. This region varies much in shape in other species of mammals, the part corresponding to the anterior wall of the tuber cinereum and uppermost part of the neurohypophysis having come to be called in most animals the median eminence (Tilney, 1936). The pituitary gland is attached to this region, either by means of a long, free-lying stalk, as in man, primates, and the rat, or with virtually no stalk, as in the cat and dog.

2. Hypothalamic Nuclei and Their Afferent and Efferent Connections:

Within the confines of this rather vague region, the hypothalamus, there are a number of very well-defined nuclear masses and a number of remarkably ill-defined nuclear groups. There are also several well-defined nerve fiber tracts and some diffuse tracts. One of the most striking nuclei to be seen in the hypothalamus is the supra-optic nucleus. This nucleus, which is extremely well defined and which has a sharp outline, straddles the rostral extremity of the optic tract, just caudal to the optic chiasm. The axons of the nerve cells of the supraoptic nucleus form a nerve tract composed of fine, unmyelinated nerve fibers which pass through the rostral part of the tuber cinereum or median eminence to run down in the neural part of the pituitary stalk into the infundibular process or neural lobe of the pituitary

gland. These axons form the supraoptico-hypophysial tract. It is clear that a large number of these axons end in the infundibular process, because when the pituitary stalk is cut many of the nerve cells in the supraoptic nucleus degenerate, and disappear.

The paraventricular nucleus lies beneath the ependymal lining of the third ventricle, dorsal and caudal to the supraoptic nucleus. The axons of the cells of the paraventricular nucleus take a curved course which passes toward the median eminence. This nerve tract composed of fine unmyelinated nerve fibers is convex rostrally and passes near or even through the supraoptic nucleus. The nerve tract formed by the axons of these paraventricular nerve cells also forms part of the hypothalamo-hypophysial tract and passes down through the median eminence into the infundibular stem and the infundibular process (Daniel, 1966).

Ramon Cajal (1911) who first described the hypothalamo-hypophysial tract, thought that it might have a sensory function. There was much uncertainty about the tract until the work of the Scharrers (1954) and Bargmann (1954) clarified its function. It was found that neurosecretory activity with specific staining properties was produced by the cells of these two nuclei and that along the nerve fibers making up the hypothalamo-hypophysial tract there could be found small bead like swellings which also gave a specific staining reaction.

A prominent landmark at the base of the human brain, lying in the interpeduncular space and immediately posterior to the tuber cinereum is made by two prominent rounded swellings, the pair of mammillary bodies. Two striking tracts of myelinated nerve fibers, which are easy to see with the naked eye and can be easily dissected

out, are related to these nuclei. First, the fornix, a massive column of myelinated nerve fibers, sweeps down obliquely through the hypothalamic region from the superior rostral area to end in the mammillary nucleus. This massive tract originates from the hippocampus and dentate gyrus. The second massive and macroscopically visible nerve tract associated with the mammillary nuclei is the mammillothalamic tract, the main out-going tract which springs from the superior aspect of the mammillary nucleus, passing upward and forward to enter and pass through the lower parts of the thalamus. It ends in the anterior nucleus of the thalamus. From the lowermost part of the mammillothalamic tract a bundle of nerve fibers leaves the main tract to pass toward the tegmental region of the midbrain as the mammillotegmental tract, which joins the dorsal longitudinal fasciculus through which some of its fibers are distributed through the reticular formation of the brain stem.

The three nuclei so far described - supraoptic, paraventricular, and mammillary, are easily identified histologically, being clearly demarcated from the adjacent areas of the hypothalamus. The remaining hypothalamic nuclei are far less well defined. In fact, some of them only have been identified by using fetal material for preliminary studies, since certain nuclei are well demarcated in the fetus although become ill-defined in the adult.

The gray matter forming the walls of the third ventricle in the region just above the tuber cinereum may be divided into two vertically flattened plate-like nuclei, the ventromedial hypothalamic nucleus and the dorsalmedial hypothalamic nucleus. These two nuclei lie in

the wall of the third ventricle between the attachment of the tuber cinereum and the site of the paraventricular nucleus.

The ventromedial and dorsomedial nuclei do not seem to have any particularly well-defined tracts of nerve fibers connected with them, but there seems little doubt that they have connections with the other hypothalamic nuclei by means of the extensive periventricular system of nerve fibers and by means of the medial forebrain bundle, an ill-defined sheet running anteroposteriorly along the length of the hypothalamus and lateral to those hypothalamic nuclei which lie beneath the ependyma of the third ventricle.

Above the mammillary nucleus and immediately caudal to the dorsomedial and ventromedial hypothalamic nuclei, lies the posterior hypothalamic nucleus also situated beneath the ependyma of the wall of the third ventricle. The posterior hypothalamic nucleus appears to make a considerable contribution to the periventricular system of fine nerve fibers. These nerve fibers appear to form a pathway connecting many of these hypothalamic nuclei with the ventricular aspect of the thalamus above and with the brain stem below, the dorsal hypothalamic nucleus perhaps providing the largest contribution. These seem to be some of the main efferent pathways of the hypothalamus.

One more nucleus of ill-defined outline and composed of small fibers may perhaps be mentioned. This is the preoptic nucleus lying anterior to and above the level of the supraoptic nuclei.

3. Hypothalamo-Hypophysial Blood Supply:

a. The Blood Supply of the Hypothalamus

The vascular supply for this region comes from small arteries arising from the vessels of the circle of Willis (le Gros

Clark, 1938) which, in effect, outlines the hypothalamic region on the base of the brain. Some of the arterial twigs come from the superior hypophysial arteries, some from the middle and anterior cerebral arteries, and some from the posterior cerebral and communicating arteries.

b. The Blood Supply of the Pituitary Gland

The earliest useful work on the blood supply of the human pituitary gland was that of Luschka in 1860, but it was not until 1930 that the subject of pituitary blood supply became of special interest. In this year, Popa and Fielding (1930) described a set of vessels running along the pituitary stalk which were of a type not previously found in relation to the nervous system. These vessels formed a connection between two capillary beds and thus had to be described as portal vessels. Popa and Fielding (1930) considered that these portal vessels carried blood from the pars distalis to the region of the hypothalamus. These studies led to much work on the blood supply of the pituitary, for it was at once obvious that a unique arrangement of blood vessels such as they had described must be of considerable functional importance. There was some evidence that the blood in the portal vessels of the pituitary stalk did not flow from the gland toward the brain, but in the opposite direction. Wislocki and King (1936) studying the blood supply of the pituitary gland of the rhesus monkey, came to the conclusion, on morphological grounds that the direction of flow must be from the capillary bed at the top of the stalk to the sinusoidal bed of the pars distalis. The direction in which blood flowed along the portal vessels of the stalk was observed in living animals, first by Houssay et al. (1935) and later by Green

et al. (1947). However, as already mentioned, there is growing evidence for some retrograde flow of blood from the pituitary to the hypothalamus.

Of all the advances in our knowledge of the pituitary gland over the last thirty years, one of the most important has been that relating to the control of its hormone secretion. The theory advanced by Harris (1955, 1960) that the control of anterior lobe hormones is exercised, at least in part, by means of neurohumors which are elaborated in the hypothalamus and carried to the cells of the pars distalis by the blood in the portal vessels, is now generally accepted. The importance of this portal system throughout the vertebrate series was shown by Green (1951) who studied a wide range of animals and found portal vessels were present in all of them.

Two small arteries, the superior hypophysial arteries, spring directly from the trunks of the internal carotid arteries in the subarachnoid space, near to the origin of the opthalmic arteries. In some cases these arteries arise not as a single small trunk, but as twin arteries. In other cases the single trunk runs medially toward the tuber cinereum or median eminence, dividing into two from an arterial ring which encircles the upper extremity of the pituitary stalk. From this arterial ring arises a series of branches, some of which supply the optic chiasm and hypothalamic region. Others downward and inward, penetrating the neural tissue of the median eminence and upper infundibular stem, where they break up into a capillary bed of complex patter. This bed forms part of the first or primary capillary bed of the portal system. The blood which has passed through these

coiled capillaries is collected into long portal vessels which pass down the stalk. These superficial long portal vessels run through the pars tuberalis. Other portal vessels pass down in the substance of the neural tissue of the stalk. When the portal vessels enter the pars distalis of the pituitary gland, they break up into the sinusoids which form the blood supply of the epithelial cells of this lobe.

The neural tissue of the infundibular process or posterior lobe of the pituitary gland is supplied by two arteries which spring from the internal carotid arteries where these lie within the cavernous sinuses, at the place where the carotid arteries turn sharply rostrally. In the human, the artery of the trabecula passes through the epithelial cells of the pars distalis to enter the lower infundibular stem, anastamosing with a branch of the inferior hypophyseal artery and usually taking part in some supply of the coiled capillaries which give rise to the short portal vessels.

4. Transmission of the Hypothalamic Impulses to the Pituitary:

When considering the connections between the hypothalamus and the pituitary, the following well-established facts should be taken into account: 1) There are no nerve endings of hypothalamic origin - or at least occurring only exceptionally - in the pars distalis of the hypophysis. An abundant hypothalamic innervation of the anterior lobe tissue has been described by earlier authors, but a careful analysis of their descriptions and illustrations proves undoubtedly that reticular fibers have been mistaken for nerve elements. 2) The principal blood supply of the anterior lobe is the portal system, which is characterized by the intimate contact of the blood vessels with the nervous

tissue of the hypothalamus. 3) Hormone secretion of the anterior lobe returns to normal after pituitary stalk section, if the vascular connections between the hypothalamus and the pituitary have regenerated, while pituitary and target organ atrophy develops, if for some reason vascular regeneration does not occur (for details see Harris, 1955; Szentágothai et al., 1968).

Based on the above-mentioned findings, Harris and Green (1947) formulated the neurovascular hypothesis of adeno-hypophysial regulation. They assumed that the hypothalamus produced some substances which, after being released in the median eminence, enter the portal circulation by which they are carried to the pituitary.

C. Hypothalamic-Hypophysiotrophic Releasing and Inhibiting Factors

It has been shown by several authors that hypothalamic extracts have a direct effect on pituitary function. Such an action was first demonstrated on ACTH secretion (Saffran and Schally, 1955; Guillemin and Rosenberg, 1955). The active principle was named corticotropic releasing factor (CRF). Further studies suggested that hypothalamic extracts contain substances which also influence the secretion of other pituitary hormones. It was reported that hypothalamic extracts stimulate the release of pituitary thyroid-stimulating hormone (TSH) (Shibusawa et al., 1956a,b; Schreiber et al., 1961; Guillemin et al., 1962), luteinizing hormone (LH) (McCann et al., 1960; Campbell et al., 1961; Nikitovitch-Winer, 1962), and follicle-stimulating hormone (FSH) (Igarishi and McCann, 1964; Mittler and Meites, 1964). These substances have been called TSH-releasing factor (TRF), LH releasing factor (LRF), and FSH releasing factor (FRF), respectively. There is also evidence

that the hypothalamus produces a substance which exerts a tonic inhibitory effect on prolactin release (prolactin-inhibiting factor, PIF) (Pasteels, 1961a,b; Meites et al., 1962; Talwalker et al., 1963), a growth hormone release inhibiting factor (GIF) (Krulich and McCann, 1968). The existence of these substances has been corroborated in various laboratories by in vivo as well as in vitro studies (see McCann and Dhariwal, 1966; Schally et al., 1968a; McCann and Porter, 1969).

The functional significance of these factors is underlined by the fact that concentration of the substances in the hypothalamus varies in connection with changes in pituitary function. It has been shown that during the sexual cycle of female rats there is a fluctuation in hypothalamic LRF content (Ramirez and Sawyer, 1965; Chowers and McCann, 1965). Suckling or estradiol treatment, besides influencing prolactin secretion, cause a decrease in PIF activity of the hypothalamus (Ratner and Meites, 1964). Hypothalamic CRF activity shows a diurnal rhythm similar to pituitary ACTH secretion. The daily peak of CRF concentration in the hypothalamus is 3 hours earlier than the daily corticosterone peak in the blood (David-Nelson and Brodish, 1969; Hiroshige et al., 1969). Thyroidectomy was reported to produce an increase in hypothalamic TRF and pituitary TSH content (Sinha and Meites, 1965-66). There is more GRF in the hypothalamus of young (30-day-old) than in old (2-year-old) rats (Pecile et al., 1965).

It appears that the hypothalamic hypophysiotropic substances are responsible for both the structural as well as functional maintenance of the cells of the anterior lobe. By transplanting it under the kidney capsule or into the anterior ocular chamber, its structure

becomes dedifferentiated and composed mainly of chromophobic cells, while its hormone content is markedly reduced (Cutuly, 1941; Cheng et al., 1949; McDermott et al., 1950; Fortier, 1951; Harris and Jacobsohn, 1952; Siperstein and Greer, 1956; Goldberg and Knobil, 1957; Nikitovitch-Winer and Everett, 1959). Normal histology as well as function of the dedifferentiated pituitary graft under the renal capsule can be restored by retransplantation under the median eminence Nikitovitch-Winer, 1958). These findings are consistent with the view that the hypothetical hypothalamic substances exert a trophic influence on the pituitary cells. It seems very likely that the action of the hypothalamic substances are not only limited to release of the trophic hormones, but that they also stimulate hormone synthesis. This is indicated by the significant increase of pituitary TSH (Sinha and Meites, 1966; Mittler et al., 1969) ACTH (Uemura, 1968), FSH (Justiz and de la Llosa, 1967), LH (Justiz et al., 1967) and GH content (Symchowicz et al., 1966; Schally et al., 1968b) when under in vitro conditions, hypothalamic extract or the appropriate releasing factor is added to the medium. Median eminence extract administered intracarotidly causes a two to five-fold increase in pituitary ACTH content (Vernikos-Danellis, 1965).

The chemical structure of three of the hypothalamic hormones has been identified to date (TRF or TRH, LRF or LRH and GIF or somatostatin). This has stimulated much investigation regarding their localization and physiological action. TRH is found throughout the hypothalamus and is not limited to any particular region. The isolation and finally the chemical synthesis in 1969 of TRH (pyro

Glu-His-Pro-NH2) (Schally et al., 1973; Guillemin et al., 1971) made it possible to develop radioimmunoassays for this compound and methods for detecting its biosynthesis. The highest hypothalamic concentration of TRH was found in the median eminence. However, it is believed that as much as 80% of TRH is found outside of the hypothalamus (Jackson and Reichlin, 1974) mostly in cortical areas.

The decapeptide LRH (pyro-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2) (Schally, 1971) is concentrated in the median eminence, extending from the preoptic area (Alpert et al., 1976) and also the premammillary area. It was found by immunocytochemical techniques that cells rostral to the anterior commissure and paraolfactory area stain with anti-LRH antibody. The lamina terminalis also contains LRH (Alpert et al., 1976).

The third hypothalamic hormone to be chemically identific is somatostatin (SRIF or GIF), a tetradecapeptide. The formula of this compound is H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH with a bridge between the two cysteine groups (Guillemin et al., 1973). In the relatively short time since its discovery, somatostatin has been found to possess a surprising range of biological effects, to have an unexpectedly wide anatomical distribution, and to be effective in all species of mammals in which it has been tested. Somatostatin has been shown to decrease TSH (Borgeat et al., 1974), ACTH (Hall, 1973), GH (Hansen, 1973), glucose and insulin (Koerker, 1974) and glucagon (Gerich et al., 1975). Extrahypothalamic sites of somatostatin include the pineal (Patel et al., 1975), pancreas, stomach, duodenum and jejunum (Arimura et al., 1975).

Blood coming from the transected pituitary stalk has been demonstrated to contain LRF (Fink et al., 1966, 1967; Fink, 1967; Kamberi et al., 1969), FRF (Kamberi et al., 1970a), TRF, GRF (Wilber and Porter, 1970), CRF (Porter, 1970) and PIF (Kamberi et al., 1970b) activity. According to Harris (1969), LRF is present in the portal blood during all phases of the sexual cycle except estrus, and electrical stimulation of the hypothalamus causes an increase in LRF activity of this blood.

It should be mentioned that in the peripheral blood of intact animals the presence of these substances has not been conclusively demonstrated, although earlier studies reported their presence in systemic blood. This was only possible under special experimental circumstances such as hypophysectomy, when presumably the releasing factors are released in larger amounts from the hypothalamus because of removal of the inhibitory "short feedback loop" from the AP hormones (CRF, Shapiro et al., 1956; Eik-Nes and Brizzee, 1958; Brodish and Long, 1962; LRF, Nallar and McCann, 1965; FRF, Negro-Vilar et al., 1968a; GRF, Krulich and McCann, 1966; Muller et al., 1967; CRF, Redding and Schally, 1969; PIF, Chen et al., 1970). If the medial basal hypothalamus is destroyed, the releasing or inhibiting activity in the peripheral blood of the hypophysectomized animals was not evident, suggesting a hypothalamic origin of the active principles.

II. Hypothalamic Monoamines

A. Pathways

The regional distribution of catecholaminergic neurotransmitters in the brain and spinal cord was determined imprecisely, using biochemical assays, many years ago; however, the more recent application

of histochemical fluorescence methods has made it possible for these compounds to be visualized within specific neuronal tracts and cell bodies (Dahlstrom and Fuxe, 1964; Fuxe, 1965; Andén et al., 1966). With the use of this approach, dopamine appears to be concentrated in three major groups of brain neurons: nigrostriatal neurons terminating in the basal ganglia; meso-limbic neurons, with cell bodies localized in the upper brain stem and axon terminals in the nucleus accumbens, olfactory tubercles, and cerebral cortex; and tuberoinfundibular neurons, located entirely within the hypothalamus and having their most prominent set of terminals in the external layer of the median eminence (Fuxe and Hökfelt, 1969; Jonsson et al., 1972). Nerve tracts that appear, by histochemical fluorescence assay to contain norepinephrine as a neurotransmitter are also grouped into three main fiber bundles; a descending system, with cell bodies localized in the medulla oblongata, whose axons course through the spinal cord to form synapses with ventral and dorsal horn neurons; and two ascending tracts, one with cell bodies located in the locus coeruleus and terminals in the cerebral cortices and the hippocampus, and a second tract with cell bodies in the medulla and pons and axons terminating largely within the hypothalamus. Within the hypothalamus, norepinephrine can be detected by microenzymatic assay in each nucleus, with the highest concentrations in the median eminence and arcuate nucleus (Palkovits et al., 1974).

Serotonin-containing neurons are only poorly visualized with the histochemical fluorescence method; however, available data seem adequate to justify the conclusion that virtually all of the serotonin cell bodies in brain are confined to the mid-line raphé nuclei of the

medulla and pons. Fibers from these cell bodies descend to terminate in the spinal cord gray matter and ascend in the brain (mainly via the medial forebrain bundle, along with dopaminergic neurons and noradrenergic axons) to innervate most of the telencephalon and diencephalon. Most hypothalamic nuclei contain serotonin (and thus, probably 5-HT terminals), as assayed by microenzymatic techniques (Saavedra et al., 1974). The highest concentrations are found in the arcuate and suprachiasmatic nuclei, with smaller, but nonetheless substantial amounts are present in the median eminence.

B. Metabolism of the Brain Amines

1. Biosynthesis

a. Catecholamines:

The synthesis of catecholamines takes place within neurons from tyrosine. The distribution of tyrosine hydroxylase which converts tyrosine to dopa parallels the distribution of catecholamines in the brain; its concentration is high in the hypothalamus, midbrain, pons and striatum and low in the cortex (McGreer et al., 1967). It is found partially free and partially bound to membranes, the latter form being more active (Costa and Meek, 1974). L-aromatic amino acid decarboxylase is found only in the soluble fraction of nerve tissue and is present in all parts of the central nervous system (CNS). Dopamine-β-hydroxylase which converts dopa to noradrenaline is associated with the synaptic vessicles in which the amines are stored. It is found in high concentrations in the midbrain and hypothalamus, but is low in the striatum (Basal ganglia) where dopamine is the main transmitter (Iversen and Glowinski, 1966).

The rate-limiting step for catecholamine synthesis depends on the activity of tyrosine hydroxylase which is under a negative feedback control exerted by "releasable" noradrenaline. When this is removed, not only does the activity increase but there is also an increase in synthesis of tyrosine hydroxylase (Spector et al., 1967; Weiner and Rabadjija, 1968). This negative feedback effect was first shown by Costa and Neff (1966); they raised noradrenaline levels in the brain by inhibition of monoamine oxidase and within 1 or 2 hours of this treatment noted a fall in turnover rate. Similar effects have been shown for dopamine (Javoy et al., 1972). Thus, it can be expected that the turnover rate will differ in the various areas of the brain, since the sizes of the storage pools are different. For example, the turnover rate for noradrenaline is slowest in the hypothalamus ($t\frac{1}{2}$ = 4 hours), where its storage and uptake is greatest (Iversen and Glowinski, 1966).

The pattern of control of catecholamine synthesis in the brain is very similar to that in the adrenal medulla, where the first investigations were made. The adrenals differ, however, in that they contain high concentrations of phenylethanolamine-N-methyl-transferase (PNMT), the enzyme which converts noradrenaline to adrenaline (Axelrod, 1962).

Although levels of adrenaline in the brain are negligible, the enzyme PNMT is present especially in the hypothalamus and olfactory bulb. In addition, an inhibitor of PNMT can be found in the same areas, which may explain the low levels of adrenaline in the brain. The presence of the enzyme and its inhibitor suggest that adrenaline is synthesized in the brain, it is locally regulated and is not stored (Ciarnello et al., 1969; Pohorecky et al., 1969).

b. 5-Hydroxytryptamine

The synthesis of 5-hydroxytryptamine from tryptophan also takes place inside the neurones, under the control of tryptophan hydroxylase and L-aromatic amino acid decarboxylase. The distribution of tryptophan hydroxylase correlates with the distribution of neurones liberating 5-hydroxytryptamine at their synapses (Ichiyama et al., 1970). The hydroxylation was thought to be the rate-limiting step of the synthesis (Moir and Eccleston, 1968), but recent evidence has shown that the rate depends on the availability of tryptophan and its transport across the neuronal membrane since normal brain levels of tryptophan do not saturate the tryptophan hydroxylase (Grahame-Smith, 1971; Tagliomonte et al., 1971).

Whether the level of 5-hydroxytryptamine at nerve terminals can exert a negative feedback effect on its own rate of synthesis is yet to be elucidated. Administration of a monoamine oxidase inhibitor reduces the conversion of ^{3}H-5-hydroxytryptophan to ^{3}H-5-hydroxytryptamine (Macon et al., 1971); however, this result has been questioned (Costa and Meek, 1974) since the change in specific activity with time was not taken into consideration. Indeed, Costa and Meek (1974) have shown that there is no change in turnover rate after increasing 5-hydroxytryptamine brain levels by monoamine oxidase inhibition.

2. Storage

The storage of amines in the brain is similar to that in peripheral nerves; they are stored either in a "bound" form or in an "easily-releasable", that is to say "functional" store (Aprison and Hingtgen, 1972). As the most newly synthesized transmitter is the most

easily released (Hamon et al., 1970; Farnebo et al., 1971), it seems that the amines must fill the "functional" store before filling the "bound" store. Electron microscope studies have shown that the amines are present in small granulated vessicles in "boutons" (swellings) of the nerve terminals, and these are presumably the storage sites (Kobayashi and Matsui, 1969), although the anatomical distribution of the bound and functional stores are not known. The storage granules are thought to be formed in the cell bodies of the neurones and to pass down the axon to the nerve terminals. This has been shown by noting changes in histofluorescence after ligaturing noradrenergic axons (Dahlstrom, 1967).

3. Release

Each amine is released from its nerve terminal after an action potential. This has been shown to occur in vitro after stimulation of cortical slices (Baldessarini and Kopin, 1967). The release of both noradrenaline and 5-hydroxytryptamine from the brain is dependent on calcium ions (Goodwin et al., 1969). Release of the amines after electrical stimulation has also been shown in vivo. Stimulation of the ventral noradrenergic bundle in the midbrain causes a fall in noradrenaline in the hypothalamus (Arbuthnott et al., 1970) and stimulation of the midbrain raphé causes a fall in 5-hydroxytryptamine in the forebrain with a concomitant rise in its degradation product, 5-hydroxyindole acetic acid (5-HIAA) (Aghajanian et al., 1967; Sheard and Aghajanian, 1968).

Electron microscopy shows that the amines are released from their storage sites by a process of exocytosis in which the contents are released into the extraneuronal space; for example, at noradrenergic

nerve terminals, noradrenaline, dopamine-β-hydroxylase and specific proteins have been shown to be discharged after stimulation (Malamed et al., 1968).

The free or active transmitter, after its release, acts on the receptor sites of the post-synaptic neuron or organ.

4. Catabolism

Noradrenaline and dopamine are removed from their sites of action mainly by an efficient uptake system back into the nerve terminals, where they are either restored or metabolized by the intraneuronal enzyme, monoamine oxidase, which is found associated with the mitochondria (Schnaitman et al., 1967). Monoamine oxidase has no regional specificity but has its highest concentrations in the hypothalamus (La Motte et al., 1969). Approximately 10 percent of the catecholamines are metabolized extraneuronally by catechol-O-methyltransferase (COMT) (Axelrod, 1959) which converts noradrenaline to normetanephrine and dopamine to 3-methoxytyramine.

The system is similar for 5-hydroxytryptamine in that there is a specific reuptake system, so that 5-hydroxytryptamine is either restored or metabolized by monoamine oxidase to 5-HIAA. There are differences as COMT does not degrade tryptophan derivatives, but enzymes are found almost exclusively in the pineal gland for converting 5-HT to melatonin.

More recently, two other enzymes that metabolize 5-HT have been found in the brain. One is N-methyltransferase enzyme (Mandell and Morgan, 1971) which has been identified particularly in the pituitary and the pineal and overts 5-hydroxytryptamine to its methyl derivatives, which may be psychogenic (Himwich, 1971). The other enzyme is 5-hydroxytryptamine sulphotransferase which has been found in the soluble

fraction of neuronal tissue and which convert 5-HT to 5-HT-O-sulphate (Hidaka et al., 1969).

5. Uptake

Uptake mechanisms for inactivation of noradrenaline were first shown in peripheral nerve endings (Iversen, 1965) but have since been shown to occur in the brain for noradrenaline, dopamine and 5-hydroxytryptamine. Uptake in vivo was demonstrated either by accumulation of radioactivity labeled amines at specific sites in the brain after intraventricular injection (Glowinski et al., 1965; Glowinski and Iversen, 1966) or by noting the increase in fluorescence in specific sites after intraperitoneal injection of the amines (Lichtensteiger and Langemann, 1966; Fuxe et al., 1968b). These sites were usually those which normally contained high endogenous levels of the particular transmitter. In the case of noradrenaline 80 percent of the uptake of exogenous amines occurs into brain nerve terminals, the rest is accumulated by a low affinity mechanism into other brain tissue (Baldessarini and Vogt, 1971).

Dopamine can be taken up by noradrenergic neurons in the central nervous system and either converted to noradrenaline within 15 to 30 minutes (Glowinski and Axelrod, 1966) or possibly act as a false transmitter (Farnebo et al., 1971). Dopamine also has a specific uptake system of its own. The uptake of 5-HT in the brain has been demonstrated in vivo and in vitro (Schanberg, 1963). The uptake occurs at two sites; in low concentrations it enters specifically into tryptaminergic nerve endings, but in higher concentrations it accumulates into catecholaminergic neurons and catecholamines and 5-HT compete for the same uptake sites (Shaskan and Snyder, 1970).

III. Hypothalamic Control of Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) Secretion

A. Neural Control Centers for Gonadotropin Release; "Cyclic" vs. "Tonic" Centers

There is considerable evidence that a structure adjacent to the hypothalamus is of importance for the normal cycling activity in female rats. This area, the parvicellular medial preoptic area (MPO), receives rich input from limbic and mesencephalic nuclei (limbic-midbrain circuit) (Nauta, 1958; Szentagothai et al., 1962; Heimer and Nauta, 1969; Nauta and Haymaker, 1969). The medial preoptic area of female rats binds labeled estradiol (Stumpf, 1971; Pfaff and Keiner, 1973) and shows in electron micrographs distinct differences in the distribution of different types of synapses when compared to the MPO of the male rats (Raisman and Field, 1971). Electrolytic lesion of the MPO stops estrous cycle activity (Everett and Radford, 1961) and electrochemical or electrical stimulation results in increased pituitary gonadotropin and prolactin release (Wuttke et al., 1972; Kalra et al., 1973). If the fibers connecting the MPO and the medial basal hypothalamus (MBH) are disconnected by the use of a small bayonet-shaped knife (Halasz-knife), normal cyclical activity also disappears (Taleisnik et al., 1970; Kobayashi and Miyake, 1971). Whereas a more rostral cut, which disconnects the fiber inputs to the MPO but leaves the fibers between the MPO and MBH intact, has no severe effects on the estrous cycle (Koves and Halasz, 1970).

For all these reasons it seems reasonable to consider the MPO as an important structure regulating the cyclical release of LH and possibly prolactin. Thus, the MPO is usually referred to as the "cyclical" center as opposed to the "tonic" center, which is located in the MBH and

regulates tonic pituitary LH release (Gorski and Wagner, 1965). Both areas, the MPO and the MBH are sites of feedback interactions (Stumpf, 1970). Lesions in the MBH (tonic center) result in anovulation associated with permanent anestrus indicating reduced pituitary gonadotropin release (Igarashi and McCann, 1964; Bishop et al., 1972). Lesions of the MPO (cyclic center) also stop cyclical activity but lead to a constant estrus syndrome with polyfollicular ovaries but no corpora lutea (D'Angelo and Kravatz, 1960; Flerko and Bardos, 1960).

The MBH is the part of the diencephalon where the releasing and inhibiting factor (RF and IF) producing neurons are considered to be located. With the help of two different methods, Halasz and associates gave evidence that the hypothalamic hormones are produced in the so-called hypophysiotropic area of the hypothalamus. Stereotaxic implantation of pituitary fragments into various hypothalamic structures of hypophysectomized and castrated rats results only in the occurrence of castration cells in the fragments if hypothalamic hormones exert their action on these fragments. Thus, regions which contain hypothalamic hormones could be mapped. It was demonstrated that only the mediobasal part of the hypothalamus has hypophysiotropic properties (Halasz et al., 1962; Knigge, 1962). Later, using his knife technique, Halasz and Pupp (1965) and Halasz and Gorski (1967) isolated the mediobasal hypothalamus from the rest of the brain. This complete deafferentation resulted in disruption of estrous cycle activity because the medial preoptic area had been disconnected from the mediobasal hypothalamus. Basal pituitary hormone secretion was unchanged though, indicating that the basal secretion of the hypothalamic hormones was still intact.

Whether the LH release controlling neurons are only present in the mediobasal hypothalamus or whether these neurons are also present in the preoptic areas as suggested by Wheaton et al. (1975) in the rat and by Barry et al. (1974) in the guinea pig remains questionable. The presence of LRH containing neurons in the preoptic area, however, does not necessarily mean that it is these neurons that are involved in preovulatory gonadotropin secretion because the synthetic decapeptide LHRH has been shown to have effects on mating behavior (Moss and McCann, 1973, 1975).

The very basal part of the MBH is the arcuate nucleus where clusters of dopaminergic neurons are located with fiber projections into the median eminence (ME) (Carlsson et al., 1962; Fuxe, 1963). Other axon terminals from noradrenergic and possibly serotonergic fibers arriving from mesencephalic structures are also found in the ME (Fuxe, 1965; Andén et al., 1966; Ungerstedt, 1971). These terminals and most likely axonal endings from hypothalamic factor-producing neurons form intimate contact with the portal vessels that run along the ME and the pituitary stalk linking the hypothalamus and the anterior pituitary gland.

It has been shown that spontaneous activation of the "cyclical" center occurs a relatively short time before the preovulatory LH surge is released. This spontaneous activation can be inhibitied by drugs such as anesthetics (e.g., pentobarbital), and also drugs that modify the metabolism of certain monoamines (Everett et al., 1949; Everett and Sawyer, 1950). If pentobarbital anesthesia is initiated prior to the activation of the cyclic center, this activation will be inhibited, the preovulatory LH and Prl surge is abolished and ovulation will not occur (Wuttke and Meites, 1970). The most effective period for pentobarbital

inhibition o f the cyclic center is the early afternoon, a few hours prior to the time when the LH surge normally occurs. The most effective period has been called the "critical period". These pentobarbital-blocked proestrous rats provide a good model to study the effects of electrical stimulation of the MPO or hypothalamic structures (Bunn and Everett, 1957). Critchlow (1958) was the first to do this type of experiment. Electrical stimulation of the MBH proved to be effective in restoring ovulation in pentobarbital-blocked rats. Later Everett and Radford (1961) demonstrated the same effect after stimulation of the MPO. It is now well established that electrical or electrochemical stimulation of the female MPO results invariably in pituitary gonadotropin release, which is probably due to increased release of hypothalamic LRH into the portal vessels (Harris and Ruf, 1970). The magnitude of response, however, varies with the stage of the estrous cycle (Kalra and McCann, 1973). Highest post-stimulatory LH levels were found in proestrous rats, which again demonstrates the priming effect of estrogens (Kalra and Kalra, 1974). It was also shown that injection of progesterone into rats on the mroning of proestrus advanced the critical period and the time of preovulatory gonadotropin release (Zeilmaker, 1966; Nallar et al., 1966; Caligaris et al., 1968; Lawton, 1972). These observations certainly suggest that differences in neuronal activity in the MPO should be demonstrable at different reproductive stages. A number of attempts have been made to record such neuronal activities under different endocrine conditions.

B. Monoamines as Transmitters of Gonadotropin Release

1. Dopamine

The results obtained after altering levels of hypothalamic dopamine fall into two categories. The earlier work of McCann and his coworkers suggested that this amine stimulates gonadotropin release, while there is now accumulating evidence suggesting it inhibits gonadotropin release.

In 1969 McCann and his coworkers started an extensive investigation into the effects of amines on gonadotropin release. They incubated rat pituitaries with each of the three amines in turn and found that dopamine had no direct effect on the release of gonadotropins but that adrenaline enhanced the release of FSH and LH, and noradrenaline only enhanced FSH release (Schneider and McCann, 1969a; Kamberi and McCann, 1969a,b; Van Loon and Kragt, 1970; Quijada et al., 1974). When portions of the stalk-median eminence area of the hypothalamus were added to the incubation, the release of both FSH and LH was enhanced by dopamine even at very low concentrations (0.5-5 μg/ml) while noradrenaline and adrenaline had no effect (Schneider and McCann, 1969b; Kamberi et al., 1970a). Dopamine must have acted in these experiments by stimulating the discharge of LH and FSH-releasing hormone from the hypothalamic fragments since it did not potentiate the action of exogenous LH/FSH releasing hormone added to the incubation mixture. α-Adrenergic and dopaminergic blocking agents (phenoxybenzamine, phentolamine and haloperidol) inhibited the release, and a β-adrenergic blocking agent (propranolol) did not (McCann et al., 1972). The prior addition of estradiol to the incubation also blocked the releasing effect of dopamine and Schneider and McCann (1970a) suggested that estradiol exerts its negative

feedback effect in physiological conditions by inhibiting the stimulating action of dopamine on LH/FSH-RH release.

The stimulating effect of dopamine has also been shown in in vivo experiments. Intraventricular injection of 1-4 μg of dopamine increased the LH/FSH releasing hormone activity in the plasma taken from the hypophysial portal system (Kamberi et al., 1969a, 1970b,c, 1971a) as well as the peripheral plasma of hypophysectomized rats (Schneider and McCann, 1970c), and this stimulatory effect was prevented by pretreatment with estradiol (Schneider and McCann, 1970c). Similarly, dopamine stimulated the release of LH and FSH into the circulating blood of intact male and female rats and in ovariectomized female rats pretreated with ovarian hormones (Schneider and McCann, 1970d; Kamberi et al., 1970a; Porter et al., 1972). In the intact female rat, dopamine was particularly effective on the second day of diestrus and on proestrus, and these effects were reversed by α-adrenergic blocking agents (Schneider and McCann, 1970d). Parallel experiments using adrenaline and noradrenaline revealed that both amines given intraventricularly stimulated gonadotropin release but that slightly higher doses (2.5-5 μg) were required and that doses of 100 μg or more were required for the two amines to be effective in intact males (Schneider and McCann, 1970b,c,d; Kamberi et al., 1970b).

When the effective intraventricular dose of dopamine was injected directly into the portal hypophysial system, release of gonadotropins was not stimulated, thus demonstrating as in the in vitro experiments that dopamine did not act directly on the pituitary (Kamberi et al., 1970b). Dopamine was also inactive when perfused into the arteries

supplying the anterior and posterior areas of the median eminence, indicating that the median eminence is probably not the site of action either (Porter et al., 1972).

Other experiments have confirmed that dopamine is of major importance for the release of gonadotropins. Kordon and Glowinski (1969, 1970) injected α-methyltyrosine and α-methyldopa into immature rats induced to superovulate by pretreatment with PMS and HCG. They found that these inhibitors of catecholamine synthesis blocked ovulation when given during the critical period but not if given a few hours before. This is strange as these compounds have an onset of action of 2-3 hours and a duration of up to 24 hours. Dopa (which restores the levels of both dopamine and noradrenaline) partially reversed the effect of α-methyltyrosine, and dihydroxyphenylserine (DOPS) (which restores noradrenaline levels only) had no effect on the ovulation. These experiments suggest that α-methyltyrosine inhibited ovulation due to a reduction of dopamine synthesis during the critical period. Kordon (1971) also found that an intrahypothalamic implantation of α-methyltyrosine inhibited ovulation, but only when placed in the arcuate nucleus-median eminence region, the site where, in fact, the dopamine neurons terminate. In a separate study pimozide (a DA receptor blocker) was shown to inhibit the release of LRH in hypophysectomized rats (Corbin and Upton, 1973).

A directly opposite role for dopamine has been suggested by Fuxe and his coworkers. They showed that there was an increased dopamine activity in the median eminence at the times when gonadotropin release was inhibited. It is possible, as has been suggested by Fuxe and

Hökfelt (1970) and even by McCann (Donoso et al., 1971), that dopamine is converted into noradrenaline after intraventricular injection and only appears to be more active than noradrenaline because injected noradrenaline is rapidly metabolized. This idea is supported by the fact that phenoxybenzamine, which blocks α-adrenergic and not dopaminergic receptors (Fuxe and Hökfelt, 1970), antagonized the gonadotropin releasing action of dopamine. Also, dopamine infusions are effective in releasing gonadotropins only when infused into the median eminence where dopaminergic nerve terminals are situated (Porter et al., 1972). Porter et al. (1972) has found that intraventricular injection of saline can induce gonadotropin release perhaps due to mechanical stretching of the ventricles. It is possible that the results obtained with dopamine in vivo are due to this artifact.

The results supporting the hypothesis that dopamine stimulates the release of the gonadotropins have been widely published, but in fact all the experiments in which dopamine itself was used, either in vivo or in vitro, came from McCann's laboratory between 1969 and 1971. In 1974 they repeated their in vitro experiments by adding dopamine to incubating pituitaries and hypothalamic fragments and found no effect on gonadotropin release; the only difference between this experiment and the previous ones was that three hypothalamic fragments were used instead of two (Quijada et al., 1974). In a similar type of experiment in which pituitaries attached by an intact portal system to a median eminence were superfused, the presence of 2.5 μg/ml of dopamine inhibited gonadotropin release (Miyachi et al., 1973).

Some in vivo experiments have also shown that dopamine can inhibit gonadotropin release. In an acute experiment, 80 μg of dopamine was

infused into the arcuate nucleus during the critical period in cyclic rats and found to inhibit ovulation (Craven and McDonald, 1973). In another report, implants of dopamine were placed into the median eminence area (as well as other parts of the hypothalamic) and were shown to have an inhibitory effect. Uemura and Kobayashi (1971), found that implants of dopamine in cholesterol (2:1) left for two weeks in the posterior part of the median eminence (which included part of the arcuate nucleus) appeared to suppress the cyclic release of LH, and both the cyclic and tonic release when the proportion of 5 to 1. These two implants were estimated to release 80-100 μg dopamine daily, respectively.

Oral administration of 500 mg L-dopa to humans caused a fall in plasma LH levels within one hour. The decline was never below the physiological range, but it was suggested that the ovulatory LH surge in women may be suppressed (Boden et al., 1972).

In conclusion, it seems that dopamine may have some inhibitory function in controlling gonadotropin release. The earlier work showing its stimulatory effect cannot, as yet, be ignored and perhaps it has a dual role depending on endogenous endocrine conditions. In addition, Ojeda and McCann (1973) obtained evidence that dopamine may be involved with FSH but not LH release. They showed that pimozide (a dopamine receptor blocker) selectively lowered plasma FSH levels in the castrated rat, while diethyl-dithiocarbamate (DDC), which lowers noradrenaline by inhibiting the conversion of dopamine to noradrenaline, lowered plasma LH. Correlated with this Choudhury et al. (1973) have shown that activating dopamine receptors with apomorphine stimulated FSH release.

2. Noradrenaline

All the results obtained after altering levels of noradrenaline in the hypothalamus indicate that it is concerned in the tonic and cyclic release of LH and that it acts as a stimulatory transmitter.

When rats are unilaterally ovariectomized, the remaining ovary undergoes compensatory hypertrophy due to an increase in gonadotropin release because of the reduction in ovarian secretion and therefore in negative feedback effects. Inhibition of catecholamine synthesis by α-methyltyrosine prevented this compensatory growth in immature rats (Müller et al., 1972) and selective destruction of noradrenergic nerve terminals by 6-hydroxydopamine prevented this growth in adult rats (Zolovick, 1972). The effect of α-methyltyrosine was reversed by both DOPA and DOPS. These results indicate that noradrenaline is involved in the tonic release of gonadotropin after unilateral castration. Similar results were obtained in bilaterally castrate males where administration of either α-methyltyrosine or DDC lowered plasma LH levels. In this preparation their effects were reversed by DOPS but not by DOPA (Ojeda and McCann, 1973).

Additional evidence supporting the noradrenergic control of tonic gonadotropin release was shown in rats in which the hypothalamus completely deafferentated. In these rats there was a fall in hypothalamic noradrenaline levels to 38 percent of the normal concentration and at the same time plasma and pituitary LH levels fell significantly, while dopamine levels remained normal. When only the anterior portion of the hypothalamus was deafferentated there was only a small decrease in

hypothalamic noradrenaline and only a slight fall in plasma LH (Blake et al., 1972; Weiner et al., 1972b).

Noradrenaline may also be concerned with the cyclic release of the gonadotropins in that Kalra and McCann (1973b) have shown that noradrenaline may be the synaptic transmitter in the pathway between the preoptic area and the median eminence, because the LH surge obtained after stimulation of the preoptic area was reduced by α-methyltyrosine (which lowers noradrenaline and dopamine levels in the hypothalamus) and by dopamine-β-hydroxylase inhibitors (which lower noradrenaline levels only). The LH surge in these animals was restored by DOPS, which selectively raises noradrenaline levels but not by a dose of DOPA which raises mainly dopamine levels. The LH surge after stimulation of the median eminence was not affected by the synthesis inhibitors (Kalra and McCann, 1973b). This type of experiment was also carried out in ovariectomized rats. When these animals are primed with estrogen and then treated two days later with a single injection of progesterone or estrogen there is a surge of LH, which can be prevented by the synthesis inhibitors and can be reversed by selectively raising noradrenaline levels, but not dopamine levels (Taleisnik et al., 1970; Kalra et al., 1971, 1972; Kalra and McCann, 1973a).

Craven and McDonald (1971) have shown that infusion of noradrenaline, but not of dopamine, into the arcuate nucleus in rats pretreated with a monoamine oxidase inhibitor can advance the critical period and therefore the time of the LH surge before ovulation. On the other hand, neither noradrenaline nor dopamine placed in the arcuate nucleus was able to overcome the anti-ovulatory effect of pentobarbital or reserpine (Craven and McDonald, 1971, 1973). They suggested that noradrenaline

may be involved in the changes occurring early on the day of proestrus leading up to the LH surge, rather than in the release of LH itself. In this connection, Kanematsu et al. (1972) found that intraventricular administration of noradrenaline stimulated the release of LH in rabbits but with a different onset and duration from that produced by coitus or electrical stimulation, suggesting that while noradrenaline was involved with LH release it was not via the same mechanisms as that producing the ovulatory surge of LH.

3. Serotonin

Inhibitory Effects

Since the administration of 5-HT caused atrophy of the reproductive organs and delayed puberty in immature mice, Robson and Botros (1961) considered it likely that 5-HT was an inhibitor of gonadotropin release. This was supported by the findings of Vaughan et al. (1970) who showed that intraperitoneal administration of 5-HT prevented the compensatory ovarian hypertrophy normally seen after unilateral ovariectomy in rats. Subcutaneous or intraperitoneal injections of 5-HT were also found to inhibit ovulation both in adult rats and immature rats induced to ovulate with PMS (O'Steen, 1964, 1965; Endersby et al., 1970; Labhsetwar, 1970; Tima et al., 1973). Similar results were obtained by raising levels of 5-HT in the body, including the brain, by administering monoamine oxidase inhibitors; this was shown in the hamster, rat and immature rat (Alleva et al., 1966; Kordon et al., 1968; Kordon, 1969; Labhsetwar, 1970).

The hypothesis that 5-HT inhibits gonadotropin release, however does not explain all the results. Most drugs that inhibit ovulation by a central action do so by suppressing the hypothalamic stimulation of

pituitary that occurs just before the critical period. However, subcutaneous administration of 5-HT had no effect just before the critical period but only inhibited ovulation when given after the critical period, or late in diestrus (Endersby and Wilson, 1973). The timing of the inhibitory effects correlates well with the times in the cycle when ovarian hormones are secreted and Wilson and McDonald (1973, 1974), suggested that the anti-ovulatory effect of the 5-HT is due to a peripheral vasoconstrictor action preventing the passage of the ovarian steroids away from the ovary to the hypothalamus. The main support of this hypothesis is that 5-HT can antagonize the ovulatory action of exogenous LH and that the anti-ovulatory effect of the 5-HT on spontaneous ovulation is reversed by a vasodilator (dipyradimole). A vasodilator compound (apresoline) has also been shown to prevent the atrophy of the reproductive organs in male rats caused by 5-HT (Boccabella et al., 1962). The experiments in which monoamine oxidase inhibitors showed an anti-ovulatory effect may be due to raised levels of peripheral 5-HT, although the authors correlate the effect with raised brain levels.

In spite of all these findings there is still much evidence that 5-HT may be a central inhibitory transmitter. Firstly, there is the presence of 5-HT nerve terminals in the hypothalamus, particularly the suprachiasmatic nuelcus, retrochiasmatic area and the median eminence; this latter area is capable of synthesizing 5-HT from tryptophan and this is known to occur only in tissues containing tryptaminergic neurons (Andén et al., 1965; Hamon et al., 1970). Secondly, there is a tryptaminergic tract from the midbrain to the hypothalamus (Ungerstedt, 1971; Fuxe et al., 1968b) and electrochemical stimulation of the ventral

tegmentum and raphé in the midbrain, or tracts leading from these areas to the hypothalamus, inhibits spontaneous ovulation in rats and also reduces plasma LH levels. This inhibitory effect can be prevented by lesioning the tracts between the two areas (Carrer and Taleisnik, 1970, 1972).

Changes in 5-HT concentrations and metabolism in the hypothalamus in different endocrine states have been noted, although the work is not as extensive as that on the catecholamines. In the intact ewe, 5-HT levels in the median eminence fall significantly just before the LH surge (Wheaton et al., 1972). When ovarian steroids were administered to castrated rats so that they exerted a negative feedback effect on gonadotropin release, there was a rise in hypothalamic tryptophan levels (Bapna et al., 1971), and also in 5-HT levels in the midbrain area (which included the hypothalamic area in this particular work) (Tonge and Greengrass, 1971). A single injection of estradiol either on day one or eleven of life raised 5-HT brain levels in immature males and females (Guilian et al., 1973). Kato (1960) found a similar effect after chronic administration of estradiol to immature rats. Conversely, ovariectomy of neonatal females reduced brain 5-HT levels. As well as brain 5-HT being influenced by the steroids, the metabolism of the steroids is altered by 5-HT. Pretreatment with 5-HTP increased the accumulation of ^{3}H-estradiol in the hypothalamus and pituitary after intravenous injection of the labeled steroid (Kordon et al., 1972). Treatment with progesterone may inhibit the synthesis of 5-HT, because accumulation of ^{3}H-tryptophan was reduced in castrated rats pretreated with progesterone (Kordon and Glowinski, 1972).

Intravenous injection of 5-HT can inhibit ovulation in rabbits if given just before the expected LH surge, indicating a central effect (Currie et al., 1969) and intraventricular administration of 5-HT inhibited LH and FSH release in both intact and castrated male and female rats (Kamberi et al., 1970b, 1971a; Kamberi, 1973; Schneider and McCann, 1970d), and caused atrophy of the gonads in immature rats (Corbin and Schottelius, 1961). 5-HT had no effect on the pituitary itself (Kamberi and McCann, 1969a,b).

Reports on the effect of intraventricular 5-HT on spontaneous ovulation differ. When high doses of 5-HT (50-200 µg per rat) were injected at various times of the day of proestrus, there was no effect on ovulation (Rubinstein and Sawyer, 1970; Schneider and McCann, 1970d; Wilson and McDonald, 1974). However, recently Kamberi (1973) found that 1-5 µg of 5-HT injected intraventricularly just before the critical period inhibited ovulation; it was ineffective when given by the intracardiac route, while its precursor 5-HTP, which passes through the blood-brain barrier, was effective. Intraventricular 5-HT also inhibited the facilitatory effect of progesterone on induced ovulation (Zolovick and Labhsetwar, 1973). Implants of a monoamine oxidase inhibitor (nialamide) in the median eminence were also effective in blocking ovulation, but this may be due to the compound itself and not the raised 5-HT levels (Kordon and Vassent, 1968).

Wilson (1974) injected 5-HT into the ventrolateral part of the anterior hypothalamus, e.g., in the medial forebrain bundle, and showed that it inhibited induced ovulation in immature rats. This suggests that the tracts from the midbrain that Carrer and Taleisnik (1970) showed

could inhibit ovulation may be stimulated by the 5-HT injections and this may be the site for inhibition of cyclic release.

In 1959, Brodie and Shore suggested that 5-HT and noradrenaline were transmitters in opposing systems, which were in dynamic balance. Lippmann (1968) suggested that gonadotropin release was controlled, not by individual levels of the amines, but their relative proportions, so that when 5-HT levels were high compared to noradrenaline, or when noradrenaline levels were low compared to 5-HT, then gonadotropin release was inhibited. Labhsetwar (1971) made a similar comment and showed that, when both amines were reduced to the same extent, there was no effect on gonadotropin release. He also showed that dopamine can reverse the inhibitory effects of 5-HT on ovulation induced by PMS and progesterone (Zolovick and Labhsetwar, 1973).

5-HT is a precursor of the pineal hormone, melatonin, which has been shown to inhibit gonadotropin release and activity (Wurtman et al., 1968; Debeljuk et al., 1970; Kamberi et al., 1971a) and also to inhibit ovulation (Longenecker and Gallo, 1971; Ying and Greep, 1973) and delay puberty (Collu et al., 1973). Conversely removal of the pineal can induce ovulation (Mess et al., 1973). Martini and his coworkers (1968) investigated the various pineal principles and noted that they had a selective effect on LH and FSH release. They found that implants of melatonin and 5-hydroxytryptophol in the median eminence of castrated male rats reduced pituitary and plasma LH but did not affect levels of FSH. On the other hand, 5-HT and 5-methoxytryptophol implants only reduced FSH levels in the pituitary. The pineal principles have a diurnal rhythm; in the dark period melatonin concentration is high and 5-HT low and during the day the levels are reversed. Fraschini (1970)

suggested that this pineal rhythm controls the rhythm seen in pituitary gonadotropins which peak daily in the afternoon. He suggested that the pineal substances travel to the basal hypothalamus via the cerebrospinal fluid and then alter releasing hormone activity.

The Stimulatory Effect of 5-HT

Although the majority of the results show that 5-HT acts as an inhibitor of gonadotropin release, there is also some evidence that 5-HT can stimulate both tonic and cyclic gonadotropin release. Most of the evidence has been obtained after administration of p-chlorophenylalanine (PCPA), a compound known to inhibit synthesis of brain 5-HT by antagonizing the action of tryptophan hydroxylase (Koe and Weissman, 1968). It has been shown that PCPA prevents the onset of puberty (Fajer et al., 1970) and the sudden release of FSH normally seen at puberty (Brown, 1971). It also reduces reproductive weights in hemicastrated male mice (Fawke and Brown, 1970) and reduces testosterone levels in the plasma of intact male rats (Bliss et al., 1972). At the same time PCPA raises pituitary FSH levels in intact and castrated male rats (Brown and Fawke, 1972). All these results indicate that the release of FSH stores in the pituitary is inhibited after PCPA and therefore the gonads are not stimulated to grow or secrete steroids. PCPA also inhibits induced ovulation if given 20 hours before the critical period (Kordon et al., 1972), that is at a time stradiol secretion is taking place, in order to eventually stimulate the release of the LH surge. Kordon suggested that 5-HT is necessary for the positive feedback effect of the estradiol, perhaps by enhancing its uptake into particular hypothalamic sites.

Complete deafferentation of the hypothalamus lowers 5-HT levels within this area by 70 percent, as well as significantly lowering noradrenaline. It is possible that the reduction in gonadotropin release seen after the operation is due to the reduction in tryptaminergic activity (Weiner, 1973).

5-HT itself has been shown to stimulate gonadotropin release. For instance, chronic subcutaneous administration of 5-HT for 12 weeks increases spermatogenesis in adult male rats (Kinson and Lui, 1973); intraocular or subcutaneous 5-HT induces ovulation in persistent estrous rats (Takahashi et al., 1973); a single intravenous injection of 5-HT raises plasma LH levels in ovariectomized rats pretreated with ovarian steroids (McCann et al., 1960).

Interesting results have been reported on the immature animal induced to ovulate with PMS. Brown (1967, 1967) showed that subcutaneous injections of 5-HT potentiated induced ovulation in immature mice, while anti-5-HT compounds, such as LSD and methylsergide, inhibited ovulation. Similar results were obtained in immature rats treated with PMS. Thirty-day old rats underweight for their age did not ovulate after PMS treatment, but if 100 mg/kg of 5-HT was given subcutaneously, just before critical period, then 50 percent ovulated (Jaitley et al., 1967). One mg/kg of 5-HT intraventricularly administered also induced ovulation in both immature and underweight rats. The stimulatory site of action of 5-HT appears to be the paraventricular nucleus, as injections of 2 μg per rat specifically into this nucleus stimulated ovulation in 77 percent of underweight immature rats; injections into the arcuate nucleus, the suprachiasmatic nucleus and the atnerior hypothalamus were ineffective (Wilson and MacDonald, 1973). In adult rats, however,

the suprachiasmatic nucleus may be the stimulatory site, as this nucleus contains high levels of 5-HT, and while lesions in this region prevented ovulation (Artnes-Rodrigues and McCann, 1967), stimulation of this nucleus induced ovulation (Critchlow, 1958).

As mentioned previously, Fraschini et al. (1971) suggested that the circadian rhythm of gonadotropin release is controlled by the pineal principles. Quay (1963) has shown 5-HT levels in the pineal are at their highest daily at midday and higher on the day of proestrus, than on other days. He also showed that hypothalamic levels of 5-HT have a circadian rhythm with a peak each day in the late afternoon, at the expected time of the critical period, and when pituitary levels of the gonadotropins are at their highest. It is possible, therefore, that 5-HT is involved in the control of the release of the ovulatory surge of LH (Quay, 1968).

A most interesting finding by Ladosky and Gaziri (1970) and also confirmed by Guilian et al. (1973) is that 5-HT may be involved in sexual differentiation. They found that between days 10-14 of life, there is a rise in brain levels of 5-HT in females which can be prevented by administration of testosterone, or by ovariectomy on day one of life. Hardin (1973a) obtained rather different results in that the 5-HT levels in the females were higher only on day two and not later in life. Injections of estradiol in the neonatal period can raise 5-HT levels in both male and female rats (Kato, 1960). The alteration in 5-HT levels may be due to the effect of the steroids on monoamine oxidase (MAO) activity; for instance, activity on day 12 is reduced by testosterone given on day one of life and raised after castration (Ladosky and Gaziri, 1970; Gaziri and Ladosky, 1973). On the other

hand, the differences may be due to the higher levels of 5-HTP decarboxylase found in 2-day-old females compared to their male litter mates (Hardin, 1973b).

Conversely, Vaughan et al. (1969) have shown that early administration of 5-HT can antagonize the androgenizing effects of neonatal testosterone in the female. This would indicate that the prevention of the cyclic release of gonadotropins by neonatal androgens, as seen in the normal male or after exogenous administration of testosterone to the female occurs via raising MAO levels and thus reducing 5-HT levels in the brain. These findings can be correlated with some fluorescent studies, which showed that when sexual differentiation is prevented in males by neonatal castration or the administration of an anti-androgen (cyproterone acetate) there was an increase in fluorescence in most hypothalamic nuclei due to raised amine levels (Hyppa and Rinne, 1971).

C. Gonadal Steroid Feedback in Regulating Gonadotropin Release

1. Negative Feedback

When the ovaries or testes are removed in the rat, both plasma and pituitary levels of LH show a marked rise (Gay and Midgley, 1969 and Yamamoto et al., 1970). In the case of FSH, the situation appears to be quite similar, the only difference being that the elevation in plasma levels of FSH is less dramatic than the elevation of LH. Also, repeated sampling and measurement by radioimmunoassay have revealed oscillations in the castrate rat (Gay and Midgley, 1969) and rhesus monkey (Dierschke et al., 1970) which have been termed circhoral fluctuations. Probably these represent a periodic pulsatile release of gonadotropin-releasing factors into the portal vessels. This effect

of ovariectomy could be inhibited by the administration of estrogen or progesterone (Ramirez et al., 1964; Smith and Davidson, 1974). The effects can be noted within one day and become maximal after two or three days. The sensitivity to the inhibitory effect of estrogen on gonadotropin release appears to be somewhat less in castrate males (Kalra et al., 1971).

2. Positive Feedback

Estrogen and administration to regular cycling rats during diestrus has been shown to advance the time of ovulation (Everett, 1948; Brown-Grant, 1969; Weick et al., 1971). This effect can be blocked as well as ovulation itself by estrogen antibody administration (Ferin et al., 1969, 1974; Knobil et al., 1974). Estrogen administration to long term ovariectomized rats causes a daily LH surge (Legan and Karsch, 1975).

Progesterone administration by itself, inhibits LH secretion in early diestrus (Everett, 1948) and interferes with the LHRH induced LH release (Martin et al., 1974). However, if progesterone is administered in an estrogen environment, such as is present in proestrus, an LH surge can be stimulated (Everett, 1948; Zeremaker, 1966; Brown-Grant and Naftolin, 1972). This facilitatory effect of progesterone upon estrogen action is seen when progesterone is given to estrogen primed, long term ovariectomized rats. Under these conditions an LH surge is seen which is similar in magnitude and timing of the preovulatory LH surge (Calgaris et al., 1971). In addition, surges of FSH and Prl accompanied this LH surge correlating it with those changes occurring during proestrus (Calgaris et al., 1974). Recent studies have shown daily morning progesterone peaks during the estrous cycle (Mann and Barraclough, 1973)

and a large progesterone surge on the morning of proestrus (Kalra and Kalra, 1974). Thus, a synergistic interaction between progesterone and estrogen may normally occur during the estrous cycle.

Under the influence of FSH (and a low basal level of LH), the ovarian follicles mature and at a certain stage secrete estradiol (Schwartz, 1969; Ely and Schwartz, 1971). Following a peak level of plasma estradiol at around midday (Shaikh, 1971) on the day before ovulation, there is a sudden surge of LH, FSH and prolactin from the pituitary in the late afternoon on the day before ovulation, lasting two to four hours (Naftolin et al., 1972; Mahesh and Goldman, 1971; Freeman et al., 1972). The surge of LH (but probably not of FSH or porlactin) is necessary for ovulation to take place. It initiates (1) the secretion of progesterone from the follicles and/or interstitial cells (Leavitt et al., 1971) and (2) changes in the follicle walls so that the follicles rupture about 12 hours later, each follicle releasing an ovum. Luteal tissue then grows to fill the follicles to form corpora lutea which are capable of producing progesterone, although they do not secrete high levels unless fertilization takes place.

In the rat the stimulation of LH release by the rise in plasma estradiol can only occur during a critical period of two hours during the afternoon of the same day. The exact time is controlled by the time of commencement of the light period for that day. For example, on a fixed regime of 14 hours of light starting at 0600 h, the critical period occurs between 1400 and 1600 h (Everett et al., 1949). The actual release of LH into the blood after stimulation occurs between 1200 and 2000 h with a peak at 1800 h.

The rise in progesterone levels occurring simultaneously, or just after the LH surge, is stimulated by the LH (Barraclough et al., 1971; Piacsek et al., 1971) and only declines slowly over the following dark period. It is thought to be necessary for inducing sexual receptivity (Boling and Blandeau, 1939) in the rat and may also help control the duration of the LH surge (Kobayashi et al., 1970). In the rat, all these changes occur over a period of four or five days (the estrous cycle).

In the human, an analogous series of change occur over a period of approximately 28 days (the menstrual cycle). During the first half of the cycle (or follicular phase) low levels of FSH and LH stimulate the ovarian follicles to secrete estrogen which reaches a peak concentration just before mid-cycle, and then, either on the same day or one day later, there is a dramatic increase in LH secretion (and to a lesser extent FSH) which lasts one or two days. This is equivalent to the LH surge in the rat and ovulation takes place at some time towards the end of this mid-cycle period. After ovulation the corpus luteum formed from the ruptured follicle secretes progesterone and also some estrogen in the second half of the cycle (the luteal phase) near the time of implantation. If fertilization does not occur, the corpus luteum regresses approximately two weeks after its formation and the steroid secretion falls to zero (Ross et al., 1970; Vande Weile et al., 1970).

3. Hypothalamic Sites of Feedback

The sites of steroidal feedback effects in the hypothalamus have been the object of much work. It is now considered that there is a tonic center in the arcuate nucleus and median eminence area

which controls low tonic secretion of gonadotropin necessary for maintaining gonadal weight and function. In the female there is an additional center in the preoptic area, which controls the cyclic release of LH just before ovulation (Gorski, 1966). It is thought that the cyclic center sends pulses at a regular time each afternoon to stimulate the tonic center in the median eminence (Tejasen and Everett, 1967; Gorski, 1968), but the latter only responds on the day before ovulation when the estradiol levels are high. It is suggested that the estradiol lowers the threshold of stimulation of the median eminence (McDonald and Gilmore, 1971; Sawyer and Hilliard, 1971). Everett (1964) has shown that there is a neural pathway connecting the two centers. Stumpf (1968) demonstrated by autoradiography that estradiol is taken up in significant quantities in the anterior hypothalamus, especially in the preoptic and median eminence areas, and Kato (1973) showed that there are significantly higher concentrations of estrogen receptors in these areas. Implants of estradiol are more effective in inducing gonadotropin release when placed in the median eminence, although implants in the preoptic area are also active in some situations (Smith and Davidson, 1967; Davidson, 1969). The site of the negative feedback effect of the steroids also appears to be at the median eminence levels (Chowers and McCann, 1967; Smith and Davidson, 1968; Taleisnik *et al.*, 1970). Possibly the steroids have biphasic effects on the threshold of stimulation of the median eminence, first lowering and then raising it (Beyer and Sawyer, 1969).

IV. The Effect of Light Upon Reproductive Function

A. General

1. Light as an Environmental Constituent

Each human organism receives a finite number of inputs from the world beyond its integument. These inputs, collectively termed "environment", include chemical, physical, biological, and informational factors; they presumably exerted the major influence on man's development throughout his evolutionary history, and continue to affect him now.

Solar radiation constitutes a ubiquitous and essential component of man's environment. Besides serving as the ultimate source of his food and energy, it has also acted directly upon man to alter his chemical composition, control the rate of his maturation, and drive or entrain his biological rhythms.

2. Characteristics of Natural Lighting

The spectral composition of sunlight at the earth's surface approximates that of the white light emitted by a theoretical black body heated to about 5600°K, minus the ultraviolet radiation below 290 nm that is unable to penetrate the ozone layer and atmospheric shield surrounding the earth. Hence the solar spectrum is continuous and the relative intensities of any of its component visible wavelengths do not differ by more than two-fold; the ratios of the radiant fluxes of red, blue, and yellow bands contained in the white light of the typical midday sun approximate 1:1:1.

Incandescent light sources emit spectra that approximate those of heated black bodies. Their color temperature (the termpature to which a theoretical black body would have to be heated in order to emit a

comparable spectrum) is considerably lower than that of sunlight, hence a larger fraction (about 90%) of their total radiant power consists of infrared radiation that provides heat rather than light (Thorington et al., 1971). Within the visible portions of incandescent spectra, the relative fluxes at different wavelengths are, as expected, maximum in the red and minimum in the blue. The identity of those wave lengths of light that are most important for the reproductive responses to light has not been established for the mammal. Allardyce et al. (1942) studied the fertility rate of rats exposed to different colors of light and found that most young were born in yellow light and none were born in blue light; the effects of red light and green light were intermediate. In the duck, Benoit and Assenmacher (1966) found that yellow light directed at the eyes did not cause gonadal stimulation but that red light was strongly stimulatory. As pointed out by Wurtman (1967), sunlight and both incandescent and fluorescent artifical lighting emit a range of wavelengths much broader than that perceived by mammalian photoreceptors, so that the quality of a light source has not been an important issue. However, certain types of fluorescent lighing that parallel more closely the emission pattern of sunlight have been shown to be more stimulating to the gonads than the usual cool-white fluorescent bulbs (Wurtman and Weisel, 1969) and thus the light source should be identified in reports of light effects.

B. Effects of Photoperiod on Reproductive Function

The system upon which light acts involves ultimately the gonads, which are producing the gametes and also the gonadal steroids. The gonads are stimulated to grow and secrete by gonadotropic hormones from the anterior pituitary gland. The pituitary synthesizes and

releases its gonadotropic hormones under the influence of neurohormones produced in the hypothalamus and released into the hypothalamic-hypophysial system in the region of the median eminence. The neurohormones may stimulate or inhibit synthesis or release of the gonadotropins from the pituitary. Although there is some evidence for a direct effect of certain treatments on the gonads, for the most part whatever acts on the reproductive system acts through an influence on the pituitary itself or on the hypothalamic control centers. The first problem then is to trace the pathway of the light stimulus from its external source to the hypothalamus.

1. Pathways of Light Impulses Affecting Reproduction

In adult mammals the receptor for light is the retina of the eye, even though measurable light does penetrate the skull (Ganong et al., 1963). Whether light is excluded by covering the whole head with a hood (Bissonette, 1936), by severing the optic nerve (Bissonette, 1938), by destroying the retina (Thomson, 1951), or by removing the eye (Corbin and Daniels, 1969; Hoffmann, 1967; Hoffmann et al., 1968) the result is the same; treated animals do not respond to altered or added external lighting. Lisk and Kannwischer (1964) have reported that implantation of glass fibers into the hypothalamus so that light could be conducted there from the outside caused some increase in ovarian weight in blinded animals exposed to constant light, compared to blinded animals in which the glass fiber was shielded. However, these experiments have not been purused further, and it is possible that energy in any form would have a stimulatory effect when conducted to appropriate areas of the hypothalamus.

Impulses initiated by light impinging on the retina are conducted back toward the brain via the optic nerves, which then join briefly at the optic chiasm. Some of the fibers cross there, and the majority of the fibers continue back toward the lateral geniculate bodies in bundles known as the optic tracts. However, some crossed fibers, which form the inferior accessory optic tract, leave the primary optic tracts immediately behind the chiasm and join the median forebrain bundles to terminate in the medial terminal nucleus of the midbrain (Hayhow, 1960). These accessory fibers have proven to be the most important fibers for reproductive responses to light. Destruction of the inferior accessory optic tract blocks the effect of added light on reproduction, whereas total destruction of the primary optic tracts does not (Critchlow and DeGroot, 1960).

Beyond this point the pathway has been established no further. That is not to say that other areas of the brain have not been implicated in responses to light, but only that clear-cut neuron-to-neuron pathways have not been traced.

Information about light ultimately reaches some center in the hypothalamus, where it is probably integrated with other information related to reproduction and translated into changes in amount and nature of neurohormones released to the pituitary.

Between the medial terminal nucleus of the midbrain and the hypothalamus the light information undoubtedly follows many pathways, and the information is probably used in different ways by many systems. One loop in this circuit that has been identified involves the pineal gland. Although the actual role of the pineal in reproduction is not yet clear, it does show morphological and functional changes in response to light, which suggest that it may mediate some of the effects of light on

reproduction. More detailed discussions of the pineal can be found in recent reviews (Quay, 1969, 1970; Reiter and Fraschini, 1969; Reiter and Sorrentino, 1970).

2. Correlation with Reproductive Activity

Bissonette (1932) in one of the earliest demonstrations of the stimulatory effects of light, showed that female ferrets came into heat in January instead of March, if they were exposed to about six hours of extra light per day in November and December. Subsequently, other workers have shown this stimulatory effect of increased photoperiod in the horse (Burkhardt, 1947), mink (Enders and Enders, 1963), racoon (Bissonette, 1937), cat (Scott, 1959), and vole (Clark and Kennedy, 1967). Conversely, keeping female ferrets in short daily photoperiods can delay the onset of estrus (VanDerWerfften Bosch, 1963). As might be expected, such species as the sheep, and goat, which normally exhibit estrus in the Fall, have been shown to advance their sexual activities when exposed to artificially shortened days and to go into anestrus earlier if photoperiod is increased in the Fall (Bissonette, 1941; Hafez, 1951).

Alternating light and dark is sometimes more stimulatory than continuous light. In the ferret, for example, exposure to 14 or 16 hours of light per day has a greater stimulatory effect than does constant exposure to light (Donovan, 1967; Hammond, 1951). This led Hammond (1951) to conclude that dark played an important role.

Some species, including the domestic rabbit (Clegg and Ganong, 1969) the cow (Asdell, 1964), and the pig (Clegg and Ganong, 1969) have been domesticated for so long that they seem to have escaped from the seasonal variations in breeding activity. Seasonal variations in fertility remain in most of these species (Clark and Kennedy, 1967) but

breeding season is no longer well defined. Laboratory animals also tend to show continuous cycles in the unvarying lighting conditions of the animal quarters; hamster (Orsini, 1961), rat (Everett, 1961), and mouse (Bingel and Schwartz, 1969).

3. Effect on Reproductive Cycle Length

The length of the reproductive cycle in the rat can sometimes be altered by the length of the photoperiod. Rats exposed to 12 hours of light and hours of darkness (12L:12D) show four day cycles while rats exposed to a (14L:10D) or (16L:8D) regimen show predominantly five day cycles (Hoffmann, 1968, 1969). In 24 hours of light per day (LL), the animals eventually go into persistent estrus. Differences in sensitivity of day length are apparent among various strains of rats (Hoffmann, 1970).

4. Effect on Sexual Maturation

An effect of photoperiod on the time of occurence of sexual maturation has been frequently demonstrated. Exposing immature female rats to constant light advances vaginal opening (Fiske, 1939; 1941; Moszkowska and DesGouttes, 1962; Piaseck and Hautzinger, 1974), and light deprivation by blinding or exposure to constant dark delays it (Fiske, 1939, 1941; Truscott, 1944). Constant light decreased the age at which large ovulatory follicles appeared therefore facilitating GnRH induced ovulation at an earlier age (Steger et al., 1975).

5. Effect on Timing of Ovulation and Mating Behavior

Probably the first indication that estrus and ovulation occurred at a specific time of day related to the light-dark cycle was the report by Dempsey et al. (1934). They found that behavioral estrus in guinea pigs occurred only in darkness and that the time of estrus

tended to shift as day length varied, so that the occurrence of estrus remained in the dark period. Subsequently, Hemmingsen and Krarup (1937) did a similar study in the rat. As in the guinea pig, estrus in the rat occurred during the dark, and a complete reversal of the light-dark schedule resulted in a reversal in the time of onset of estrus within a few cycles. In a similar experiment with the mouse, Snell et al. (1966) not only found that mating behavior predictably occurred at a particular time of day but also that ovulation occurred between midnight and 0400 h. Later, Everett, and Sawyer (1949, 1950) found that the release of ovulating hormone in the rat could be checked by barbiturates but only during a limited period of time--between 1400 and 1600 h on the day of proestrus (lights on at 0500 h and off at 1900 h; 14L:10D). As long as the time is measured from the midpoint of dark, the critical period for ovulation blockade occurs between 1400 and 1600 h, whether the length of the daily photoperiod is 12, 14 or 16 hours. Thus, the onset of the critical period and the resulting ovulation seem to be associated with the midpoint of dark or light, rather than with the beginning of dark or light (Alleva et al., 1970; Hoffmann, 1969). When the light to dark ratio is held constant but the photoperiod is shifted relative to solar time, the time of maximum ovulation blockade (Everett, 1952; Everett and Tajasen, 1967) and the time of ovulation (Alleva et al., 1970; Austin and Braden, 1954; Carlysle and Carter, 1961) shift a corresponding number of hours.

Psychoyos (1966) has suggested that the lighting schedule can also affect the sensitivity of the ovary to LH. He gave PMS to 28-day-old rats, placed them in conditions varying from 0 to 24 hours of light per day, and administered exogenous LH 2 days later. All the animals

ovulated, but the 18L:6D group shed twice as many ova, when compared to all the others.

6. Effect on Luteal Phase of the Reproductive Cycle

Little, if any, data are available to suggest that the length of the luteal phase in infertile cycles is affected in any way by light. The length of pregnancy, however, can sometimes be altered by light. In most cases the reason for the effect is not yet known. In the mink, delayed implantation is quite common, and females mating early in the season tend to have a longer delay and therefore a longer total gestation than those breeding later. Furthermore, increasing the photoperiod to which mink are exposed can cause earlier implantation (Pearson and Enders, 1944). This is also true of the marten and the sable (Farner, 1961). The exact mechanism of the delay in these species is not clear. In the rat delayed implantation is seen during lactation, and implantation can be induced by giving a single injection of estrogen (Krehbiel, 1941); however, no hormonal regimen has been successful in inducing implantation in the wild species that normally show the delay (Canivenc and Bonnin-Laffarque, 1963; Hammond, 1951). All that can be said is that the corpora lutea remain in a state of quiescence until some external signal or internal clock turns them on.

The total length of gestation in the horse is known to vary with the season. Mares delivering in winter have a gestation period as much as 20 days shorter than mares delivering in spring (Asdell, 1964). Whether this is due to a difference in time of implantation or to rate of development of the foal is not known.

C. Effect of Constant Light or Constant Dark on Reproduction

In the complete absence of a light-dark alternation--in constant light or constant dark--reproductive cycles may continue undisturbed or may be profoundly altered. The rat and mouse go into a state or persistent estrus fairly soon after exposure to constant light (mouse, Chu, 1965; Murthy and Russfield, 1970; rat, Browman, 1937; Everett, 1939, 1942; Hardy, 1970; Hoffmann, 1970; Jochle, 1956; Lawton and Schwartz, 1965, 1967; Takahashi and Suzuki, 1969). Follicles develop to the preovulatory stage and secrete estrogen, but ovulation does not occur, so that after a time the ovary contains no corpora lutea. Only the follicles in various stages of development are seen, and the constant secretion of estrogen causes a continuous vaginal cornification, a high uterine weight, and almost continuous sexual receptivity. If, instead of constant light, the animals are in constant dark (by being housed in lightless quarters or by blinding), the picture is entirely different. Browman (1937) reported that rats kept in continuous dark showed normal estrous cycles, but he apparently kept them in these conditions for only a few weeks. Jöchle (1956) kept animals in constant dark for 80 days and reported that their cycles became longer, with more diestrus smears. Hoffmann (1967) found that 20 percent of female Wistar rats blinded or placed in continuous darkness as adults went into complete anestrus. Those animals showed continuously leukocytic smears and at autopsy had very small ovarian and uterin eweights, no recent corpora lutea, and only small follicles in the ovaries. Females that were blinded at 21 days of age, on the other hand, showed only a slight delay in reaching puberty and later showed completely normal estrous cycles and organ weights. It is important to note that light deprivation can have

quite different effects depending on the origin of the rats under study and the age of the animals when treatment beings (Hoffmann, 1970).

In the hamster, constant light was first reported to have no effect on the estrous cycle (Greenwald, 1963). However, the animals were studied for only 60-70 days (16-18 cycles). When exposure was continued for 6-14 months, hamsters also showed persistent estrus and failure of ovluation (Kent et al., 1968). In constant dark, 1L:23D or after blinding, male and female hamsters show a very severe atrophy of reproductive organs (Reiter, 1974). This greater sensitivity to light deprivation in comparison to the rat is perhaps a reflection of the more recent domestication of the golden hamster or perhaps due to the fact that the hamsters studied have been pigmented animals, whereas most of the rats studied have been albino strains.

Relatively little information is available on the effects of constant light or dark in other species. Dempsey et al. (1934) reported that guinea pigs continued to cycle in constant dark and reproduced normally, although the onset of heat no longer could be correlated with a specific time of day. Terry and Meites (1951) reported that sheep kept in constant light for 7 weeks in the summer continued to cycle. Waddill et al. (1968) exposed young pigs to constant light for one complete estrous cycle and found no difference in ovulation rate.

In the experiments of Mennin and Gorski (1974), constant light exposed ovariectomized rats did not respond to the positive feedback action of estrogen and showed a different response to progesterone priming than LD ovariectomized rats in terms of their serum LH release.

Constant light exposure may act by increasing serum levels of FSH (Fink, 1975) or by increasing estrogen levels (Negro-Vilar et al.,

1968). The effect of estrogen in increasing the pituitary response to GnRH has been demonstrated *in vivo* and *in vitro* (Libertun *et al.*, 1974; Arimura and Schally, 1971). The fact that estradiol benzoate accelerates vaginal opening more effectively in LL than LD rats (Piaseck and Struer, 1975) suggests that the LL female is more sensitive to estrogen than the LD female. However, constant light inhibited the ovulatory response to PMS in the immature rat (Steger *et al.*, 1975) suggesting that LL alters the ability of the hypothalamus to regulate endogenous GnRH injection. In a later study, Steger *et al.* (1976) have shown that LL enhanced the pituitary's LH response to synthetic GnRH in the immature rat. However, in the mature female rat (Smith and Davidson, 1974) an ovulation stimulus such as mating does not result in high LH release due to a sensitivity of the pituitary to LHRH.

Even though female rats show continual vaginal cornification and cease ovulation a few weeks after they are exposed to continuous light, this condition is not irreversible. Takahashi *et al.* (1977) found that when these animals were replaced in the darkness for ten hours, 80 percent of the animals ovulated approximately 46 hours later. Furthermore, according to Brown-Grant (1974) light induced persistent estrous rats show an extensive loss of photoreceptors but nevertheless resume regular ovarian cycles when returned to a light-dark regimen. Apparently retinal changes are not necessary to cause persistent estrus. Pigmented rats may become anovulatory without any retinal changes at the light microscopic level. Lambert (1975) has found that constant light intensities of 5 or 25 $\mu w/cm^2$ significantly prolonged estrus but at these levels of LL no retinal pathology was apparent with the light microscope in an albino strain of rat. Both white LL and red LL

prolong vaginal cornification at 100 $\mu w/cm^2$ but only white LL produces degeneration of retinal photoreceptors.

There are other changes in the hypothalamo-hypophysial-gonadal axis which occur in response to constant light exposure. Fink (1974) reported that the pituitary of LL exposed rats was just as sensitive as that of the LD exposed rat on the morning of proestrus in terms of LH response to a single injection of synthetic GnRH.

The generalization has frequently been made that many if not all effects of light upon reproductive processes occur through changes in the release of various pineal substances which in turn affect the homeostasis of the hypothalamo-hypophysial-gonadal axis. In the phenomenon of light induced persistent estrus in the rat, this is probably not the case (Reiter, personal communication). Light itself creates changes in the functioning of the neuroendocrine system that are independent of involvement of the pineal gland (Ifft, 1962). Light may induce a deficiency of catecholamines and an excess of serotonin in hypothalamic areas a shypothesized by Kledzik and Meites (1974) in light light persistent estrous female rats.

MATERIALS AND METHODS

I. Animals, Treatments and Blood Collection

Mature female rats used in these studies were obtained from Spartan Research Animals, Haslett, MI. Animals were housed in light 14 h on, 10 h off (14L:10D) and temperature (25°±1°C) controlled rooms and provided with Purina Rat Chow (Ralston Purina Co., St. Louis, MO) and tap water ad libitum. For the induction of persistent estrus, a group of female rats was kept under constant illumination with an intensity of 10-15 foot candles as measured at floow level of plastic cages (Weston, Model 915 light meter). After at least 5 weeks of constant light exposure, those animals showing 10 consecutive days of vaginal cornification were considered to be in persistent estrus and selected for these experiments. Animals kept under 14L:10D conditions which showed regular 4-day cycles were selected on the day of proestrus unless otherwise indicated.

Blood samples were taken by decapitation or cardiac puncture under light ether anesthesia. Blood samples were stored at 4°±1°C for 24 h to allow clot formation and serum was separated by centrifugation and stored at -20°C until assayed for hormone concentration.

Alpha-methyl-para-tyrosine methyl ester HCl (Regis Chemical Co., Morton Grove, IL) and pargyline hydrochloride (Sigma Chemical Co., St. Louis, MO) were dissolved in 0.85% NaCl just before use. Estradiol benzoate (EB) and progesterone (P) (Nutritional Biochemicals Corp.,

Cleveland, OH) were dissolved in corn oil. Synthetic gonadotropin releasing hormone (GnRH, Abbot, Chicago, IL) was dissolved in 0.85% NaCl.

II. Radioimmunoassay of Serum Hormones

Serum concentrations of luteinizing hormone (LH), follicle stimulating hormone (FSH) and prolactin (Prl) were determined using standard double antibody radioimmunoassay procedures. Serum prolactin was assayed using the method of Niswender et al. (1969), while serum LH and FSH were determined by the methods described in the NIAMDD kits. Hormone concentrations are expressed in terms of the standard reference preparations NIAMDD rat-prolactin-RP-1, NIAMDD rat -LH-RP-1, and NIAMDD rat-FSH-RP-1. All serum samples were assayed in duplicate. Samples from individual experiments were all tested in the same assay to avoid inter-assay variability.

Methods used for ether extraction of plasma estradiol, progesterone and testosterone, separation of bound from free steroids by charcoal dextran and scintillation counting were previously described by Campbell et al. (1977). Anti-progesterone-11-BSA, GDN #337, 1:2500; anti-estradiol-6-BSA, GDN #244, 1:20,000, and anti-testosterone-11-BSA, GDN #250, 1:40,000; were provided through the courtesy of Dr. G.D. Niswender of Colorado State University. Specificity of the estradiol anti-serum was determined by Campbell et al. (1977).

III. Assay of Hypothalamis Luteinizing Releasing Hormone (LHRH)

A. Isolation and Preparation of Brain Tissue

Rats were decapitated and their brains immediately removed from the cranium. A block of hypothalamic tissue constituting the region lying between the rostral borders of the optic chiasm and

mammillary bodies and medial from the optic tracts were dissected to a depth of about 2 mm. This was considered to be the medial basal hypothalamus (MBH). Another block of tissue anterior to the MBH along the same lateral borders and posterior to the anterior commisure was dissected and considered to be the preoptic-anterior hypothalamic area (AHA) (Figure 1). The average weight of each of these hypothalamic pieces was about 19 mg. Samples were stored on dry ice following their dissection and then weighed and homogenized in 1 ml of 0.1 N HCl and neutralized with 1 ml of 0.1 N NaOH. After centrifugation at 5000 g for 20 minutes in a Sorvall refrigerated centrifuge at 4°C, the supernatants were diluted with 0.1% gelatin in a phosphate-buffered saline (PBS) to an appropriate concentration. Both serum and hypothalamic extracts were stored at -20°C until assay.

B. Radioimmunoassay of LHRH

Hypothalamic LHRH was measured by the double antibody RIA described by Nett et al. (1973). Anti-GnRH serum, R-42 pool, was provided by Dr. G.D. Niswender (Colorado State University), and was used at a final dilution of 1:280,000. Synthetic LHRH (Lot α, CN-79, 479-11K, TM 10455 x 151-2, Parke-Davis Co., Detroit, MI) was used as a reference preparation.

IV. Assay of Dopamine (DA), Norepinephrine (NE), and Serotonin (5-HT) in Brain Tissue

A. Isolation and Preparation of Brain Tissue

Following decapitation, brains were removed and placed on ice. The medial basal hypothalamus (MBH) and preoptic anterior hypothalamic area (AHA) was dissected and frozen on dry ice. Tissue samples

were then weighed and homogenized in 20 µl of 0.4 N perchloric acid (containing 10 mg EGTA/100 ml). The MBH used in this study corresponded roughly to that described in Materials and Methods, section III. Cuts were made at the hypothalamic sulci, the mammillary bodies and caudal to the optic chiasm. The AHA included the area medial to the hypothalamic sulci, and caudal from the anterior commisure to the MBH (Figure 1).

B. Radioenzymatic Assay of Catecholamines

Dopamine (DA) and norepinephrine (NE) were assayed by the radioenzymatic method of Coyle and Henry (1973) using catechol-O-methyltransferase (COMT) isolated from the rat liver by the method of Nikodijevic et al. (1970). The assay was sensitive to 320 pg DA and 500 pg NE and linear to at least 4 ng for both catecholamines. The presence of tissue has been reported to reduce the activity of COMT (Coyle and Henry, 1973). Since samples of hypothalami were all diluted to the same concentration (1 mg/10 µl), values were not corrected for tissue inhibition. Results are expressed as ng DA or NE per gram wet weight.

Normetanephrine and methoxytyramine were separated utilizing the solvent extraction and thin layer chromatography method of Ben-Jonathan and Porter (1976). Amine content of samples were determined after separation by counting chromatographic spots containing the ^{3}H-labeled metabolites in glass scintillation vials containing 10 ml of Scintiverse (Fisher Scientific Products, Livonia, MI). Samples were counted in a New England Nuclear, Mark II, scintillation counter.

C. Radioenzymatic Assay of Serotonin

Hypothalamic 5-HT concentrations were assayed according to the method of Saavedra et al. (1973). Tissue samples were homogenized in

100 µl 0.1 N HCl (containing 10 mg EGTA/100 ml). Rat liver N-acetyl transferase was prepared by the method of Weissbach et al. (1961). Hydroxyindole-O-methyl transferase extracted from bovine pineals (Pel-Freez Biologicals, Inc., Rogers, ARK), was prepared by the method of Axelrod and Weissbach (1961). Results are expressed as ng 5-HT per gram wet weight.

Data were analyzed statistically by analysis of variance and the Student-Neuman Keuls multiple range test (Sokal and Rohlf, 1969). The level of significance chosen was $P<0.05$.

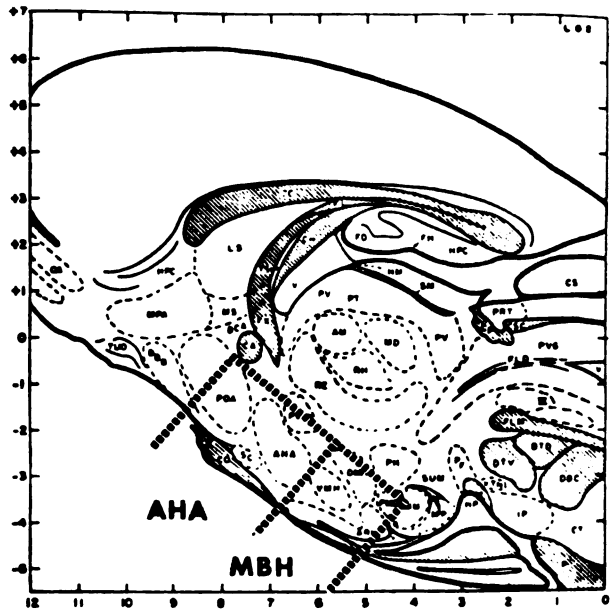

Figure 1. DeGroot Atlas Section showing Preoptic-Anterior Hypothalamic Area (AHA) and Medial Basal Hypothalamus (MBH).

Tissue sample weight was 18±2.4 mg (AHA) and 20±1.8 mg (MGH) (N=54).

EXPERIMENTAL

I. Effect of Constant Light on Morning and Afternoon Serum Levels of Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH) and Prolactin (Prl) in the Mature Female Rat

A. Objective

Critchlow (1963) postulated that exposure of rats to constant illumination blocks the cyclic mobilization of LH essential for ovulation, and that the retained follicles probably are responsible for the continuous secretion of estrogen which causes persistent vaginal cornification. A temporary state resembling these prolonged estrous conditions can be produced in the rat by neural blocking agents presented before the time of onset of the critical period for LH release (2-4 PM). If such injections are repeated for several days, the animals appear to continue secreting estrogen until atresia occurs (Everett, 1961).

Light induced persistent estrus appears to resemble the proestrous stage of the estrous cycle, except for the cornified vaginal cytology. With a strong stimulus such as mating, ovulation occurs in these animals. Therefore it was of interest to determine whether light induced persistent estrous rats showed daily patterns of LH, FSH, and Prl release similar to those seen in proestrous.

B. Methods and Materials

Mature Sprague-Dawley female rats weighing 225-275 grams were either housed under 14:10 light:dark conditions or under constant light

as described in the Methods section. Rats kept in the light:dark regimen (LD) were selected on the day of proestrous of a normal 4-day estrous cycle. Animals kept in constant light (LL) for at least 5 weeks and showing 10 consecutive days of vaginal cornification were considered to be in persistent estrus, and used for this experiment. Blood samples were collected by cardiac puncture under light ether anesthesia in the morning (0900-1000 h) and afternoon (1600-1700 h). Serum levels of LH, FSH, and Prl were determined by radioimmunoassay as described in the Materials and Methods section.

C. Results

Morning levels of serum LH were not significantly different in LL and LD rats (22±10 ng/ml vs. 20±8 ng/ml), respectively (Figure 2). A significant increase in LH was seen in the LD proestrous rat in the afternoon but not in the LL rat (508±115 ng/ml vs. 24±6 ng/ml (Figure 2) ($P<0.05$). These results indicate that the times measured, no preovulatory LH surge was seen in rats exposed to constant light.

Morning levels of serum FSH were slightly higher in LL rats than in LD rats (233±16 ng/ml vs. 196±8 ng/ml) (Figure 2) ($P<0.05$). However, both groups of animals showed an afternoon rise in FSH which was of equal magnitude (510±26 ng/ml vs. 526±54 ng/ml). These results demonstrate that unlike LH, constant light apparently does not suppress the ability of FSH serum levels to surge in the afternoon. Prl morning serum levels were elevated in LL rats as compared to LD rats (38±8 ng/ml vs. 7±4 ng/ml) (Figure 2) ($P<0.05$). In contrast to the afternoon surge of Prl seen in the LD rats (317±124 ng/ml), LL rats showed no significant difference between AM and PM serum levels. These results suggest that although LL may induce elevated basal levels of serum Prl

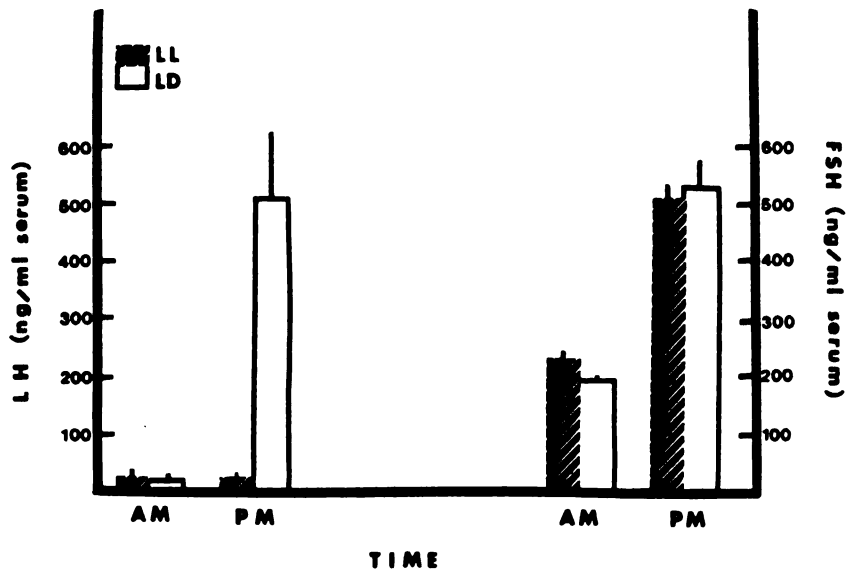

Figure 2. The Effect of Constant Light on Serum LH and FSH Levels in the Morning (AM) and Afternoon (PM).

Serum LH and FSH concentrations at 0900-1000 h (AM) and 1600-1700 h (PM) were compared in proestrous rats kept under 14L:10D conditions (LD) (open bars) and light induced persistent estrous rats (LL) (striped bars). Each bar represents the mean determination of 6-8 rats. Vertical lines indicate ±1 SEM.

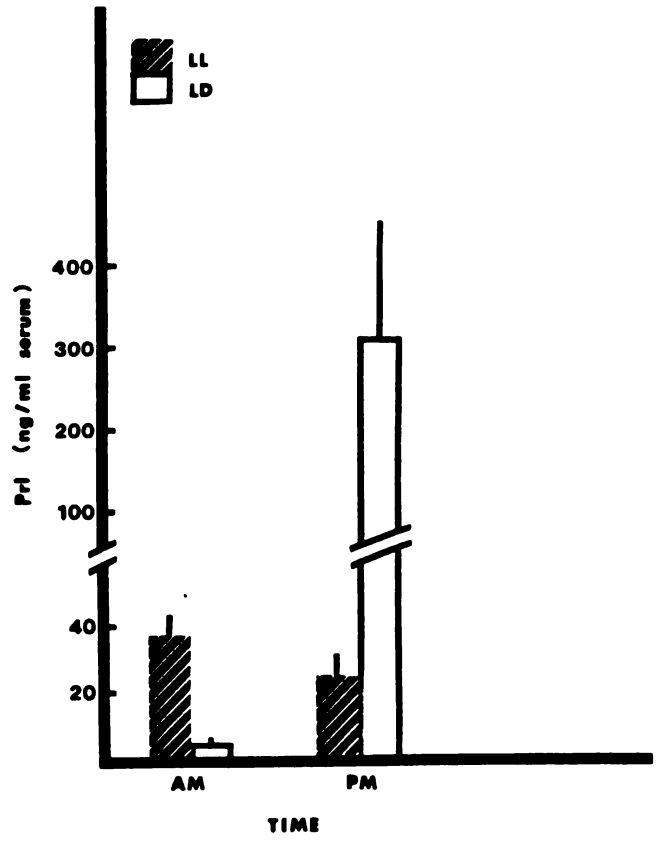

Figure 3. The Effect of Constant Light on Serum Prl Levels in the Morning (AM) and the Afternoon (PM).

Serum Prl concentrations at 0900-1000 h (AM) and 1600-1700 h (PM) were compared in proestrous rats kept under 14L:10D conditions (LD) (open bars) and light induced persistent estrous rats (LL) (striped bars). Each bar represents the mean determination of 6-8 rats. Vertical lines indicate ±1 SEM.

as compared to LD proestrous rats, the afternoon Prl surge was not seen at this sampling time. It is however, possible that a Prl surge may have occurred at some other time.

D. Discussion

From these data, it might be suggested that constant light exposure may have a differential effect on LH and FSH release. The characteristic proestrus afternoon surge of LH was not seen in the LL rat. This is what might be expected based on the previous observations of Daane and Parlow (1971) who reported a greatly reduced incidence of the normal proestrous LH surge even during the first vaginal cycle following exposure to constant light. Serum FSH levels have been reported to be elevated in LL rats (Fink, 1975), but the present data are believed to be the first observations of a proestrous type afternoon "surge" of FSH in LL rats. Since all of the LL rats showed a rise in FSH on the same day, it is likely that this may be a daily occurrence in light induced persistent estrus. Perhaps this daily pulse of FSH release participates in maintaining a constant secretion of estrogen. This effect could in turn disrupt gonadal steroid positive feedback of LH release if the control mechanism for that release was rate dependent.

The differential effect of LL on LH and FSH release is also interesting from the standpoint that it supports the theory for separate releasing factors for LH and FSH rather than a common one. This observation might also suggest that constant light may be selectively affecting hypothalamic neuronal centers associated with LH and Prl positive feedback. Possibly these separate gonadotropin control systems are desynchronized and unmasked by the LL treatment. In support of the

differential effect which LL has on gonadotropin release are the observations of Piacsek et al. (1966). They observed a release of FSH from pituitary transplants in hypophysectomized rats when placed in constant light. These investigators also found that serum levels of FSHRH (as measured by bioassay) were elevated in those rats.

Anterior hypothalamic lesions can accelerate the onset of the breeding season in ferrets (Donovan et al., 1956). It is possible that the lesions produce estrus by the destruction of a neural mechanism which, during sexual quiescence, would restrain gonadotropin secretion. Stimuli such as constant light, might have this same effect by depressing this neural mechanism, and a discharge of gonadotropin (primarily FSH) in an amount sufficient to stimulate the gonads would follow.

According to Legan and Karsch (1975), a neural signal for the LH surge is emitted each day throughout the estrous cycle of the rat, and prolonged maintenance of elevated circulating estradiol is essential for the expression of these signals. Perhaps a rate sensor exists for the rapid increase in circulating estradiol which can be remembered for at least several days afterwards.

Unlike the proestrous rat, no afternoon Prl surge was seen in the LL rat. It is not clear what role Prl plays in maintenance of the estrous cycle. Therefore, the significance of the absence of a Prl surge in the LL rat is difficult to determine. It cannot be concluded at this point whether the lack of a Prl surge is the result or one of the contributing factors in the light induced persistent estrous syndrome.

Although light induced persistent estrus is somewhat similar to proestrus, the afternoon surges of LH and Prl are not seen in this

condition. In contrast, a surge of FSH is seen in the afternoon of LL rats. Although the etiology of light induced persistent estrus is still unclear, the changes in gonadotropin release observed in this experiment probably reflect effects rather than causes of this condition.

II. Effect of Constant Light on the Sensitivity of the Pituitary to Exogenous GnRH In Vivo in the Mature Female Rat; Single and Multiple Injections of Synthetic GnRH

A. Objective

In normal cycling mature female rats the responsiveness of the anterior pituitary to exogenous or endogenous luteinizing hormone releasing hormone (LHRH) changes with the various stages of the estrous cycle (Aiyer et al., 1973, 1974; Cooper et al., 1973; Fink and Aiyer, 1974; Gordon and Reichlin, 1974). The timing and magnitude of the increase in pituitary responsiveness indicates that it plays a major role in the development of the LH surge. Conceivably, constant exposure of rats to light may also lead to an increase in pituitary responsiveness to LHRH which would facilitate the rapid reflex release of LH after mating (Brown-Grant et al., 1973) and the postulated increase in the secretion of FSH (Critchlow, 1963) in this type of animal. Fink (1975) has shown that the pituitary of the constant light exposed rat responded to synthetic doses of LHRH similarly to regular cycling rats on the morning of proestrus. The purpose of this study was to further investigate the possibility that increased pituitary responsiveness to LHRH occurs in constant light, especially the pituitary response to single and multiple priming doses of synthetic LHRH.

B. Materials and Methods

Experiments I and II

Female Sprague-Dawley rats weighing 225-275 g were exposed to constant light for about five weeks. Only rats showing at least 10 consecutive days of vaginal estrus were considered to be in persistent estrus and used in this study. Control animals of this study were female rats of the same age, strain and weight as above and selected on their ability to show a regular 4-day estrous cycle under 14L:10D lighting conditions. The control group was divided into proestrus, estrus, and diestrus (I and II). All experimental procedures were done between 0900 and 1000 h.

Synthetic gonadotropin releasing hormone (GnRH) (Abbot, Chicago, IL) was dissolved in 0.89 percent NaCl in concentrations of 450 ng or 50 ng GnRH/0.1 ml. After pretreatment blood samples were obtained by cardiac puncture under light ether anesthesia, the animals were given a single injection of 450 ng GnRH/100 g body weight (Experiment I) or six injections of 50 ng GnRH/100 g body weight every 30 minutes for a period of two and one-half hours (Experiment II). All injections were administered subcutaneously in a volume of 0.1 ml/100 g body weight.

In Experiment I, blood samples were taken at 45 minutes prior to a single injection of 450 ng GnRH/100 g body weight and at 15 and 75 minutes thereafter.

In Experiment II, serial blood samples were taken at -15, +30, 60, 120, and 180 minutes following the initial injection of 50 ng GnRH/100 g body weight. An additional proestrous group was added and was bled and injected according to the same schedule as above between 1500 and 1700 h.

Serum LH and FSH were measured by standard double-antibody radioimmunoassay procedures using NIAMDD kits. Results are expressed as ng/ml serum in terms of the reference standards, NIAMDD rat LH-RP-1 and NIAMDD rat FSH-RP-1.

C. Results

Effect of a Single GnRH Injection on Serum LH in LL and LD Rats

Pre-injection serum LH levels were not significantly different among any of the groups in this experiment. The LH response to a single injection of 450 ng GnRH/100 g body weight is shown in Figure 4. Serum LH rose in the LL rats from 21±5 ng/ml to 442±19 ng/ml at 75 minutes after injection of GnRH. Proestrous and estrous rats showed an LH response of similar but lesser magnitude at 75 minutes (19±4 ng/ml to 234±23 ng/ml and 18±2 ng/ml to 240±45 ng/ml, respectively). In contrast, the diestrous group responded to the GnRH injections with a serum LH increase from 19±1 ng/ml to 51±12 ng/ml at 75 minutes. There was no significant difference in serum LH concentration changes among control groups between 15 and 75 minutes after GnRH injection. Serum LH levels of LL rats, however, appeared to continue rising even at 75 minutes (442±19 ng/ml) and was significantly greater than serum LH levels of any of the control groups ($P<0.05$) at that time. This would indicate a greater pituitary LH response to GnRH in LL rats.

Effect of Multiple GnRH Injections on Serum LH in LL and LD Rats

The effects of a series of six injections of 50 ng GnRH/100 g body weight are shown in Figure 6. Serum LH rose from 19±3 ng/ml to 1226±206 ng/ml in LL rats and from 19±4 ng/ml to 1128±194 ng/ml in proestrous (PM) rats in 180 minutes following the initial GnRH injection.

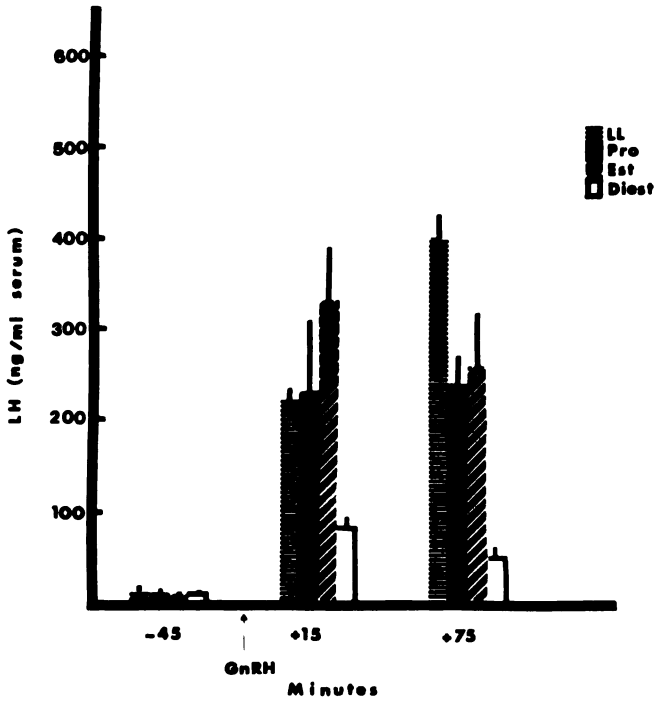

Figure 4. The Effect of Constant Light on Pituitary LH Response to a Single Synthetic GnRH Injection In Vivo.

Synthetic GnRH (450 ng/kg body weight) was injected s.c. into constant light (LL), and in proestrous (Pro), estrous (Est) and diestrous (Diest) rats (14L:10D). Each bar represents the mean determination of 6-8 rats. Vertical lines indicate ±1 SEM.

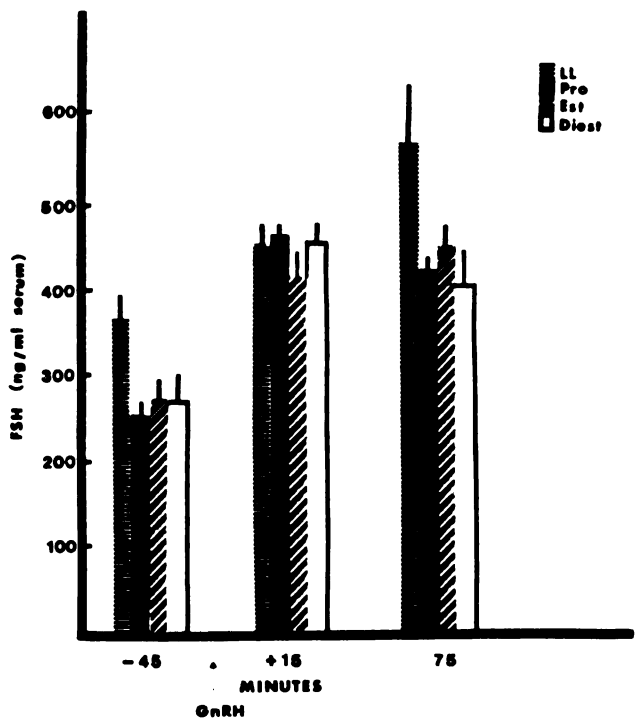

Figure 5. The Effect of Constant Light on Pituitary FSH Response to a Single Synthetic GnRH Injection In Vivo.

See Figure 4 for explanation.

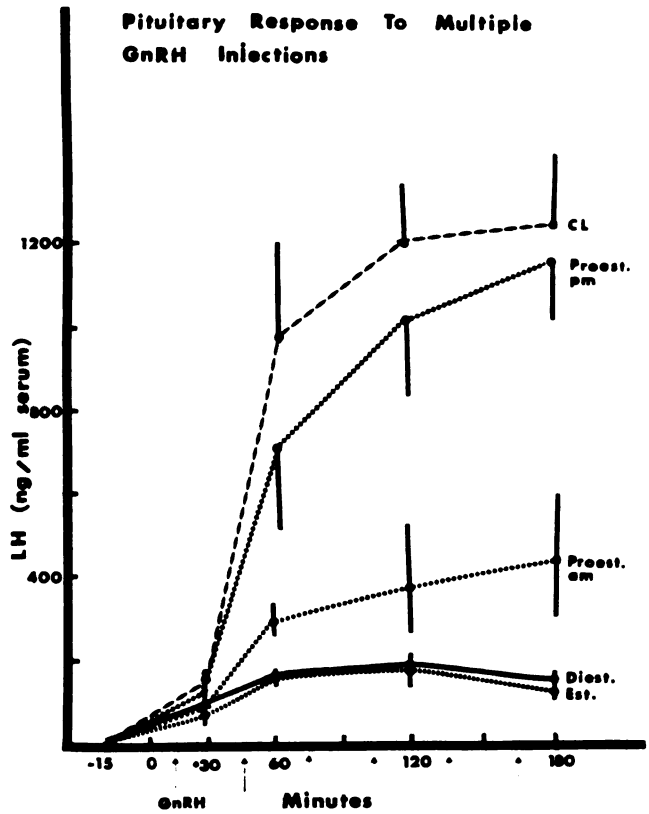

Figure 6. The Effect of Constant Light on Pituitary LH Response to Multiple Synthetic GnRH Injections In Vivo.

Synthetic GnRH (50 ng/kg body weight x 6) was injected s.c. into constant light (CL) rats and in proestrous (AM), (PM), estrous, and diestrous rats kept under 14L:10D conditions. Blood samples were collected at -15, +30, 60, 120, and 180 minutes following the initial injection of a series of six GnRH injections given every 30 minutes (arrows). Each point represents the mean determination of 6-8 rats. Vertical lines indicate ±1 SEM.

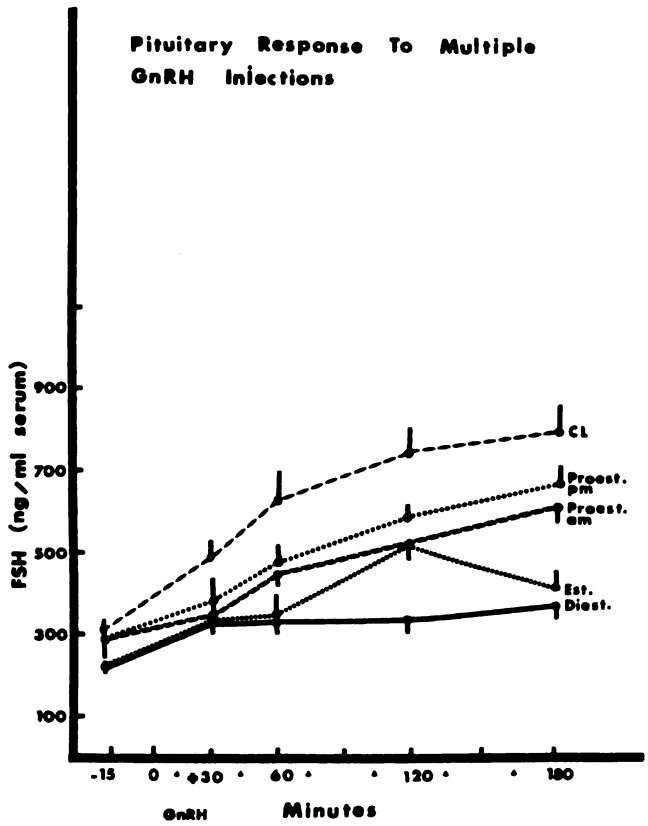

Figure 7. The Effect of Constant Light on Pituitary FSH Response to Multiple Synthetic GnRH Injections In Vivo.

See Figure 6 for explanation.

The LH responses of these two groups to the GnRH injections were not significantly different from one another. Pretreatment serum LH levels of proestrous (AM) (16±2 ng/ml), estrous (14±3 ng/ml), and diestrous (12±3 ng/ml) rats rose to 413±185 ng/ml, 130±7 ng/ml, and 152±6 ng/ml, respectively, in 180 minutes following GnRH injections. This would suggest that the priming effect of GnRH is greater in LL rats and proestrous (PM) rats than in proestrous (AM), estrous and diestrous rats in terms of pituitary LH response.

Effect of a Single GnRH Injection on Serum FSH in LL and LD Rats

Basal levels of serum FSH in the control groups of animals were not significantly different from one another. Basal levels of LL rat serum FSH was significantly greater than those of any of the control groups (Figure 5). There was no difference in the magnitude of increase in serum FSH in any of the groups 15 minutes following a single injection of GnRH. However, 75 minutes after the GnRH injection LL rats had significantly higher ($P<0.05$) serum FSH levels (561±91 ng/ml) than proestrous (428±22 ng/ml), estrous (456±31 ng/ml) or diestrous (412±43 ng/ml) rats.

Effect of Multiple GnRH Injections on Serum FSH in LL and LD Rats

Pituitary FSH response to six injections of 50 ng GnRH/100 g body weight in LL and LD rats is shown in Figure 7. Basal levels of serum FSH in LL rats was 310±20 ng/ml which was not different from proestrous (PM) (291±62 ng/ml), proestrous (AM) (288±88 ng/ml) but greater than estrous (225±18 ng/ml) or diestrous (227±7 ng/ml) rats ($P<0.05$).

At 180 minutes after the start of the GnRH injection series, serum FSH levels rose to 770±72 ng/ml in LL rats. This was not different from

proestrous (PM) rats (652±48 ng/ml) but significantly greater than proestrous (AM) (581±33 ng/ml), estrous (404±41 ng/ml) or diestrous (345±28 ng/ml) rats.

D. Discussion

The LL rats showed a greater pituitary LH and FSH response to a single injection of 450 ng GnRH/100 g body weight than any of the LD control groups. Furthermore, there appeared to be a difference in the time course of the response of pituitary FSH and LH to single GnRH injection between LL and LD control groups. While LD control rats showed their maximum serum levels of FSH and LH within 15 minutes following GnRH injection, the highest serum levels of FSH and LH did not appear until 75 minutes following the GnRH injection in LL rats. This difference may suggest a greater pituitary reserve of LH and FSH in the LL rat but not necessarily a greater rate of release of these gonadotropins. In contrast, Fink (1975) found that the increments in plasma LH and FSH of LL rats 60 minutes following a 50 ng dose of synthetic GnRH were similar to those of proestrous animals. The difference in results of these two experiments could be due to differences in the doses of GnRH used or to route of administration or blood sampling times.

In animals exposed to constant light, mating causes a prompt increase in serum LH which results in ovulation (Brown-Grant et al., 1973; Smith and Davidson, 1974). However, this particular sensitivity of the LL rat to ovulation-inducing stimuli is not the result of a supersensitive pituitary to LHRH (Smith and Davidson, 1974). In order to elicit an LH response to GnRH in LL rats which was comparable to

that response seen in the LD rat during the proestrous preovulatory period (PM), Fink (1975) found that it was necessary to inject 4-5 times the dose of GnRH. Therefore, Fink suggested that in contrast to the normal animal, in which only a small fraction of the readily releasable pool of LHRH is discharged during the spontaneous preovulatory surge, the mating reflex surge of LH in LL rats involves the release of most of the readily releasable pool of the hypothalamic hormone. According to Aiyer et al. (1974), the magnitude of the LH response is related in a positive manner to the secretory activity of the pituitary before administration of GnRH. This relationship may be due to a priming effect of GnRH on the pituitary.

The priming effect of GnRH on pituitary LH and FSH release was also compared between LL and LD control rats in this study. LL rat pituitary LH and FSH release was not significantly different from that of proestrous (PM) rats in response to multiple priming doses of GnRH but significantly greater than proestrous (AM), estrous, or diestrous rats. Therefore, the magnitude of the LH and FSH response to priming doses of GnRH in LL rats is comparable if not greater than regular cycling animals in proestrus.

Fink (1975) suggested that the increased responsiveness of the anterior pituitary in rats exposed to constant light may be related to the levels of circulating estradiol which are higher than at estrus but lower than at proestrus in rats with 4-day cycles (Naftolin et al., 1972).

The results of this study would argue against the possibility that in the constant light exposed rat, the failure of spontaneous

preovulatory LH release and corresponding ovulation is due to a decreased pituitary sensitivity to LHRH.

III. Effect of Constant Light on the Content of LHRH in the Anterior Hypothalamic Area (AHA) and Medial Basal Hypothalamus (MBH) of the Mature Female Rat on Morning and Afternoon Time Periods

A. Objective

Exposure of the pituitary to increased amounts of hypothalamic LH releasing hormone (LHRH) is believed to be a direct stimulus for the preovulatory LH release in the normal cycling female rat. Unfortunately, LHRH secretion cannot be measured without severely stressful hypophysial portal vessel cannulation procedures. An alternative approach to investigating the role of LHRH is to measure its content in the hypothalamus. A depletion of LHRH content in the hypothalamus occurring prior to an event such as the preovulatory LH surge may suggest release of hypothalamic LHRH into the portal circulation to stimulate the LH surge.

Since light induced persistent estrous rats fail to show any LH surge to stimulate ovulation, it was of interest to test the hypothesis that these animals had either a deficiency of LHRH in the hypothalamus, or that the hypothalamic LHRH was not being released into the portal vessels to stimulate pituitary LH release.

B. Methods and Materials

Mature female Sprague-Dawley rats weighing 225-275 g were kept under constant light for 5 weeks. Those animals showing at least 10 consecutive days of vaginal estrus were considered to be in persistent estrus and used in this experiment. A control group of rats was kept under 14L:10D light conditions and selected on the day of proestrus of a regular 4-day cycle.

Rats were killed by decapitation in the morning (0900-1000 h) or afternoon (1600-1700 h). Brains were removed from the cranium immediately after killing and dissected according to the procedure outlined in the general Materials and Methods section. Hypothalamic LHRH was measured by the double antibody RIA method of Nett et al. (1973).

C. Results

Anterior hypothalamic area (AHA) concentrations of LHRH in LL rats was higher than LD proestrous rats in the AM (817±96 pg/hypothalamus vs. 494±24 pg/hypothalamus) and in the PM (551±68 pg/hypothalamus vs. 244±20 pg/hypothalamus) (Figure 8). Medial basal hypothalamus (MBH) concentrations of LHRH were also higher in the LL rat than LD control at both AM (6.4±0.4 ng/hypothalamus vs. 4.8±0.2 ng/hypothalamus) and PM (6.5±0.7 ng/hypothalamus vs. 3.8±0.2 ng/hypothalamus) time periods (Figure 8).

These results suggest that no deficiency of LHRH exists in the LL rats as compared to the LD proestrous rat. In fact an apparent surplus of LHRH is seen in both areas of the hypothalamus examined in the LL rat.

AHA LHRH concentrations were significantly smaller in the PM than AM (244±20 pg/hypothalamus vs. 494±24 pg/hypothalamus) ($P<0.05$) in the LD proestrous rats. This same pattern was seen in the AHA of the LL rat. Afternoon (PM) concentrations of LHRH were smaller than AM concentrations (551±68 pg/hypothalamus vs. 817±96 pg/hypothalamus) ($P<0.05$) in LL rats (Figure 8). These results suggest that AHA LHRH concentrations change with time in both types of animals. This change in content with time may imply movement of LHRH from one area of the

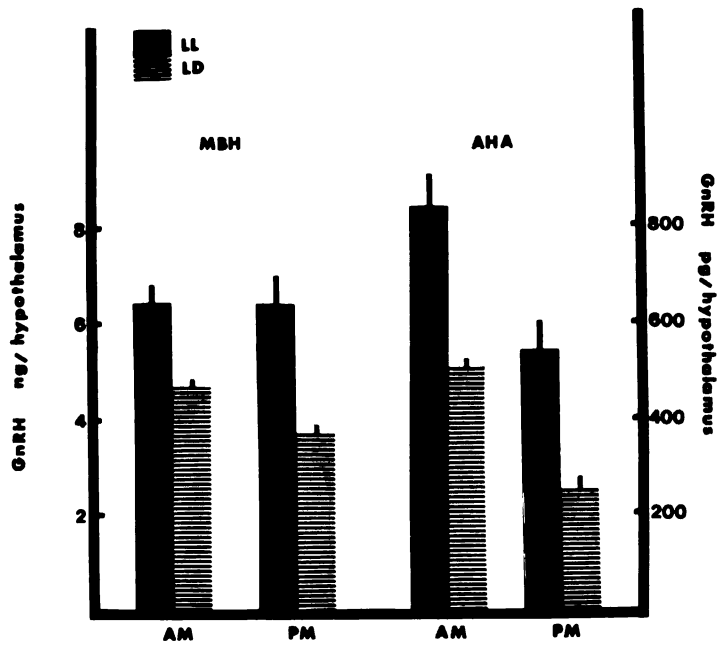

Figure 8. Effect of Constant Light on LHRH (GnRH) Content of the Preoptic-Anterior Hypothalamic Area (AHA) and the Medial Basal Hypothalamus (MBH) in the Morning (AM) and Afternoon (PM).

GnRH content of both hypothalamic areas was measured in constant light (LL) rats and proestrous rats kept under 14L:10D conditions (LD) in the morning (AM) and afternoon (PM). Each bar represents the mean determination of 6-8 rats. Vertical lines indicate ±1 SEM.

hypothalamus to another during the time course of hypothalamic events associate with the estrous cycle.

Medial basal hypothalamic LHRH content was smaller in the PM than AM (3.8±0.2 ng/hypothalamus vs. 4.8±0.2 ng/hypothalamus ($P<0.05$) in the LD controls (Figure 8). This pattern was not seen in the MBH of LL rats comparing PM to AM levels of hypothalamic LHRH (6.5±0.7 ng/hypothalamus vs. 6.4±0.4 ng/hypothalamus). These results suggest that while LHRH is removed from the MBH by the afternoon time period (i.e., presumably partially released to the portal vessels) in the LD proestrous rat, no such release of LHRH was seen in the LL rat.

D. Discussion

The results of this experiment suggest that LHRH is present in the hypothalamus of LL rats in quantities which exceed those of the LD proestrous rat. However, the data also infer that unlike the LD rat, the LL rat showed no apparent "release" of LHRH from the MBH. It should be noted that since LHRH was measured at only one time in the PM in this experiment, a different time course of LHRH release in the LL rat cannot be ruled out. It is unlikely that this occurred since ovulation did not follow.

As an alternative to increased LHRH release, the preovulatory surge of LH may result from an increase in pituitary sensitivity to LHRH. This hypothesis was tested and discussed in Experimental section II of this thesis. To summarize, it was found that the pituitary of the LL rat was equally or more sensitive than the pituitary of the LD proestrous rat to priming doses of GnRH *in vivo*. Therefore, endogenous hypothalamic LHRH content of LL rats was examined in this experiment and compared to that of the proestrous rat.

One feature of the rat exposed to constant light is that is is quite sensitive to ovulation-inducing stimuli. Smith and Davidson (1973) found that 100 percent ovulation occurred following stressful stimuli in these rats. They observed a decline in LHRH activity of the basal hypothalamus of LL rats which reached a low point 20 minutes after the onset of mating, followed by a return toward the initial level, suggesting rapid release of stored LHRH followed by resynthesis. These observations would support the hypothesis that no deficiency of hypothalamic LHRH exists in the LL rat but that its "spontaneous" (but not "reflex") release may be impaired.

Another characteristic of constant light exposure is the advancement of the average age of vaginal opening and the first appearance of large follicles in immature rats (Steger et al., 1975). These effects may be associated with alteration in follicle stimulating hormone releasing hromone (FSHRH) levels in the immature LL rat as described by Negro-Vilar et al. (1968). These workers found elevated levels of FSHRH activity in plasma of hypophysectomized rats exposed to constant light which were greater than that of LD hypophysectomized rats. Increased FSHRH activity could presumably release FSH which could stimulate follicular growth and estrogen production by ovaries to result in advancement of vaginal openings, but ovulation does not occur. This latter effect may be due to an inability of the LL rat to spontaneously release LHRH. Since FSH serum levels are elevated in the adult LL rat while no change in LH serum levels is seen, this differential release of gonadotropin releasing factors may be occurring in the adult LL rat as well.

This experiment has presented at least some indirect evidence which suggests that LHRH is present but not released from the hypothalamus in light induced persistent estrus.

IV. Effect of Constant Light on Hypothalamic Sensitivity to Positive and Negative Feedback by Gonadal Steroids: LH and FSH Response to Estradiol Benzoate (EB) and Progesterone (P) Priming

A. Objective

There is abundant evidence that ovarian steroids can facilitate or inhibit luteinizing hormone (LH) secretion and ovulation depending on the dose administered and the physiological conditions at the time of the hormone treatment (Everett et al., 1969; Brown-Grant, 1974). The preovulatory release of gonadotropin in cycling rats is also influenced by a neural mechanism which is cued by the light-dark cycle (Everett et al., 1949; Critchlow, 1963). Phase shifts in the onset of the light period cause a corresponding temporal shift in the activation of the control mechanism and consequently of pituitary LH discharge. In ovariectomized rats, the timing of estrogen-induced LH release can also be altered by advancing the onset of the light period (Columbo et al., 1974). Thus, the environmental light cycle acts as an external signal for synchronizing the pituitary discharge of LH in both cycling and estrogen-primed ovariectomized animals.

The preovulatory rise in estrogen titers is known to be an important factor in spontaneous LH release and ovulation (Brown-Grant, 1974; Schwartz, 1969). A number of factors could affect estrogen secretion rate and LH-release threshold, although no specific data are available to distinguish between these two sites of action. The effects of photoperiod on cycle length might be due to changes in the LH-release

threshold (Hoffmann, 1975). In the extreme conditions of constant light, the LH release threshold may be raised and estrogen secretion rate slowed.

It was the objective of these experiments to compare the thresholds for positive and negative feedback of estrogen and progesterone on LH and FSH release between ovariectomized rats exposed to constant light and ovariectomized rats exposed to a 14L:10D light cycle.

B. Methods and Materials

Female Sprague-Dawley rats weighing 225-275 g were housed under constant illumination for a period of about five weeks. A second group of Sprague-Dawley females was kept in a 14L:10D lighting regimen. Those animals showing persistent estrus in the constant illumination or those control animals showing regular cycles in the 14L:10D regimen were then ovariectomized. Two to three weeks later, blood samples were taken by cardiac puncture under light ether anesthesia. Serum was separated by centrifugation and stored at -20°C. Serum LH and FSH was measured by RIA as outlined in the general Materials and Methods section.

Estrogen and Progesterone Priming

Experiment I - Twenty µg of estradiol benzoate (EB) in 0.2 ml corn oil was administered S.C. to each group of rats. Seventy-two hours later a second injection of 20 µg EB was given. Thirty hours after the second injection, a blood sample was taken by cardiac puncture under light ether anesthesia.

Experiment II - Twenty µg of EB in 0.2 ml corn oil was administered S.C. to each group of rats. This was followed seventy-two hours later by 5 mg progesterone (P) administered S.C. in 0.2 ml corn

oil. Six hours after the P injection, a blood sample was taken by cardiac puncture under light ether anesthesia.

C. Results

Experiment I

The effects of castration and EB positive and negative feedback on serum levels of LH and FSH in LL and LD rats is seen in Figure 9. Post-castration levels of LH were not different between LL and LD rats (560±161 ng/ml and 419±92 ng/ml, respectively). Seventy-two hours following 20 μg EB, serum LH was depressed to a lower level in LD rats (86±17 ng/ml) than LL rats (352±170 ng/ml ($P<0.05$). In response to a second injection of EB, LD rats showed a small but significant increase in serum LH (273±167 ng/ml) while serum LH levels continued to fall in LL rats (104±39 ng/ml) at this time.

No differences were seen in serum FSH levels between LL and LD rats in response to castration nor after negative or positive EB stimuli.

Experiment II

The effects of castration, EB negative and P positive feedback on serum levels of LH and FSH in LL and LD rats is seen in Figures 10 and 11. Serum LH levels of LL and LD rats did not differ prior to nor at 1 or 12 days postcastration (Figure 10). Although LL rats showed a greater EB negative feedback on serum LH than LD rats, comparing post-castrate and post-EB serum LH levels, both groups of rats responded similarly to progesterone treatment with an elevation of serum LH (Figure 10). This would indicate that progesterone can overcome any difficulty in positive feedback of serum LH release under constant light conditions.

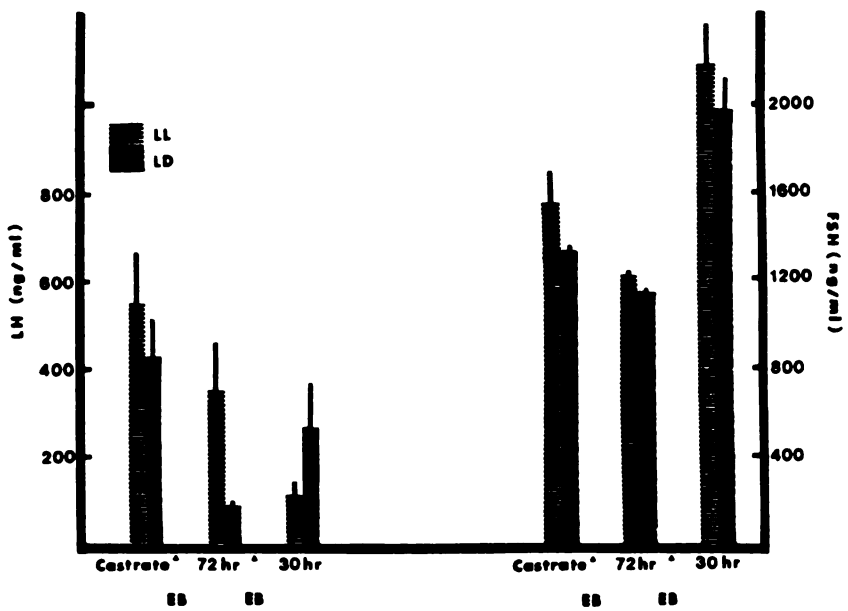

Figure 9. The Effect of Constant Light on Estradiol Benzoate (EB) Induced Negative and Positive Feedback Effects on Hypothalamic Control of Pituitary LH and FSH Release.

Constant light rats (LL) and rats kept under 14L:10D conditions (LD) were ovariectomized and injected with EB (20 μg) s.c. at 72 hr intervals after which gonadotropin response to negative (72 hr) and positive (30 hr) feedback of EB was measured. Each bar represents the mean determination of 6-8 rats. Vertical lines indicate ±1 SEM.

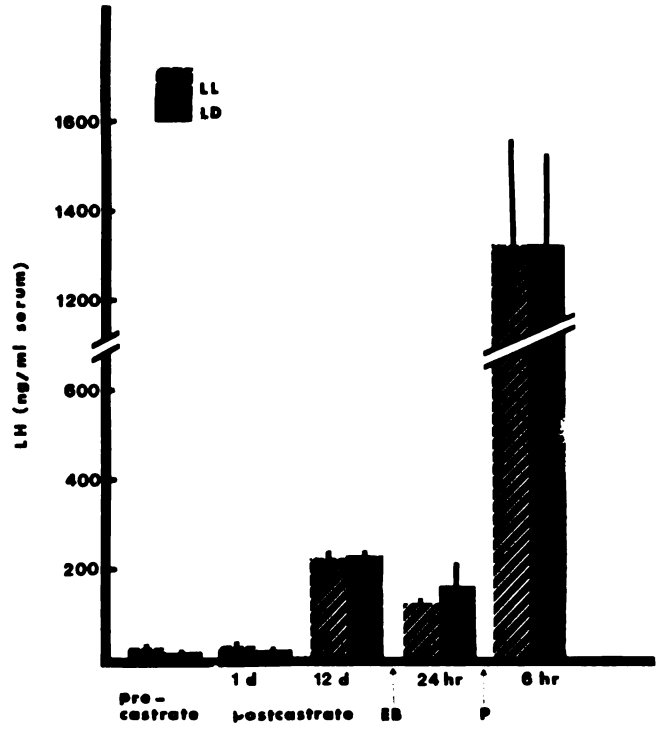

Figure 10. Effect of Constant Light in Progesterone (P) Induced Positive Feedback on Hypothalamic Control of Pituitary LH Release.

Constant light (LL) rats and rats kept under 14L:10D conditions (LD) were ovariectomized and injected with estradiol benzoate (EB) (20 µg) followed 24 hr later by progesterone (P) (5 mg) s.c. Gonadotropin response to P positive feedback was measured 6 hr following P injection. Each bar represents the mean determination of 6-8 rats. Vertical lines indicate ±1 SEM.

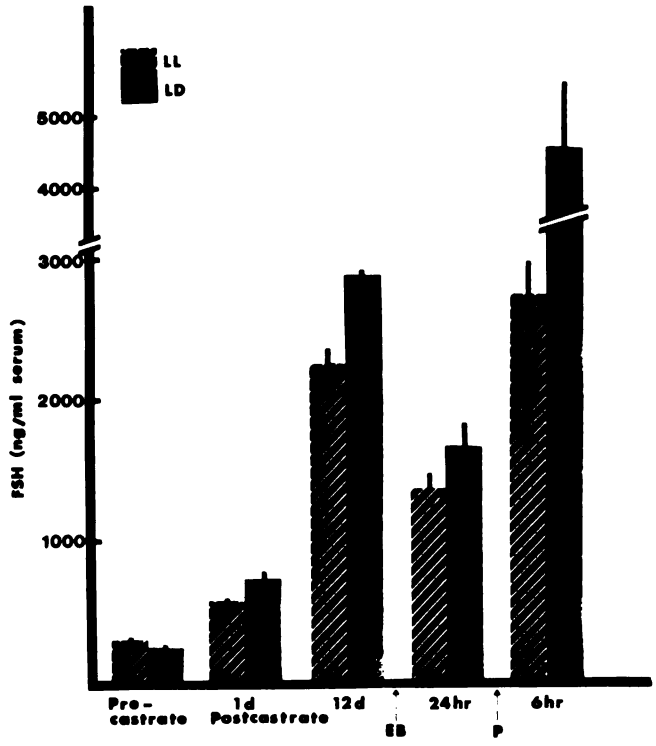

Figure 11. Effect of Constant Light in Progesterone (P) Induced Positive Feedback on Hypothalamic Control of Pituitary FSH Release.

See Figure 10 for explanation.

Basal serum FSH levels were not different between LL and LD rats (Figure 11). However, 1 day following castration LD rats had higher serum FSH levels than LL rats (755±44 ng/ml vs. 568±31 ng/ml). Similarly at 12 days post-castration serum FSH levels in LD rats were still higher than those of LL rats (2912±39 ng/ml vs. 2256±93 ng/ml). Six hours following an injection of P (5 mg) serum FSH levels of LD rats rose to a higher level than in LL rats (4637±872 ng/ml vs. 2727±226 ng/ml). This would suggest that, at six hours after progesterone injection serum FSH levels of LL rats were not as sensitive to positive feedback by progesterone.

D. Discussion

Since spontaneous ovulation does not occur in light-induced persistent estrus, it was of interest to test the response of gonadotropin release in the LL rat to negative and positive feedback effects of gonadal steroids.

The ability of EB to exert a negative feedback effect on the post-castration serum LH rise was demonstrated in LD rats but not LL rats in exerpiment 1 (Figure 9) 72 hr after injection. In contrast, the results of experiment 2 show the opposite LH response but at 24 hr after EB injection (Figure 10). The discrepancy in LH response between these two experiments is probably due largely to a difference in sampling times following EB injection. This also suggests that in the LL rat, there may be a different time course for steroid negative feedback on LH release. Alternatively, if LH responses between LL and LD rats were compared only at 72 hr after EB, the data may suggest that the sensitivity of the LH control system to the negative feedback effects of gonadal steroids was decreased in constant light. This latter

alternative is in agreement with the findings of Hoffmann and Cullin (1977) demonstrating a decreased negative feedback sensitivity of the LH control system to estrogen negative feedback with increased photoperiod length. Unlike LH, the FSH response to negative feedback by EB was not different between LL and LD rats.

Estrogen not only has negative feedback effects on LH release, but also can induce the release of a surge of LH comparable to the ovulatory surge in rhesus monkeys (Karsch et al., 1973; Legan et al., 1975). Differences between the LH response to the positive feedback effects of estrogen were also seen between LL and LD rats. In contrast to LD rats, LL rats failed to show a serum LH increase following a second EB injection in experiment 1 (Figure 9). Since serum was collected at only one time period following the second EB injection, it cannot be concluded whether or not the sensitivity of LH release control to positive feedback by EB is decreased in the LL rat or whether the time course of LH response was different in the LL rats. However, in a related study, Mennin and Gorski (1975) found no elevation of serum LH following a second EB injection in ovariectomized light-induced persistent estrous rats at time periods ranging from six to fifty-six hours following the EB injection.

Mann et al. (1976) have shown that in the ovariectomized rats in a 14L:10D light cycle: a) EB produces an LH rise in the absence of any circulating progesterone (P), b) and P potentiates and advances the serum LH surge in response to EB. These findings are in agreement with our observations in experiment 2. In addition, Mann et al. (1976) have shown that rats maintained in constant light were less sensitive to the positive feedback actions of EB and P than LD rats. In

experiment 1, the LL rats failure to respond to the positive feedback of EB is in agreement with the results of Mann et al. (1976). However, in experiment 2, in contrast to the findings of Mann et al. (1976), a decreased sensitivity to the positive feedback effect of P in LH was not seen. A possible explanation for this discrepancy may be the difference in doses of P used in the experiments of Mann et al. (1976) and experiment 2. Since almost twice as much P was used in our experiment 2 as compared to theirs, a higher serum level of P may have been able to overcome a decreased sensitivity of the LL rat to the positive feedback effects of P on LH.

It is interesting to note that in the studies of Mann et al. (1976), the degree to which sensitivity of positive feedback of EB and P on LH was reduced was dependent on whether or not the animals had adrenal glands. Rats that were both ovariectomized and adrenalectomized showed an enhanced sensitivity to EB and P in both LL and LD rats. No explanation was given for this finding.

The results of these experiments would suggest that LL induces an alteration in the sensitivity of the control system for LH release to negative and positive feedback by gonadal steroids. This may contribute to a lack of spontaneous ovulation in the light induced persistent estrous rat.

V. Effect of Constant Light on Morning and Afternoon Plasma Levels of Estradiol, Progesterone and Testosterone in the Mature Female Rat

A. Objective

The sequence of changes in plasma gonadal steroids which occurs in the normal cycling female rat are believed to contribute to positive

and negative feedback effects on gonadotropin secretion (Everett, 1964; Kalra and Kalra, 1974). They participate in the regulation of hormonal events occurring during the estrous cycle. The persistent estrus resulting from constant light exposure is associated with an absence of the preovulatory surge.

It was of interest to measure plasma estradiol, progesterone and testosterone levels in light induced persistent estrous rats. It has been suggested that the receptivity of the constant light exposed rat to a mating stimulus and its effectiveness in inducing ovulation may be due to suprabasal but constant secretion of estrogen maintaining the estrous vaginal cytology and priming the pituitary to respond to an LHRH stimulus (Smith and Davidson, 1974).

B. Methods and Materials

Mature Sprague-Dawley female rats were maintained under 14L:10D or constant light (LL) conditions. LD rats showing a regular 4-day estrous cycle were selected for this experiment on the day of proestrous of the estrous cycle. LL rats which showed ten consecutive days of vaginal estrus were also selected for this experiment. Blood samples were collected by cardiac puncture under light ether anesthesia in the morning (0900-1000 h) and afternoon (1600-1700 h). Plasma steroids were measured according to the procedure described in Appendix D.

C. Results

Plasma estradiol levels of LL rats were not significantly different from LD proestrous rats in the AM (34±4 pg/ml vs. 28±8 pg/ml). However, while estradiol levels in proestrous rats rose to 44±7 pg/ml in the afternoon, LL rats showed no significant change in estradiol levels at this time (Figure 12).

Plasma progesterone levels were significantly lower in LL rats than proestrous rats at morning (12±4 ng/ml vs. 26±8 ng/ml) ($P<0.05$) and afternoon (16±4 ng/ml vs. 34±9 ng/ml) ($P<0.05$) time periods (Figure 12).

Plasma testosterone levels were higher in LL rats than LD proestrous rats in the morning (203±38 pg/ml vs. 84±12 pg/ml) ($P<0.05$), but this relationship was reversed in the afternoon when LL plasma testosterone levels fell to 130±9 pg/ml while LD proestrous levels rose to 324±41 pg/ml (Figure 13).

D. Discussion

Data on plasma estradiol levels in this experiment show that at those time periods when samples were collected, LL rats had plasma estradiol levels which were neither much higher nor lower than the range of estradiol concentrations found in the normal cycling rats. This observations is supported by the findings of Takahashi et al. (1977) where ovarian vein plasma levels of estradiol were of an intermediate value between those of diestrous night and proestrous morning in normal cycling rats.

The interpretation given by various investigators concerning the basis for persistent estrus that develops after exposure to constant light centers around the concept of a persistent mild elevation in gonadotropin secretion (FSH) (Daane and Parlow, 1971; Mann et al., 1973, 1974) that drives a slightly elevated estrogen secretion (Naftolin et al., 1972) hovering at a level intermediate between the peaks and troughs of the normal cycle. There is also a reduction in estrogen binding capacity at various estrogen target tissues in long-term light-sterilized rats (Allei-Donhoffer et al., 1974) which may be related to a failure of the normal feedback regulation of ovulation.

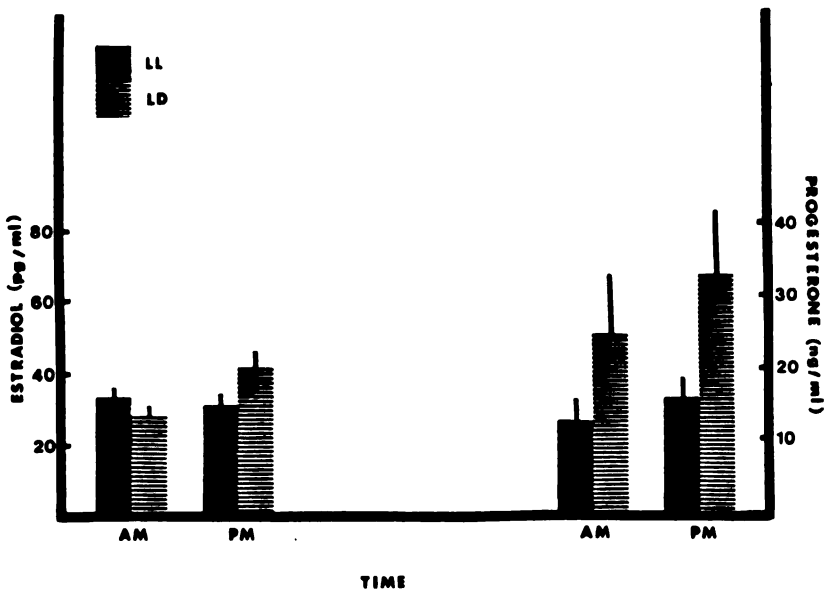

Figure 12. Effect of Constant Light on Plasma Estradiol and Progesterone Levels in the Morning (AM) and Afternoon (PM).

Morning and afternoon levels of plasma estradiol and progesterone were measured in constant light (LL) rats and in proestrous rats kept under 14L:10D conditions (LD). Each bar represents the mean determination of 6-8 rats. Vertical lines indicate ±1 SEM.

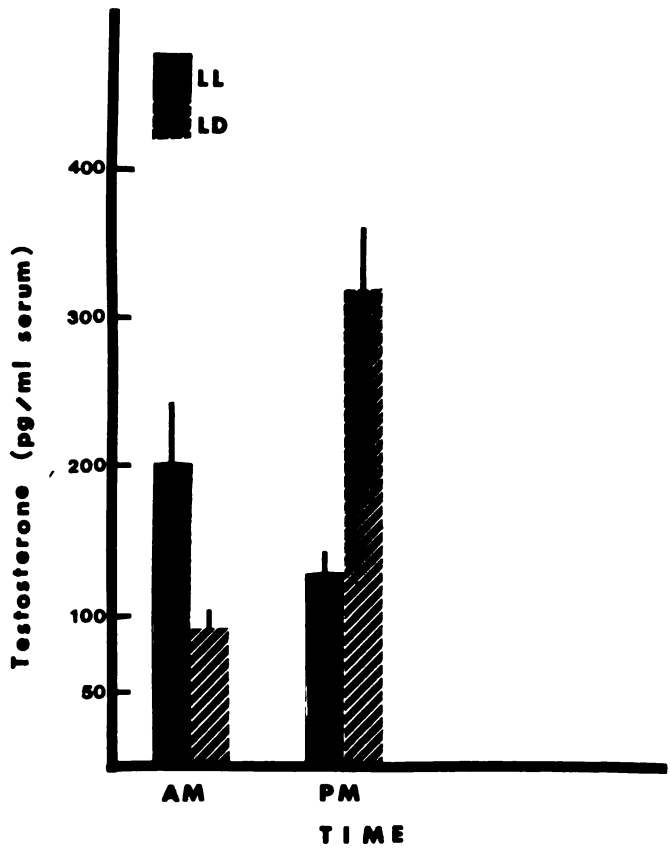

Figure 13. Effect of Constant Light on Plasma Testosterone in the Morning (AM) and Afternoon (PM).

See Figure 12 for explanation.

Not only are minimum levels of plasma estradiol necessary for positive feedback effects on preovulatory LH release, but dynamic changes in estradiol may play a significant role as well. Kalra (1975) found that following the initial "priming" of central sites with low levels of circulating estradiol during diestrus II, rapid elevations in the ovarian estrogen secretion between 2300 h of diestrus II and 0300 h of proestrus facilitated the neural "trigger" of pituitary LH release during the critical period on proestrus. In addition, Turgeon and Barraclough (1977) have studied the possible consequences of the abrupt decrease in estradiol secretion that occurs following the initiation of the gonadotropin surge. They found that the pituitary responsiveness to exogenous LHRH was doubled after decreased circulating estradiol secondary to ovarian removal. The abrupt decrease in estradiol coupled with the degree of LHRH presensitization of the pituitary may determine the magnitude of the LH surge. The above types of dynamic changes in estradiol were not examined in this experiment and thus cannot be ruled out as possible contributory factors in light induced persistent estrus.

The results of this experiment regarding plasma progesterone levels also suggest that LL rats may have an insufficient level of progesterone released as compared to the LD proestrous rat. This is in agreement with the findings of Takahashi et al. (1977) who also found very low levels of ovarian vein plasma progesterone but elevated levels of 17-OH-progesterone in LL rats. These comparative steroid levels (continuous but low levels of estrogen and low levels of progesterone) are in accord with the morphological state of the ovary in LL rats since no new corpora lutea are observed and follicles are at the preovulatory state.

Ramaley and Bartosik (1975) found that the rhythmic release of progesterone and corticosterone which are first seen in immature female rats at 29 days of age was eliminated by exposing the rats to constant light from weaning age. Since progesterone levels in their control rats were greatly reduced by adrenalectomy, they believed the source of the progesterone rhythm was probably the adrenal.

Nequin and Schwartz (1971), Feder et al. (1971) and Lawton (1972) suggested that the adrenal secretion of P before the critical period is involved in facilitating the release of LH on the evening of proestrus. Progesterone injection into light induced persistent estrous rats re-initiates cycling (Everett, 1948). It might be implied that if plasma progesterone levels reached some threshold level in the LL rat, preovulatory LH surges should have occurred.

The pattern of changes in plasma testosterone levels in LL rats appeared to be opposite to that in LD proestrous rats. It might be implied that plasma testosterone changes in the LL rat are out of phase with the LD proestrous rat. These are only speculations since only two time periods were sampled. The role of testosterone in the normal estrous cycle has not been clearly defined. Mori et al. (1977) found that injection of antibodies produced against testosterone into HCG primed rats decreased the number of ova per ovulation to normal. Androgen has been implicated in the control of follicular maturation (Louvet et al., 1975). Production of androgen in the follicular fluid may participate in the regulation of oogenesis in the rat.

Changes in plasma estradiol, progesterone and testosterone secretion seen in LL rats could be a response to constant light or a

contributing factor in light induced persistent estrus. Due to the high degree of integration of the various components of the estrous cycle, it may be difficult to separate cause from effect unless a thorough time course of these and other component changes are investigated.

VI. Effect of Constant Light on Hypothalamic Monoamine Metabolism on Morning and Afternoon Time Periods: Determination of Turnover Index of Norepinephrine (NE), Dopamine (DA) and Serotonin (5-HT) in the AHA and MBH

A. Objective

After exposure to constant illumination (LL), female rats exhibit an alteration in gonadotropin release, and enter a persistent estrous state (Browman, 1937; Hemmingsen et al., 1937; Everett, 1942). The control of gonadotropin release and their corresponding regulation of ovarian function is in turn influenced by hypothalamic monoamines (Coppola, 1968; Kamberi et al., 1970; Schneider and McCann, 1970). Kledzik and Meites (1974) have shown that light induced persistent estrous rats could be induced to resume regular estrous cycles by injection of L-DOPA and PCA (a drug combination which increases catecholamine metabolism and decreases serotonin metabolism). These workers have hypothesized that constant illumination may induce a persistent estrous condition by causing a depletion of catecholamine and an excess of serotonin activity.

In order to investigate this hypothesis, norepinephrine (NE), dopamine (DA) and serotonin (5-HT) turnover index (TI) were measured in the preoptic-anterior hypothalamic area (AHA) and the medial basal hypothalamus (MBH) of light induced persistent estrous rats on morning and afternoon time periods.

B. Methods and Materials

Mature Sprague-Dawley rats were housed under constant light conditions for 5-7 weeks. Only those animals exhibiting 10 consecutive days of vaginal estrus were selected for this experiment. A control group of rats was housed under 14L:10D lighting conditions. Control animals used in this study were rats showing a regular 4-day cycle and selected on the day of proestrus.

Measurement of Hypothalamic Monoamines

Catecholamines: Rats were injected intraperitoneally with 200 mg α-methyl-para-tyrosine (αMPT)/kg body weight or its vehicle, 0.85% NaCl. One hour later, the rats were killed by decapitation, brains were removed and placed on ice. The medial basal hypothalamus (MBH) and preoptic-anterior hypothalamic area (AHA) were dissected and frozen on dry ice. Tissue samples were then homogenized in 20 µl of 0.4 N perchloric acid (containing 10 mg EGTA/100 ml).

Dopamine (DA) and norepinephrine (NE) were assayed by the radio-enzymatic method of Coyle and Henry (1973) as described in the general Materials and Methods section. The turnover index of catecholamines was calculated as the percent depletion of NE or DA one hour after α-MPT injection.

Serotonin: Rats were injected intraperitoneally with 75 mg pargyline-HCl/kg body weight or its vehicle, 0.89% NaCl. Thirty minutes after injection, animals were killed by decapitation, the brains removed, and the hypothalamus was dissected as above and frozen on dry ice. Tissue samples were homogenized in 100 µl of 0.1 N HCl (containing 10 mg EGTA/100 ml). Hypothalamic 5-HT was assayed according to the

radioenzymatic method of Saavedra et al. (1973). The turnover index of serotonin was calculated as the percent accumulation of 5-HT following pargyline injection.

C. Results

Although DA concentrations were significantly greater in the AHA of LL rats as compared to LD rats (Figure 14) ($P<0.05$), DA depletion one hour following α-MPT injection was in most cases not significantly different between LL and LD rats. The exception to this was found in the MBH at the AM measurement where DA depletion was higher in LD than LL rats (57.8±1.9% vs. 46.0±1.9%)($P<0.05$). This may suggest that LL rats have a lower DA metabolism than LD rats in the MBH in the morning.

The AHA norepinephrine concentration was significantly greater in the AM of LD rats as compared to the PM (Figure 15) ($P<0.05$). The opposite trend in AHA NE concentration was seen in LL rats (Figure 15). Likewise, one hour following α-MPT injection, LD rats showed a greater AHA NE depletion in the AM than PM (45.9±5.7% vs. 21.3±7.7%) ($P<0.05$). In contrast, AHA NE depletion was lower in the AM than PM (7.5±3.9% vs. 22.2±3.3%) ($P<0.05$), in LL rats. Furthermore, LD rats showed a greater NE depletion than LL rats in the AHA (45.9±5.7% vs. 7.5±3.9%) ($P<0.05$) in the AM, and in the MBH (55.4±6.5% vs. 16.4±6.0% ($P<0.05$) at this time. These results suggest that a decreased NE metabolism exists in LL rats in the AHA and MBH during the morning.

The concentration of AHA serotonin was greater in LD than LL rats in the AM (Figure 17) ($P<0.05$). MBH serotonin concentration was greater in LL than LD rats at the PM sampling time (Figure 16) ($P<0.05$). Thirty minutes following pargyline injection, LD rats showed a greater AHA accumulation of 5-HT in the PM than the AM (64.5±9.1% vs. 19.2±3.0%)

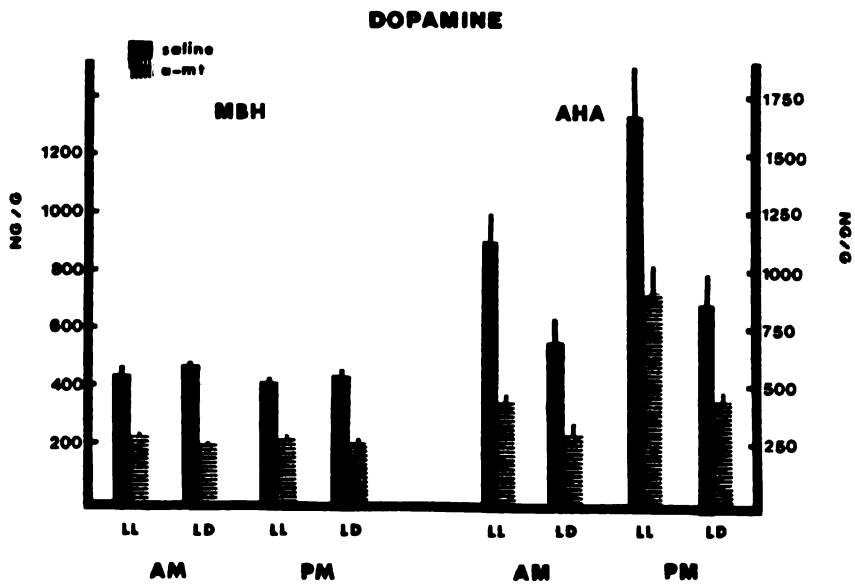

Figure 14. Effect of Constant Light on Dopamine Turnover Index in Two Areas of the Hypothalamus in the Morning and Afternoon.

Catecholamine content was measured in the preoptic-anterior hypothalamic area (AHA) and the medial basal hypothalamus (MBH) of constant light (LL) rats and proestrous rats kept under 14L:10D (LD) conditions. Morning (AM) and afternoon (PM) measurements were made 60 minutes after alpha-methyl paratyrosine (α-MPT) (200 mg/kg) i.p. or its vehicle 0.89% saline. Turnover index was calculated as percent depletion of amine after α-MPT. Each bar represents the mean determination of 6-8 rats. Vertical lines indicate ±1 SEM.

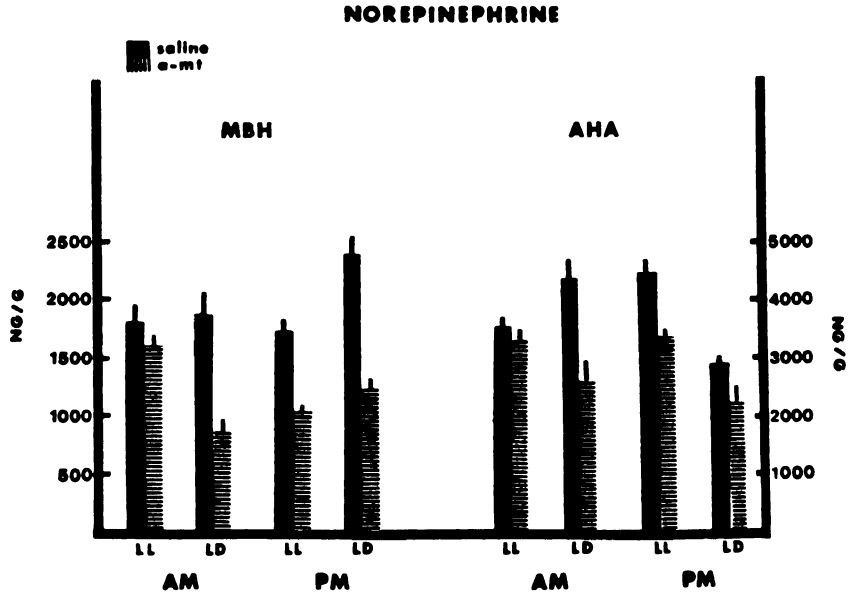

Figure 15. Effect of Constant Light on Norepinephrine Turnover Index in Two Areas of the Hypothalamus in the Morning and Afternoon.

See Figure 14 for explanation.

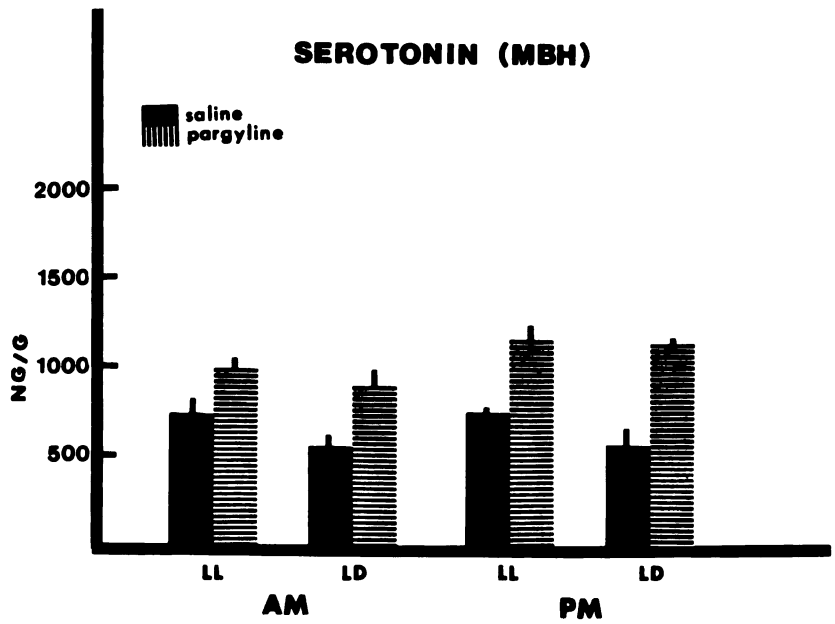

Figure 16. Effect of Constant Light on Serotonin Turnover Index in the Medial Basal Hypothalamus in the Morning and Afternoon.

Serotonin content was measured in the medial basal hypothalamus (MBH) of constant light (LL) rats and proestrous rats kept under 14L:10D (LD) conditions. Morning (AM) and Afternoon (PM) measurements were made 30 minutes following pargyline i.p. (75 mg/kg) or its vehicle 0.89% saline. Turnover index was calculated as percent accumulation of amine following pargyline. Each bar represents the mean determination of 6-8 rats. Vertical lines indicate ±1 SEM.

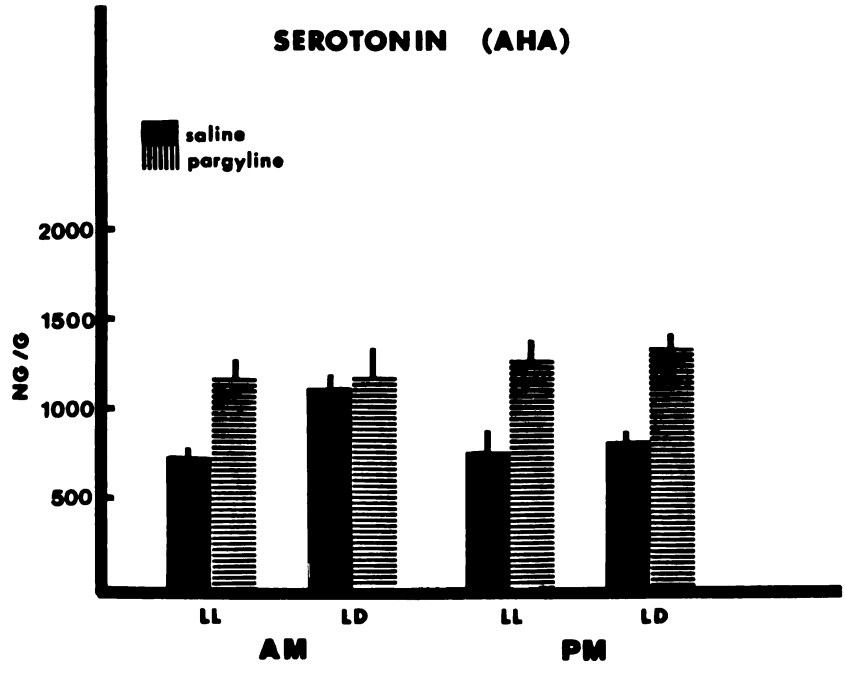

Figure 17. Effect of Constant Light on Serotonin Turnover Index in the Preoptic-Anterior Hypothalamic Area (AHA) in the Morning and Afternoon.

See Figure 16 for explanation.

($P<0.05$). This was also true of PM vs. AM accumulation of 5-HT in the MBH (94.7±8.0% vs. 57.5±18.4% ($P<0.05$) of LD rats. No such changes in 5-HT turnover index were seen in PM vs. AM of LL rats. LL rats showed a greater 5-HT TI in the AM of the AHA than LD controls (61.8±10.0% vs. 19.2±3.0%), but a smaller 5-HT TI in the PM of the MBH than LD controls (54.6±11.2 vs. 94.7±8.0%). These results suggest an apparent attenuation of the diurnal 5-HT metabolic rhythm in rats exposed to constant light.

D. Discussion

In general, the present results indicate that a depression in NE and an increase in 5-HT metabolism may occur during the morning in the AHA of light induced persistent estrous rats. Also a depression in NE (AM) and 5-HT (PM) metabolism was seen in the MBH together with a DA (AM) metabolic decrease of questionable significance in this condition.

Few differences in DA turnover were seen between LL and LD controls rats in this study. However, DA steady state concentrations were higher in LL rats than in LD rats. A possible explanation for this may be that in rats exposed to constant light, the rate limiting enzyme for DA synthesis, tyrosine hydroxylase, may be less sensitive to end produce inhibition, i.e., DA accumulation, together with a decreased release or decreased reuptake of the amine. In addition, LL may induce a decrease in the activity of MAO or COMT in this DA system. This selective effect of constant light on the AHA DA metabolism might also suggest that the activity of the incerto-hypothalamic DA system in the AHA is affected by the constant illumination more than the MBH tubero-infundibular DA system.

The LD control rats exhibited a greater morning NE turnover index than LL rats in either MBH or AHA. Changes in hypothalamic concentration and metabolism of NE have been reported to occur during stages of stimulatory steroid feedback. On proestrus of the estrous cycle, anterior hypothalamic (Stefano and Donnoso, 1967; Coppala, 1969) and median eminence (Selmanoff et al., 1976) NE concentration and whol brain NE turnover increase (Zacharek and Wurtman, 1973). During the first estrous cycle, hypothalamic NE turnover increases during early proestrous and decreases in late proestrous and estrus (Advis et al., 1978). The data of this experiment on AM vs. PM NE turnover index in LD rats is in agreement with the above reports. Most studies indicate that NE has a stimulatory effect on LH secretion (Sawyer, 1975; Krieg and Sawyer, 1976; Kalra et al., 1972). The absence of this stimulatory hypothalamic input in the constant light exposed animal may contribute to the persistent estrous condition in which the stimulation for ovulation, i.e., LH release, is also missing.

Comparing the AHA diurnal rhythmi of NE turnover index in the LL rats with the LD controls, it appears that constant light results in a phase shift of the NE metabolic rhythm during the day (Figure 15). Since measurements of NE TI were made at only two time periods, this hypothesis needs to be tested further at additional time periods. Perhaps the light-dark cycle synchronizes central neuronal activity rhythms which could involve NE turnover. Induction of an LH surge during the estrous cycle may depend on the synchronous activity of multiple monoaminergic neurons. The absence of the external signal entraining these rhythms may result in failure of a preovulatory LH surge to occur.

The diurnal rhythm in serotonin turnover index (low in the AM and high in the PM) seen in both areas of the brain in LD rats was not apparent in the LL rats in this study. These data are in agreement with the observations of Meyer and Quay (1976a) who reported that a diurnal variation in 5-HT uptake occurs in the suprachiasmatic nuclear region (SNR) of male and female rats _in vitro_, with a peak during the late light to early dark period. They suggested that this increase in SNR uptake of 5-HT may serve to limit free 5-HT and its inhibitory effects on the gonadotropin release hormone system. In addition, Meyer and Quay (1976b) have found that rats exposed to continuous light show no significant difference in serotonin uptake by the SNR at 1000 or 2200 h. In the present study, the AHA 5-HT turnover index was greater in the LL than LD rat in the AM. In contrast to the LD rat, the LL rat showed no change in AM vs. PM serotonin turnover index. This elevation in morning serotonin TI and/or arrhythmic diurnal variation in the LL rat may have an inhibitory influence on LH release.

Kueng _et al_. (1976) examined 5-HT concentrations in 18 brain regions during the estrous cycle of the rat. They found that in the lateral preoptic area, the 5-HT concentration was elevated in the morning of proestrus and then decreased in proestrous afternoon, estrus and metestrus. The LD rat serotonin concentrations in the AHA showed the same pattern of changes during the day in these studies. Kueng _et al_. (1976) proposed that there may be increased release of 5-HT in the afternoon of proestrus. In support of their hypothesis, these studies also suggest an increased 5-HT turnover in proestrous afternoon, but a constant high level of 5-HT turnover during the day in LL rats.

In constrast to the AHA, LL rat 5-HT turnover index was lower than that of LD rats in the MBH. These differences in 5-HT metabolism in two areas of the brain in the same type of animal appear very unusual but are not necessarily mutually exclusive. Just as different areas of the brain may have opposite effects on a physiological function, the same neurotransmitters in those areas could have opposite effects as well. Perhaps the AHA 5-HT center is inhibitory to LH release during most of the estrous cycle while the MBH 5-HT center is stimulatory. Alternatively, MBH 5-HT metabolism may not play any significant role in control of the preovulatory LH surge.

In any brain region, the metabolism of a neurotransmitter is influenced by the activity and metabolism of other neurotransmitters, and by the activity of free amino acids which may act both as putative neurotransmitters and as precursors for monoamine transmitters. It is therefore difficult to ascribe abnormalities in brain function to alterations in a single specific neurotransmitter substances inasmuch as overall brain activity ultimately depends on the sum of changes in all neurotransmitters.

It has been postulated that the hypothalamus exerts a dual control over ovulation--its inhibitory influence being transmitted via a 5-HT pathway and a stimulatory one through a catecholaminergic (CA) pathway. It is believed that the balance between these contrasting influences determines the occurrence of ovulation--a critical balance in favor of the CA pathway promoting ovulation, and the opposite inhibiting it (Labhsetwar and Zolovick, 1973).

There appear to be similarities in neuroendocrine changes occurring in the reproductive state of constant estrus as induced either by old age or constant light in the rat. In general, the results of these studies suggest that a depression in AHA NE metabolism and MBH 5-HT and NE metabolism but an increase in AHA 5-HT metabolism (AM) may occur in light induced persistent estrus. Huang (1978) showed that old (20 month) female rats in constant estrus show a lower turnover index of NE in the AHA than younger regular cycling animals on proestrus or estrus. Previously it has been observed that chronic stimulation of catecholamine or depression of serotonin metabolism in old female rats in constant estrus results in improved reproductive function (Clemens et al., 1969; Quadri et al., 1973). Finally, Everett (1970) has shown that when old constant estrous rats were placed in an environment with less than 10 hours of light, regular cycling resumed. Although it would be tempting to speculate that the light induced persistent estrous condition may be a useful model in which to study the effects of aging on neuroendocrine control of reproduction, further work is necessary to evaluate the possible relationship between these two phenomena.

DISCUSSION

This report explains some of the changes which occur at various levels of the hypothalamo-hypophysial-gonadal axis in the light induced persistent estrus rat. A common neuroendocrine change seen in various states of noncyclicity (such as light induced persistent estrus) is the absence of a preovulatory LH surge. Therefore, attention was focused on those processes directly or indirectly involved in initiating and maintaining this surge and considers possible mechanisms for the effect of constant light on reproductive function in the rat.

Based on experiments where the daily administration of a barbiturate delayed ovulation by 24 h intervals, Everett and Sawyer (195) concluded that preovulatory secretion of LH in the rat displayed in a definite 24 h periodicity. Since the time of the preovulatory surge of LH in plasma was advanced or delayed in response to corresponding shifts of the photoperiod (Everett, 1970; Blake, 1975), it became obvious that the 24 h periodicity was synchronized to the light-dark schedule. The fact that cyclic ovulation occurred for long periods in rats kept in continuous darkness suggests that daily light-dark cues are not essential for maintaining the cyclic mechanism of the LH surge and suggests the synchronizer may be endogenous. In the absence of a light-dark cycle, changes in temperature or humidity (Wurtman, 1967) may furnish sufficient stimulus for establishing the phase of the estrous cycle. The possibility of a spectrum or hierarchy of environmental cues

complicates claims of inherent rhythmicity of reproductive functions. McCormack and Sridaran (1978) found that after exposing rats which had been allowed to "free run" in an environment of very dim constant light, to a light:dark regimen, the rats resynchronized to the new lighting schedule. This may suggest that the light:dark regimen may "cue" some endogenous synchronizer for LH release or that it is a stronger synchronizer than some other environmental cue.

Whereas rhythmic changes in environmental lighting exert a synchronizing effect on estrous cycles, conditions of continuous light or dark may result in aberrant vaginal cyclicity. Rats under constant illumination, with possible strain differences in sensitivity, commonly show persistent vaginal cornification (Hoffmann, 1973). These distrubances in the estrous cycle resulting from constant illumination or dark suggest that light is more intimately involved in mechanisms controlling gonadotropin secretion than is generally implied in its accepted role as synchronizer or monitor of reproductive functions in mammals (Critchlow, 1963).

Among the effects of constant light on reproduction is the elevation in serum FSH and Prl levels while serum LH levels are decreased or unchanged. In accordance with previous studies (Kledzik and Meites, 1974) no preovulatory LH surge was seen in LL rats in Experiment I. Morning serum FSH levels were not elevated in LL rats but rose in the afternoon, just as in LD proestrous rats. This differential effect of constant light on gonadotropin release is interesting in that it supports the theory for separate hypothalamic releasing hormones for LH and FSH. It also suggests that constant light may be selectively

inhibiting neuronal areas which are more closely involved with LH release.

Administration of testosterone proprionate (TP) to 5-day old female rats produces a syndrome characterized by anovulatory ovaries and vaginal cornification (Barraclough, 1961). Barraclough (1966) also has demonstrated that neonatal TP treatment deleteriously affects neural mechanisms responsible for the cyclic discharge of LH. These effects are similar to those found in constant light induced persistent estrus. Recent studies by Chappel and Barraclough (1976) have shown that electromechanical stimulation of the medial preoptic area (MPOA) induces the release of both LH and FSH whereas a selective release of pituitary FSH can be elicited by dorsal anterior hypothalamic area (DAHA) stimulation. Chappell and Barraclough (1976b) have also shown that neonatal steroid exposure does not affect those DAHA components involved in FSH release but significantly less LH was released after MPOA stimulation in these animals. Perhaps constant light is selectively affecting these same hypothalamic areas in a similar pattern.

The present studies have ruled out the possibility of a decreased pituitary sensitivity to GnRH as a probable cause for absence of a preovulatory LH surge in the LL rat. On the contrary, the LL rat pituitary was found to have comparable sensitivity to GnRH as the LD proestrous rat pituitary as reported by Fink (1975). This increased pituitary sensitivity would in turn explain the greater effectiveness with which ovulation occurs in LL rats in response to mating (Smith and Davidson, 1974). This ability to ovulate in response to mating, to a brief period of darkness (Takahashi et al., 1977) or even to stress (Smith and Davidson, 1974) also suggests that LHRH is still synthesized

in the hypothalamus of LL rats and furthermore can be released to affect pituitary LH release under these conditions. In an LL rat not exposed to these stimuli, however, spontaneous release of LHRH into the pituitary portal system would not be expected to occur. The results of hypothalamic LHRH content measurements in the LL rat (Experimental III) suggest that this may indeed by the case.

One of the factors involved in the physiological regulation of spontaneous and cyclic LHRH release is the sensitivity of the hypothalamus to positive and negative feedback by estrogen (E) and progesterone (P). Hoffmann (1973) has postulated that in light induced persistent estrus, just as in persistent estrus due to old age, the hypothalamic sensitivity to estrogen positive feedback may be decreased. Huang et al. (1978) have shown that a decreased positive feedback sensitivity to estrogen occurs in old (24 month) constant estrous rats. Similarly, neonatal androgen induced persistent estrous rats fail to demonstrate positive feedback release of LH in response to estrogen priming (Mennin and Gorski, 1974). The same effect was seen in light induced persistent estrous rats in Experimental section IV in this thesis. Furthermore, in all three of the above types of persistent estrous states, estrogen priming followed by progesterone administration was found to be effective in inducing a positive feedback release of LH. It would appear then that progesterone was able to overcome whatever changes occurred in hypothalamic positive feedback sensitivity to estrogen in these persistent estrous states. Presumably then, if progesterone were present in sufficient levels at the proper time, spontaneous LH release should occur in these animals. Exogenous progesterone administration at appropriate times during the 4 or 5 day estrous cycle will advance

ovulation 24 hours in intact rats (Everett, 1948; Brown-Grant, 1969). Progesterone synergizes with subthreshold dosages of ethanol estradiol to induce an ovulatory LH surge in castrate female rats (Swerdloff et al., 1972).

Ovarian progesterone secretion does not increase prior to the proestrous release of gonadotropin (Barraclough et al., 1971; Piacsek et al., 1971). Consequently, if this steroid synergizes with estrogen to facilitate this event, it must originate from some extra-ovarian source. Feder et al. (1968) have demonstrated that the rat adrenal glands secrete substantial amounts of progesterone. Furthermore, in a recent investigation Mann and Barraclough (1973) have shown that a diurnal rhythm exists in adrenal progesterone secretion during the rat estrous cycle; being high during the early morning hours (0100-0500 hr) and then falling to a nadir between 1000 and 1400 hr. A possible role of adrenal progesterone in the regulation of LH secretion on proestrus in the rat has been proposed by several investigators (Nequin and Schwartz, 1971; Feder et al., 1971; Lawton, 1972). Mann and Barraclough (1976) have reported that progesterone was necessary in order to stimulate an LH rise in EB primed ovariectomized-adrenalectomized rats. Moreover, these investigators have proposed that adrenal progesterone secretion is responsible for synchronizing the timing of the LH surge and thus the critical period. Since adrenal progesterone secretion is controlled by ACTH (Resko, 1968) and release of this trophic hormone is synchronized by the light-dark cycle, then perhaps the timing of the critical period for LH release actually is indirectly regulated via circadian adrenal progesterone rhythms.

Persistent estrus induced by old age or constant light is accompanied by an apparent cessation in the rhythm in steroid secretion. Huang et al. (1978) observed low and arrhythmic secretion of estradiol and progesterone secretion in old constant estrous rats as compared to young regular cycling rats. Takahashi et al. (1977) reported that constant light induced persistent estrous rats had plasma estradiol levels which were intermediate between proestrus and diestrus levels but also very low levels of progesterone. Constant light has been shown to abolish the rhythm of corticosterone secretion, which is presumably a response to a change in ACTH release in this condition (Critchlow, 1963; Takahashi et al., 1977). In accordance with the results of Takahashi et al., 1977), intermediate estradiol levels and low levels of progesterone were seen in light induced persistent estrous rats in Experimental section V of this thesis. Since both adrenal corticosterone and progesterone release respond to ACTH secretion, which is in turn synchronized to the light-dark cycle, constant light might be expected to result in a disturbance in the progesterone rhythm. The low levels of progesterone seen in light induced persistent estrous rats in Experimental section IV may be one of the major contributing factors inducing the persistent estrous condition. Progesterone injection has been reported to induce cycling in LL rats (Everett, 1948) and old constant estrous rats (Huang et al., 1976). Perhaps under conditions such as constant light, hypothalamic sensitivity to positive feedback by estrogen is decreased and therefore the facilitatory support of progesterone becomes necessary although absent in this condition.

The sensitivity of the hypothalamus to positive and negative feedback by steroids may be interpreted mechanistically as the effectiveness with which steroids affect hypothalamic monoaminergic neuronal activity. The activity of these neurons directly or indirectly affects GnRH neurosecretory neurons and their corresponding release of gonadotropins. Kalra et al. (1971) have shown that the stimulatory effects of progesterone on LH and FSH release involve noradrenergic neurons. In Experimental section VI, it was seen that light induced persistent estrous rats showed a decreased morning NE turnover index in the anterior hypothalamic area and medial basal hypothalamus as compared to LD proestrous controls. Also, morning 5-HT turnover index was higher in the AHA of LL rats along with an apparent loss of daily 5-HT metabolic rhythm. These results support the hypothesis of Kledzik and Meites (1974) that LL may result in decreased catecholamine and increased serotonin metabolism. They were able to reinitiate cycling in LL rats by administration of catecholamine precursor and 5-HT inhibitor drugs. Not unlike light induced PE, Huang (1978) demonstrated that old constant estrous animals showed a decreased hypothalamic NE turnover index as compared to young proestrous or estrous rats.

NE has been reported to stimulate release of gonadotropins (Kalra and McCann, 1973b) and 5-HT has been reported to be inhibitory (Kamberi et al., 1970b) or stimulatory to LH release (Takahashi et al., 1973). Therefore an alteration in the function of these hypothalamic neuronal systems in response to constant light exposure, as seen in Experimental section VI, could result in the blocking of the preovulatory LH surge. It is possible that constant light could directly affect the function of these neuronal systems and thus affect gonadotropin release.

Alternatively, it is also possible that constant light could alter ACTH release rhythm and its corresponding influence on adrenal progesterone rhythm which in turn could alter activity of hypothalamic neuronal systems to block preovulatory LH release. Regardless of which of these routes constant light takes to affect gonadotropin release, its initial effect on reproduction appears to be at the level of the brain.

Reviewing the data presented in this thesis, no clear hypothesis regarding a cause and effect relationship between constant light and its effect on reproductive cyclicity can be made. Although some mechanisms for this effect have been ruled out in these experiments, only correlations between hypothalamic and hormonal changes in light induced persistent estrous can be made at this time. Due to the complex integration of components involved in maintaining this "cycle", it may be difficult to distinguish cause from effect after persistent estrus is established. Nevertheless, these experiments have shed some light on which pieces of this puzzle are of importance and will hopefully stimulate further inquiry into the etiology of light induced persistent estrus.

Some effects of light and photoperiod appear to have a direct influence on human reproductive cycles (Dewan, 1967) and on domestic animals important as a human food source (Peters et al., 1978). Investigation of the full potential of light in these circumstances could be very beneficial. Since it would be important to know about the mechanisms by which light may induce these responses, a study of the mechanisms in light induced persistent estrus may offer some clues about the mechanism involved in the above light effects as well.

APPENDICES

APPENDIX A

Coyle and Henry Catecholamine Assay Procedure

1. Homogenize pieces of brain tissue in desired volume of 0.4 N perchloric acid (plus 10 mg% EDTA) using matched glass microhomogenizers (Micrometric Instruments, Cleveland, Ohio).

2. Transfer homogenate to microcentrifuge tubes (Kew Scientific, Inc., Columbus, Ohio) and centrifuge for 45 sec in a microcentrifuge (Coleman Instruments, Oak Brook, Illinois).

3. Transfer 10 µ of supernatant (or of working NE and DA standard solution) to glass culture tubes and add 25 µl of the following mixture:

Reagent	Proportion
20 mM EGTA-Na salt (0.760 gm/100 ml H_2O and pH to 7.2)	1
Pargyline Solution (to 4 mg pargyline add 25 µl β-mercaptoethanol and 225 µl H_2O)	1
1 M Tris base (with 3 mM $MgCl_2$) (to 6.05 gm Tris add 50 ml H_2O plus 30.5 mg $MgCl_2$)	6.5
S-adenosyl-l-methionine(Methyl-^3H) [11.6 Ci/m mole in Sulfuric acid:ethanol solution (90:10, v:v), pH 1-3]	3.0
Catecholamine-o-methyl transferase (COMT, partially purified by the method of Nikodijevic et al., 1970)	2.5
1 mM sodium phosphate buffer	2.5

4. Incubate for 40 min at 37°C.

5. Add 30 µl of mixture of 5 volumes 0.45 M borate buffer (pH 10.0) and 1.0 volumes of carrier methoxyamine mix prepared as follows:

   add 5.0 ml H_2O to the following salts:
   50 mg 3-methoxytyramine, 50 mg DL-metanephrine, 50 mg DL-normetanephrine and 5 mg Na-bisulfite

6. Add 500 µl to toluene:isoamyl alcohol solution (3:2, v:v), vortex for 30 sec and centrifuge for 5 min at 3,000 RPM (RC2-B, Sorvall, Dupont Instruments, Newtown, Connecticut).

7. Transfer 400 µl of organic phase to conical centrifuge tubes containing 400 µl borate buffer (pH 10.0), vortex for 30 sec and centrifuge at 5/7 speed in IEC clinical centrifuge (International Equipment Co., Needham Hts., Mass.).

8. Transfer 300 µl of organic phase to conical centrifuge tubes containing 500 µl of 0.1 N HCl, vortex for 30 sec and centrifuge as in step 7.

9. Aspirate organic phase.

10. To remaining aqueous phase add 7 ml toluene:isoamyl alcohol (3:2, v:v), vortex, centrifuge as in step & and discard organic phase.

11. To remaining aqueous phase neutralize with 500 µl of 0.5 M sodium phosphate buffer (pH 7.5), add 50 µl of 3% sodium metaperiodate wait 2 min and add 50 µl of 10% glycerol.

12. Add 10 ml toluene, vortex for 30 sec, centrifuge as in step 7.

13. Transfer 9 ml of organic phase to conical centrifuge tubes containing 1 ml of 1 N NaOH for final extraction of NE metabolites. Vortex for 30 sec, centrifuge and discard organic phase. Add 100 µl glacial acetic acid, 10 ml Scintiverse (Fisher Scientific, Livonia, Michigan) and transfer to 20 ml glass scintillation vials for counting.

14. From the remaining aqueous phase of step 12, the residue toluene is aspirated, 500 µl of 1 M borate buffer is added and tubes are vortexed. Add 8 ml of toluene:isoamyl alcohol (3:2, v:v), vortex and centrifuge. 0.6 ml of the organic phase is added to 10 ml of Scintiverse in 20 ml glass scintillation vials and counted for dopamine.

APPENDIX B

Ben-Jonathan and Porter Catecholamine Assay Procedures

1. Same as step 1 in Appendix A.

2. Same as step 2 in Appendix A.

3. Transfer 10 µl of supernatant (or of working NE and DA standard solutions) to conical centrifuge tubes and add 25 µl of the following mixture:

Reagent	Proportion
20 mM EGTA-Na salt (0.760 gm/100 ml H_2O and pH to 7.2)	1
Pargyline solution (to 4 mg pargyline add 25 µl β-mercaptoethanol and 225 µl H_2O)	1
1 M Tris base (with 3 mM $MgCl_2$) (to 6.05 gm Tris add 50 ml H_2O plus 30.5 mg $MgCl_2$)	6.6
S-adenosyl methionine (Methyl-^3H) (11.6 Ci/m mole diluted 1:3.5 with H_2O)	3.0
Catecholamine-o-methyl transferase (COMT; partially purified by the method of Nikodijevic et al., 1970)	5.1

4. Incubate for 60 min at 37°C.

5. Add 30 µl of 0.45 M borate buffer (pH 10.0) and 5 µl of carrier methoxyamine mix (50 mg 3-methoxytyramine, 50 mg DL-metanephrine and 50 mg DL-normetanephrine; 10 mg of each amine/ml of 0.1 N HCl). Add 500 µl of toluene:isoamyl alcohol (3:2, v:v), vortex for 30 sec, and centrifuge at 5/7 speed on IEC clinical centrifuge (International Equipment Co., Needham Hts., Mass.).

6. Transfer 400 µl of organic phase to conical centrifuge tubes containing 40 µl of 0.1 N HCl. Vortex for 30 sec and centrifuge as in step 5. Carefully aspirate organic phase.

7. Apply 25 µl of acid phase to LQ-60 silica gel plates previously spotted with 5 µl of carrier methoxyamine mix. Allow plates to dry.

8. Place plates in thin-layer chromatography tanks containing chloroform, ethanol and methylamine (40:18:5 by volume). Allow plates to run 1-1/2 to 2 h and remove from tank to allow for drying.

9. Visualize and outline spots under ultraviolet light.

10. Scrape plates and place scrapings into scintillation vials containing 1.0 ml of ethylacetate, acetic acid and H_2O (3:3:1 by volume) and shake for 30 min. Add 10 ml of Scintiverse and count.

APPENDIX C

Serotonin Microassay

Assay:

1. Homogenize tissue in 100 µl 0.1 N HCl. (with 10 mg EGTA/100 ml)
2. Centrifuge 6,000 RPM (RC2-B) 5 min.
3. Add 10 µl supernate to 5 ml disposable culture tube containing 10 µl PO_4 buffer - NaOH.
4. Add 20 µl of a mixture of equal parts of Acetyl CoA, 1 mg/ml (PO_4 buffer) + NAT.
5. Incubate 37°C/30 min.
6. Add 25 µl of a mixture containing 14.5 parts PO_4 buffer, 10 parts HIOMT, 0.5 parts SAM-H^3.
7. Incubate 37°C/10 min.
8. Stop reaction: note - stop ASAP. Place on ice and add 100 µl Borate Melatonin (5 mg/ml).
9. Extract in 2 ml toluene.
10. Vortex 30 sec.
11. Spin at 6,000 RPM/5 min (RC2-B).
12. Aspirate 1.5 ml supernate (organic phase) and transfer to a scintillation vial containing 2 ml fresh toluene.
13. Dry overnight at 80°C (hot plate - time - 25%) in hood (in pan).
14. Add 1 ml EtOH + 10 ml scintiverse.
15. Count.

Std.:

Serotonin 10 µg/ml 0.1N HCl (free base). Weigh out 21.48 µg serotonin creatinine sulphate/ml 0.1N HCl.

Working Soln. Take 5 µl stock serotonin:
5 µl -qs → 1 ml (0.5 ng/10 µl)
10 µl -qs → 1 ml (1.0 ng/10 µl)
20 µl -qs → 1 ml (2.0 ng/10 µl)

Reagents:

PO_4 buffer 0.2M pH 7.9 Titrate dibasic (2.84 g/100 ml) with Monobasic (2.76 g/100 ml) such that pH = 7.9

Borate Melatonin Mix 9 parts Borate 0.5M pH 10 (6.2 g/200 ml H_2O brought to pH 10 with 5N NaOH pellets) with 1 part Melatonin 5 mg/ml (50 mg Melatonin/2.5 ml EtOH....bring up to 10 ml with H_2O).

0.1N HCl (containing 10 mg EDTA/100 ml).

PO_4 buffer - NaOH (22.5 ml PO_4 buffer + 2.5 ml 1N NaOH).

Scintiverse (Fisher).

APPENDIX D

Steroid Radioimmunoassay Protocol

A. Extraction of Serum.

1. Pipette desired amount of serum into 18x150 mm test tubes.
2. Add 1 ml ether/0.1 ml serum to serum tubes.
3. Vortex (30 sec).
4. Place tubes in dry ice-ethanol bath until serum fraction is frozen.
5. Decant ether fraction (which now contains the extracted steroids) into 16x125 mm test tubes.
6. Dry ether fraction with air.
7. Wash tube walls with ether (about 1 ml) and dry with air.
8. Add 1 ml PBS-gel to tubes when completely dry.
9. Vortex tubes for 30 seconds and allow to stand at room temperature for 30 min.
10. From the 1 ml PBS-Gel, pipette desired dilution into assay PBS-Gel so that the total volume is .5 ml.

B. Recovery, Tracer and Solvent Blank Tubes.

1. Tracer tubes: add 100 µl labeled steroid to scintillation vials, then add 100 µl PBS-Gel and 5 ml counting fluid.
2. Recovery tubes: add 100 µl labeled steroid to serum samples not to be assayed and extract as above (steps 1-9, use 200 µl).
3. Solvent blank: extract distilled water (1 ml) as in steps 1-9.

C. Standard (Estradiol, Progesterone and Testosterone).

1. Standards are made in 100% EtOH (5 pg/µl).

2. Standard curve: 2.5, 5, 10, 20, 40, 60, 80, 100, 200, 400, 800 pg.

3. Dry standard in air.

4. Add 0.5 ml PBS-Gel to all standard tubes. Add 0.6 ml PBS-Gel to total count and non-specific binding tubes.

D. Hormone Assay.

1. Add 100 µl antibody to assay tubes and standard, except TC and NSB tubes.

2. Vortex and incubate at room temperature for 30 minutes.

3. Add 100 µl of labeled steroid to all tubes.

4. Incubate at 4°C overnight.

5. Add 200 µl charcoal dextran on ice (vortex).

6. Incubate 10 minutes on ice.

7. Centrifuge at 1000 g in 4°C.

8. Take 0.5 ml supernatant and add to 3 ml scintillation fluid.

9. Count.

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Education:	University of Toledo, 1969, B.S., Biology University of Toledo, 1971, M.S., Biology (C.J.V. Smith) Michigan State University, 1978, Ph.D., Physiology (J. Meites)
Positions Held:	1) Graduate Research Assistant, Physiology Department, Michigan State University, 1975 to present. 2) Graduate Teaching Assistant, Physiology Department, Michigan State University, 1974 to 1975. 3) Research Technician (Sgt., United States Army), Biology Department, Division of Medicinal Chemistry, also Neuroendocrinology Department, Division of Neuropsychiatry, Walter Reed Army Institute of Research, Washington, D.C., 1972 to 1974 (Honorable Discharge). 4) Research Assistant, Biochemistry Department, Medical College of Ohio at Toledo, June 1971 to October 1971. 5) Graduate Teaching Assistant, Biology Department, University of Toledo, September 1969 to June 1971.
Research Interests:	Mechanisms of hypothalamic and extrahypothalamic control of anterior pituitary hormone secretion, particularly the role of biogenic amines in response to various environmental stimuli, reproductive states, and the aging process.

Research Techniques: Radioimmunoassay; LH, FSH, Prl, TSH, GH, LHRH estradiol, progesterone and testosterone.
Radioenzymatic assay; NE, DA, 5-HT.
Stereotaxic and general endocrine surgical produres.
Column and thin layer chromatography.

Publications:

Mioduszewski, R., L. Grandison and J. Meites. Stimulation of prolactin release in rats by GABA. Proc. Soc. Exp. Biol. Med. 151: 44-46, 1976.

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Campbell, G.A., C.A. Hodson, R. Mioduszewski and J. Meites. Increased estrogen suppression of LH in starved, ovariectomized rats. Endocrine Society Proceedings, 1977 (Abstract).

Hodson, C.A., R. Mioduszewski and J. Meites. Effects of adrenergic agonists and antagonists on growth of DMBA-induced summary tumors and serum prolactin levels. ASCO Abstracts 18: 636, 1977 (Abstracts).

Bruni, J.F., J.W. Simpkins, R.J. Mioduszewski and J. Meites. In vivo and in vitro stimulation of LH release by thyroid hormones. (Submitted to Life Sciences).

Hodson, C.A., R. Mioduszewski, and J. Meites. Effects of catecholaminergic and anti-catecholaminergic drugs on growth of carcinogen-induced mammary tumors in rats. (Submitted to Proc. Soc. Exp. Biol. Med.)

Mioduszewski, R., H.T. Chen, C.A. Hodson, R.W. Steger and J. Meites. Changes in hypothalamic biogenic amine turnover index in light induced persistent estrus. (In preparation).

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Mioduszewski, R. Hypothalamic control of carbohydrate metabolism in the diabetic rat: blood glucose and plasma growth hormone levels following bilateral electrolytic lesions of the ventromedial hypothalamus. Masters Thesis, University of Toledo, 1971.

Mioduszewski, R. The effect of constant illumination of the hypothalamo-hypophysial-gonadal axis of the female rat. Ph.D. Dissertation, Michigan State University, 1978.

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