STUDIES ON THE NEUROENDOCRINE CONTROL OF GONADOTROPIN RELEASE

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Joseph Meites
Major professor

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ABSTRACT

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OF GONADOTROPIN RELEASE

by Bèla Emery Piacsek

The effects of various internal and external environmental factors on hypothalamic control of gonadotropin release were investigated. Special emphasis was placed on the changes in hypothalamic content of luteinizing hormone releasing factor (LRF). Whenever possible, in vitro methods were used and the gonadotropin concentrations were determined by the ovarian ascorbic acid depletion method for LH and by the Steelman-Pohley method for FSH. The following results were obtained:

1. A suitable method was designed to study release of LH from the pituitary by in vitro techniques. Adult male pituitaries were incubated in medium 199 in an atmosphere of 95% O_2 and 5% CO_2 for a period of one hour. The medium was assayed for LH by the ovarian ascorbic acid depletion method. Significant increases in LH release were obtained when the pituitaries were incubated in a medium to which neutralized acid extracts of hypothalamic tissue were added.

2. A dose-response relationship was demonstrated between the amount of hypothalamic extract added to the incubation medium and the amount of LH released into the medium.

This relationship was found to be linear when the amount of LH released was related to the logarithm of the dose of hypothalamic extract used. An inverse dose-response relationship was found between the logarithm of the dose of hypothalamic extract used and the amount of LH remaining in the incubated pituitary explants.

3. The effects of ovariectomy and estrogen treatment on the hypothalamic content of luteinizing hormone releasing factor (LRF) were determined in three separate experiments. Ovariectomy was found to reduce the amount of hypothalamic LRF by 50-70%. Estrogen treatment in the ovariectomized rat caused a further reduction in hypothalamic LRF content to approximately 50% of the ovariectomized value. A fourfold increase in pituitary LH content was observed after ovariectomy, while the administration of 0.8 ug/day of estradiol benzoate almost completely prevented this increase. It was concluded that estrogen depressed the synthesis of LRF by the hypothalamus. The effects of ovariectomy were attributed to an increased release of LRF from its hypothalamic storage site. If the release of LRF were greater than synthesis, even though synthesis was also increased, this could result in decreased hypothalamic content.

4. The ability of progesterone and combined estrogen-progesterone treatment to alter hypothalamic LRF activity was studied. Progesterone (4 mg/day) alone in the

ovariectomized rat did not alter hypothalamic LRF content. When progesterone (4 mg/day) was administered with estrogen (0.8 ug/day) in the ovariectomized rat, it counteracted the depressing effect of estrogen on hypothalamic LRF content and caused a slight increase in LRF.

5. Studies were conducted to determine the effects of orchidectomy and testosterone on hypothalamic LRF concentration. In two separate experiments, orchidectomy was found to produce a threefold increase in hypothalamic LRF activity. The administration of 1 mg/day of testosterone propionate completely prevented this increase. This supports the view that testosterone in the male rat can inhibit synthesis of LRF.

6. The effects of half-normal feed intake alone, as well as the effects of constant light, epinephrine and acetylcholine in rats on half-normal feed intake were studied with respect to changes in ovarian function, secretion of pituitary gonadotropins and hypothalamic LRF content. Estrous cycles ceased in all animals on reduced food intake within 2 weeks. Ovarian and uterine weights, and histological examination of these organs, indicated an almost complete cessation of pituitary gonadotropin release as a result of chronic underfeeding. No changes in pituitary FSH content were found but a significant reduction was found in pituitary LH and hypothalamic LRF content. The FSH in the pituitary apparently was not

released, in agreement with the observations of earlier workers.

Both exposure to constant light and twice daily injections of 0.25 mg epinephrine caused a significant increase in the ovarian weight in the half-fed rats, but only constant light increased uterine weight. No changes in either ovarian or uterine weights were found with twice daily injections of 2.5 mg of acetylcholine. Pituitary FSH and LH concentrations remained unchanged with all three treatments, as did hypothalamic LRF activity. Examination of ovarian and uterine histology indicated that exposure to constant light resulted primarily in an increase in follicular development, while administration of epinephrine resulted in induction and maintenance of luteinization of the ovaries. This suggests that constant light induced primarily FSH release whereas epinephrine elicited primarily LH and prolactin release. These results indicate that constant light or epinephrine can partially overcome the depressing effects of reduced food intake on ovarian function.

7. The effects of constant light on the gonadotropic function of transplanted pituitaries were studied in mature hypophysectomized female rats, in three separate experiments. Hypophysectomized rats bearing two subcutaneous pituitary transplants were exposed to constant illumination for 21 days. Some of these rats were injected with human chorionic gonadotropin (HCG) during the last 3 days of treatment. Exposure to constant light produced an increase in both the

ovarian and uterine weights of the animals bearing pituitary transplants. No differences in ovarian or uterine weights were found between hypophysectomized controls (without pituitary transplants) and the hypophysectomized rats (without pituitary transplants) exposed to constant light.

Histological examination of the ovaries of rats with pituitary transplants exposed to constant light indicated an increased follicular development, suggesting that the increase in gonadotropic activity was primarily due to FSH. The transplanted pituitaries of the rats exposed to constant light were larger, showed many more viable cells and took up more stain than transplanted pituitaries of rats not exposed to constant light. Stimulation of the ovaries in hypophysectomized rats bearing pituitary transplants and exposed to constant light, is believed to be due to increased release of hypothalamic FSH-RF into the systemic circulation, which stimulates pituitary FSH release.

STUDIES ON THE NEUROENDOCRINE CONTROL OF GONADOTROPIN RELEASE

By

Béla Emery Piacsek

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Dedicated

to

my Wife

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INTRODUCTION

Since the early work of Harris and co-workers (1937), the importance of the hypothalamus in the control of anterior pituitary function has become well established. Recent *in vivo* and *in vitro* investigations have pointed to the existence of specific neurohumors secreted by the hypothalamic portion of the brain, which enter the portal vessels and stimulate or inhibit secretion of the anterior pituitary hormones. This evidence is based on studies involving pituitary stalk section, placement of hypothalamic lesions, stimulation of various areas of the brain, *in vitro* incubation of pituitary tissue and administration of crude or partially purified hypothalamic extracts into animals.

The existence of regulatory neurohumors has now been reported for each of the six anterior hypophysial hormones. In the case of prolactin, the neurohumoral factor inhibits release and synthesis of this hormone by the pituitary. In the case of all other anterior pituitary hormones, the hypothalamic factors have been shown to elevate release and probably also increase the synthesis of these hormones. Because these factors have an important regulatory influence on the function of the anterior pituitary, recent investigations in our and other laboratories have centered on the

role of these neurohumors in the mediation of various environmental and other physiological influences.

Other investigations have centered on the effects of the gonadal steroids, estrogen, progesterone and testosterone, on secretion of FSH and LH. These hormones have been shown to be very potent modifiers of gonadotropic activity, but their exact sites of action remain unknown. Conflicting reports have attributed inhibition of gonadotropin release by steroids either to a direct action on the pituitary or to an action on the hypothalamus. It was part of the intention of the present study to elucidate this problem further.

Stimuli from the external environment apparently exert their influences through the central nervous system (CNS), and their effects would be superimposed on the pituitary-target organ feedback mechanism. There is lack of adequate information on the effects of external environmental factors on gonadotropin release. It was of special interest to study the effects of underfeeding, light and drugs on the hypothalamic content of LRF. It was also of interest to study the effects of these agents on the control of release of follicle stimulating hormone (FSH).

<u>In vivo</u> methods do not give a clear separation of the effects of steroids on pituitary and hypothalamic functions, since the portal vessels form a circulatory link between these systems. Furthermore the direct effects of steroids

on the pituitary can not be evaluated, since there is a possibility of metabolic changes which these steroids can undergo in the body. It was decided therefore to study the problems by in vitro techniques. The effects of steroids on the hypothalamic content of LRF could thus be determined, as well as the direct effects of steroids on the pituitary. Attempts were also made to elucidate the hypothalamic involvement in the effects of underfeeding, light and drugs on the hypothalamic content of LRF.

Studies were also initiated to study the effects of light on the gonadotropic function of transplanted pituitaries. The purpose of these experiments was to determine whether gonadotropin releasing hypothalamic neurohumors in the general circulation could be raised to such a level that gonadotropic function by pituitary transplants would be stimulated without the presence of the portal circulation.

To summarize the objectives of this thesis, the following problems were investigated: (1) A study was conducted to determine the effects of castration and gonadal steroids (estrogen, testosterone and progesterone) on the hypothalamic content of LRF, (2) The effects of underfeeding on FSH and LH release, as well as on the hypothalamic LRF content, were investigated in female rats. An attempt was made to reinitiate release of gonadotropins in underfed female rats by light, epinephrine and acetylcholine, (3) The effects of constant light on the gonadotropic function of transplanted pituitaries was studied in female, hypophysectomized rats.

REVIEW OF LITERATURE

I. Evidence for Hypothalamic Control of Anterior Pituitary Function

Reports which suggested possible hypothalamic involvement in pituitary control first appeared in the early years of this century. Aschner (1912) reported disturbances in hypophysial function in dogs with hypothalamic lesions, and hypophesized a possible role for the autonomic nervous system and the tuber cinereum in regulation of endocrine functions. Further evidence was presented for central nervous system control of anterior pituitary function by Hohlweg and Junkmann (1932). Using hypophysial transplants and parasympathetic inhibitors, they reported evidence for involvement of the central nervous system and even predicted that a negative feedback system exists between the target organs and pituitary function.

Probably the first direct evidence for stimulation of pituitary function by the central nervous system was presented by Marshall and Verney (1936) and Harris (1937). Marshall and Verney (1936) induced ovulation in rabbits by massive electrical stimulation of the head, and Harris (1937) produced pseudopregnancy in the rat by electrical stimulation of the head, and induced ovulation by direct stimulation of the hypothalamus (Harris, 1937a).

Supportive evidence for hypothalamic control of gonadotropin release also comes from histological studies of the pituitary-tuberal area in the mammal. The earliest evidence for the presence of a local portion circulation between the median eminence region and the anterior hypothysis was reported by Popa and Fielding (1930). Wislocki and King (1936) studied the blood supply of the hypothalamo-hypophysial area with vital dyes. The portal vessels were found to drain the stalk and infundibular region. No vascular connections were found between the infundibular stem and the upper hypothalamic regions. This supports the hypothesis that the neurohumors are carried from the median eminence region to the hypophysis via the portal circulation. Any transfer of a humoral substance from an upper nucleus to the median eminence area would have to be via neuronal transfer. An exact and precise description of the anatomy of the hypothalamic region was presented by Rioch et al. (1940). Their description supports a neurohumoral mechanism.

Further study indicated that nervous connections to the adenohypophysis are extremely scanty (Green and Harris, 1947). The same report also showed that vascular connections between the median eminance and adenohypophysis are very prominent in many mammalian species. Nerve fibers coming from the hypothalamo-hypophysial tract come in close contact with the portal vessels. This type of arrangement gives strong support to a neurohormonal theory. Green (1948)

presented evidence in the rat and man that the direction of blood flow in the portal vessels is downward from the median eminence to the adenohypophysis. Regeneration of portal vessels after sectioning was clearly demonstrated by Harris (1949). Confirmation in the primate was also observed by Harris (1950). This report also shows that the nerve fibers in the stalk do not regenerate and are not essential to anterior pituitary function. Harris (1950a) also demonstrated that stalk-sectioned female rats can resume cycling after regeneration of the portal system. Insertion of a paper block between the section prevents regeneration, leaving the rats in anestrus for months. Regeneration can occur in as short a time as one day.

An excellent comparative study of the pituitary and hypothalamic regions of more than 70 vertebrate species by Green (1951) indicated great similarity from Amphioxus to man. The independent vascularization of the posterior pituitary does not appear until the amphibious stage. Innervation is scanty in all vertebrates. The lack of good innervation was confirmed by Green (1951a).

Worthington (1960) studied the vasomotor responses in the superior hypophysial artery and the portal vessels, and concluded that adrenergic and cholinergic drugs could act on the hypophysis by producing vasomotor changes in these vessels. An increase in the number of neurosecretory granules in the latero-dorsal interstitial nucleus has been

observed to follow castration (Barry and Torre, 1962). Their work suggests that castration causes an increase in the synthesis of the LH regulating neurohumor. Kobayashi et al. (1963a) found vesicles of 300-500 A diameter in the fibers terminating in the infundibular wall. Their position suggested that these vesicles could be released into the portal vessels. Rinne (1960) found AF positive material around portal vessels in the median eminence, but no PAS positive material could be found.

Evidence for the hypothalamic control of pituitary function has involved all of the anterior pituitary hormones. Some evidence was presented for the control of adrenocorticotropic hormone (ACTH) release by the hypothalamus (DeGroot and Harris, 1950). Further studies with hypothalamic lesions indicated a definite role for the central nervous system in the control of ACTH release (DeWied, 1961). Early workers in this area thought that the releasing factor for corticotropin was identical with vasopressin and a strong case for this theory was advanced by McCann and Brobeck (1954). Hilton and his co-workers (1959), however, showed evidence for the direct action of vasopressin on the adrenals, thereby placing the validity of this theory in doubt. They showed good evidence for the involvement of the supraoptic system in the control of ACTH release. Proof for the existence of a releasing factor of separate identity from vasopressin was first presented by Saffran and his co-workers (1955).

Greer (1951) presented some evidence from studies on hypothalamic lesions for hypothalamic control of thyrotropic hormone (TSH) release. Shibusawa and co-workers (1956) first reported the effectiveness of a "TSH releasing principle" in rats with pituitary stalk sections. However, their work could not be confirmed by other investigators (see D'Angelo, 1963). Studies with pituitary transplants tend to support the view that a hypothalamic neurohumor is necessary for TSH release and possibly synthesis (Knigge, 1961). Partially purified and active hypothalamic polypeptides with TSH releasing activity were first convincingly demonstrated by Guillemin et al. (1963). In vitro TSH releasing activity by hypothalamic extracts has also been reported by Sinha and Meites (1965).

Considerable literature is available on the influence of the hypothalamus on prolactin release, although the influence appears to be an inhibitive one. Transplantation of pituitary glands to sites distant from the hypothalamus does not reduce their ability to secrete prolactin (Everett, 1954; Desclin, 1956). Further evidence from pituitary transplantation experiments can be found in the work of Nikitovitch-Winer and Everett (1960). More conclusive evidence, with specific bioassay for prolactin was reported by Nicoll and Meites (1961,1962) on prolactin release and synthesis. Their work was conducted in organ cultures, thereby demonstrating the release of pituitary prolactin in

an *in vitro* system. The fact that prolactin release under these conditions was not reduced but significantly increased, presents a strong case for the existence of some type of hypothalamic inhibition of prolactin secretion in the intact system. Definitive evidence for the existence of a prolactin inhibiting factor (PIF) in acid extracts of hypothalamus was presented by Meites and his co-workers (Meites et al., 1962; Talwalker et al., 1963). Acid extracts of beef, sheep and pig hypothalami were also shown to have PIF activity (Talwalker et al., 1963, unpublished; Schally et al., 1965).

The evidence for the role of the central nervous system in control of growth hormone (GH) secretion is less abundant and more indirect than for other pituitary hormones. Reduced body growth in rats with hypothalamic lesions has been shown by Cahane and Cahane (1938) and confirmed by Bogdanove and Lipner (1952), but the results of such experiments can be interpreted in many ways. Transplantation of the pituitary from its normal location under the median eminence to other sites also resulted in reduced body growth (Goldberg and Knobil, 1957). Multipituitary transplants have been shown to partially stimulate growth in young hypophysectomized rats (Hertz, 1959). Meites and Kragt (1964) observed significant weight gains in young hypophysectomized rats given a single subcutaneous pituitary transplant. *In vitro* studies by Meites and co-workers (1962) have

indicated that the rat anterior pituitary is capable of secreting appreciable amounts of GH in tissue culture in the absence of hypothalamic influence. Convincing *in vitro* evidence for the existence of a growth hormone releasing factor (GHRF or SRF) was first presented by Deuben and Meites (1963) and later confirmed (Deuben and Meites, 1964; Schally et al., 1965). Work on the purification of the growth hormone releasing factor is in its initial phases at the present time (Dhariwal et al., 1965).

Circumstantial evidence for control of FSH release by the hypothalamus is abundant and was reviewed by Flerko (1963). More direct evidence has recently been presented by Igarashi and McCann (1964) and by Mittler and Meites (1964). Both groups of investigators presented evidence for the presence of an FSH releaser (FSH-RF) in acid extracts of rat hypothalamus. These two groups provided *in vitro* and *in vivo* evidence of the existence of an FSH-RF. Further evidence for the existence of hypothalamic control over the release of FSH will be presented in the review on hypothalamic control of LH release.

II. Hypothalamic Control of Luteinizing Hormone (LH) Release

A. Neurogenic Control of Ovulation

The first evidence for involvement of the central nervous system (CNS) in ovulation was presented by Marshall and Verney (1936). They found that electrical stimulation

of the central nervous system could elicit ovulation in a rabbit in heat. Such a response could be elicited by stimulating both the brain and the lumbar-sacral portion of the spinal cord. Ovulation was not immediate but invariably occurred within 17 to 24 hours after stimulation, indicating a delay in action and suggesting the presence of a hormonal mechanism. Pseudopregnancy occurred in some but not all of the animals. A year later this work was confirmed by Harris (1937) and Haterius and Derbyshire (1937). Harris (1937a) found that stimulation of the hypothalamus, as well as the hypophysis, produced ovulation and formation of cystic, hemorhagic follicles. The results with direct stimulation of the pituitary are hard to explain. It is possible that some of the stimulating current spread to the hypothalamic areas. Ovulation following direct hypophysial stimulation was not confirmed by more recent work by Harris (1948). Stimulation of the pituitary stalk, on the other hand, resulted in genital atrophy. Haterius and Derbyshire (1937) found activity limited to an area of the hypothalamus two to three millimeters in diameter regardless of the strength of the stimulus. Ovulation followed stimuli of varied intensity.

Conclusive proof for the role of the CNS was presented by Sawyer et al. (1949) by showing that ovulation could be induced and blocked by various chemical agents. Direct pituitary infusions of epinephrine produced ovulation in the rabbit in heat, while the copulation-induced ovulation

in the estrous rabbit could be blocked by rapid post-coital injection of dibenamine (Sawyer et al., 1949). Adrenaline was later shown to act through the hypothalamus (Sawyer et al., 1950). From these results Sawyer and his co-workers (1949) concluded that the ovulatory response has an adrenergic component. Further studies by the same investigators demonstrated the presence of a cholinergic component as well, since ovulation could be blocked by the administration of atropine. They concluded, however, that the cholinergic component precedes the adrenergic one, since atrophine had to be given earlier than dibenamine to be effective.

Evidence for the cyclicity of the ovulatory mechanism was presented a year later (Everett et al., 1949) when it was shown that in rats on a 5:00 a.m. to 7:00 p.m. lighting schedule, the critical time for the blocking of ovulation occurred on the day of proestrus between two and four o'clock in the afternoon. Injection of dibenamine at 2:00 p.m. blocked ovulation but was completely ineffective if given at 4:00 p.m. Since ovulation did not occur until 2:00 a.m. of the following morning, a humoral mechanism was suspected. Dibenamine not only blocked the ovulation but also the associated cholesterol depletion as judged by histochemical techniques. From this it was concluded that some type of a trigger mechanism is activated during this critical period and stimulates the release of an "ovulating hormone" from the pituitary. Once this mechanism has been

stimulated, further administration of blocking agents appeared ineffective.

The role of the central nervous system in the induction of ovulation was further demonstrated by Everett and Sawyer (1950), who showed that Nembutal and other barbiturates active at the CNS level, were also very effective in blocking the ovulation mechanism during the critical two p.m. period. However, these same barbiturates were ineffective if administered after 4:00 p.m. Blocking the ovulatory response on the day of proestrus delayed it 24 hours, and resulted in persistence of grafian follicles. Markee et al. (1952) reviewed the subject of ovulation and concluded that a neurohumoral substance was involved in the ovulatory mechanism, probably of an adrenergic nature. They also concluded that nerve fibers reaching the anterior pituitary are inactive in ovulation. It now appears doubtful that such nerve fibers exist.

Subsequent studies by Everett and Sawyer (1953) limited the time of the ovulatory stimulus to an even narrower range. Using atropine as a blocking agent, they showed that the duration of the neurogenic stimulus lasts about 20 to 35 minutes, with a 12 to 14 hour delay in ovulation. Progressive increases in percentage of ovulations were shown when hypophysectomy or atropine administration was performed at half hour intervals between 2:00 and 4:00 p.m. (Everett, 1956). This indicated that the anterior pituitary is activated by

some neural trigger at this time and releases its quota of "ovulatory hormone", probably LH.

Direct proof of CNS involvement was obtained by Critchlow (1958) when he showed that lesions in the mammillary peduncle on the day of proestrus prevented ovulation in an overwhelming majority of rats. In those animals where ovulation was not blocked, it was delayed 24 hours. Sawyer (1959) presented additional proof of the role of the hypothalamus in the activation of the ovulatory response. He showed by diagrams the nervous connections of the possible center(s) to other areas of the brain, and demonstrated that the threshold for electrical excitation of this center can be modified by influences from the rhinencaphalon and the reticular formation, as well as by ovarian steroids and possibly anterior pituitary hormones. These influences could be of both a facilitatory and inhibitory nature.

Pushing deeper into the details of the mechanism of ovulation, Everett (1961) showed that the preoptic region of the hypothalamus remains highly sensitive to electrical stimulation even in the presence of nembutal anesthesia. Stimulation of this area on the last day of diestrus advanced ovulation by 24 hours. Everett (1961b) also showed that the stimulatory threshold was lower on the day of proestrus than during diestrus.

The hypothesis of electric stimulation of the hypothalamic area was corrected by Everett (1961a) when he

demonstrated that platinum electrodes did not induce ovulation, while stainless steel electrodes did. He concluded, that the stimulation was electrolytic rather than electric, caused by an irritative deposit of iron at the site of application. Finally it was shown by Everett (1964) that the fibers which are effective in eliciting ovulation have a diffuse origin in the septal region which finally merge in the tuberal region. The results of the long and extensive research on the control of ovulation indicates that the central nervous system is definitely involved, and that its effects are mediated through a neurohumoral agent rather than through direct nervous stimulation of the adenohypophysis.

B. Effects of Electrical Stimulation, and Placement of Lesions

1. Electrical stimulation

Some of the strongest evidence for hypothalamic control of LH release comes from studies on electrical stimulation of various parts of the brain. It was demonstrated early that stimulation of the pituitary stalk produced changes in the reproductive system despite the fact that the pituitary itself was not reactive (Ashner, 1912). Harris (1937) induced psuedopregnancy in the rat by stimulation of the head. It was shown shortly thereafter that certain peripheral receptors could also be involved in mediating environmental stimulation to the central nervous system in the control of the anterior pituitary. It was

found that cervical sympathectomy reduced the number of pseudopregnancies in the rat produced by cervical stimulation (Friedgood and Bevin, 1941). The release of LH following electrical stimulation of the tuber cinereum has been reported in unanesthetized rabbits by Harris (1948).

Increased electrical activity in the brain has been observed in the cat following vaginal stimulation by mechanical means (Porter et al., 1957). Such activity could be observed only in animals that were in estrus. Animals in anestrus did not show changes in the electrical activity following such stimulation. Sawyer and Kawakami (1959) found reproducible changes in the EEG patterns of the postcoital rabbit. They also found that these changes were too late to be functional in the induction of LH release. Instead they appeared to be produced by the hormones that were released by the pituitary and the ovaries.

Taleisnik and Caligaris (1962) suggested that the cortex might normally be inhibitory to the release of LH. They found that a spreading depression induced by the application of 25% KCl to the cortex produced a release of LH from the pituitary. Induction of LH release as indicated by ovarian ascorbic acid depletion (OAAD), has also been shown by stimulation of the cortex by both mechanical and electrical means (Taleisnik et al., 1962). However, the use of the OAAD method by this group was questionable. These results on the role of the cortex are interesting, but the methods

used by these investigators can hardly be considered physiological. Inhibition of LH release by cerebral cortical lesions has also been reported (Soulairac and Soulairac, 1958).

2. Placement of Lesions

More abundant evidence for hypothalamic control of LH release has come from placing lesions in various areas of the brain, either electrolytically or surgically. Some of these lesions were produced by tumors in the hypothalamic area. Precocious puberty has been reported by Weinberger and Grant (1941) in at least 28 patients with hypothalamic tumors. They concluded that the tumors destroyed some mechanism, which normally inhibited gonadotropin release. One must be very careful in interpreting the results of such clinical observations, since these lack adequate controls and are mainly descriptive in nature.

Dey (1941) observed sterility in guinea pigs following artificial lesions. They observed three types of sterile animals, as follows: those with normal appearing reproductive organs but without a mating reflex; those with mainly follicular ovaries, lacking corpora lutea; and those with completely atrophic ovaries. The last group had lesions invariably in the median eminence area, suggesting that this region of the hypothalamus is the primary channel for mediation of the neurohumors controlling pituitary function. Further localization of the area controlling LH secretion

was reported by Dey (1943). He found that lesions in the caudal portion of the chiasmatic region produced primarily follicular ovaries without any evidence of ovulation.

Hillarp (1949) further probed the problem of localization of the gonadotropin controlling area in the hypothalamus. He concluded that the LH controlling area was located in the anterior hypothalamus, ventral to the paraventricular nucleus. Lesions in this area produced constant estrus and follicular ovaries. Sectioning of the pituitary stalk in male rats by surgical techniques caused a dedifferentiation of the cells of the adenohypophysis and prevented almost completely the post-castration rise in basophils (Barnett and Greep, 1951). This suggested that the portal vessels in the stalk carry some humoral substance which controls the differentation and formation of basophil cells primarily responsible for secretion of gonadotropins. Similar results were also reported in the female rat (Greep and Barnett, 1951). Stalk sectioning was followed by general ovarian and uterine atrophy. The authors attributed these changes to pituitary infarction due to lack of sufficient vascularization. Subsequent revascularization of non-portal origin did not reverse the degenerative changes.

Gonadal atrophy was found following placement of bilateral hypothalamic lesions in male rats (Bogdanove and Halmi, 1953). These same authors also found that animals exhibiting complete gonadal atrophy, failed to respond to

castration by a typical rise in basophils (Bogdanove et al., 1955).

The importance of normal CNS function was also evaluated in infant male rats (Riss, 1956). By placing lesions in various areas of the brain, the time of the first fertile mating could be advanced or retarded. In the male rat, gonadal atrophy can not always be used as an indicator of reduced gonadotropin secretion. Bogdanove (1957) found that half of a group of lesioned rats showed normal testis development but had impaired gonadotropin secretion.

The effects of CNS lesions have been demonstrated in species other than the rat and the rabbit. Herbert and Zuckermann (1958) induced precocious heat in ferrets in the anestrous state by placing lesions in the caudate nucleus and adjacent areas. Assenmacher (1957) showed that lesions in the supraoptic and paraventricular region of the duck resulted in gonadal atrophy. Gonadal atrophy was accompanied by a depletion of neurosecretory material in the median eminence area. Neural regulation of the gonadotropes in the duck has been extensively reviewed (Assenmacher, 1958). Aron and Petrovic (1960 and 1960a) demonstrated a basal level of hormonal activity even after stalk section in the male guinea pig. This was found to be true of both the male and the female. It is possible, however, that the male reproductive organs are more sensitive to gonadotropic stimulation. In the dog, posterior median eminence lesions

resulted in general testicular and prostatic atrophy (Davidson and Ganong, 1960).

Many confirmatory reports can be found in the literature on precocious development of gonads of lesioned rats. Precocious follicular growth and vaginal manifestation of estrogen were found in rats with hypothalamic lesions by Bogdanove and Schoen (1959). Female rats bearing lesions in the arcuate nucleus of the posterior tuberal region have been shown to mature significantly sooner than intact controls (Gellert and Ganong, 1960). Vaginal opening occurred earlier in female rats lesioned at four days of age than in sham operated controls (Horowitz and van der Werff ten Bosch, 1962). The lesions were found to be effective if located near the paraventricular nucleus reaching upward to the anterior commissure. The significance of these findings remains uncertain. Riss et al. (1963) found retardation of sexual development both in the gonads and behaviourally in young rats bearing lesions in the hypocampal region, but accelerated sexual activity in those having pyriform lesions. Bar Sela (1964) placed hypothalamic and amygdaloid lesions in young male rats and concluded that the amygdala was probably inhibitory to gonadotropin release, since the lesioned animals exhibited ventral prostate enlargement at age 40 to 50 days.

The induction of persistant estrus by hypothalamic lesions was studied by Kobayashi et al. (1959). They found

that electrolytic lesions induced constant estrus in the rat, but ovarian weight was smaller than in the controls due to lack of corpora lutea. The common location of the lesions in such animals was between the supraoptic nucleus and the rostral part of the median eminence. Flerko and Bardos (1959) found two distinguishable effects with hypothalamic lesions. With suprachiasmatic lesions they obtained constant estrus, cystic ovaries and no corpora lutea. Lesions immediately dorsal to the paraventricular nucleus produced anestrus with large numbers of corpora lutea. They hypothesized that the constant estrus region contains either an FSH inhibitory area or an LH stimulatory area in the intact rat. These results are in general agreement with the work of Kobayashi et al. (1959).

Most recent reports are more specific in evaluating the effects of lesions. Changes in gonadotropin secretion were evaluated by specific bioassays. Davidson et al. (1960) measured the FSH and LH content of the pituitaries of dogs bearing lesions in the posterior median eminence, and found a depression in content of both hormones. Taleisnik and McCann (1961) found that rats bearing median eminence lesions failed to show the usual elevation of pituitary LH content after ovariectomy. These rats also exhibited a constant diestrous type of smear. These results indicate that the lack of a specific neurohumor in the hypothalamo-pituitary portal circulation reduced both synthesis and release of LH

by the anterior pituitary. Somewhat different results were obtained by van der Werff ten Bosch et al. (1962). These investigators found that rats which exhibited prolonged or constant estrus had a high pituitary content of LH but reduced plasma activity when compared with control values. This could imply that different factors are involved in the stimulation of synthesis and release of LH by the pituitary. One must question, however, the sensitivity of their bioassay in determining plasma LH levels. Variable results were reported by Bogdanove et al. (1964) on the effects of hypothalamic lesions on pituitary content of LH. The lesions did not prevent the post-castration changes in the pituitary. No plasma LH activity was detected after lesions, as evaluated by the much more sensitive OAAD assay for LH. These results agree partially with those of van der Werff ten Bosch et al. (1962).

C. Evidence from Hypophysial Transplants

Hohlweg and Junkmann (1932) were the first to use hypophysial transplants to demonstrate CNS control over gonadotropin release. They also postulated the existence of a negative feed back system for the action of the target organs on the control of pituitary hormones. Greep (1936) confirmed the importance of the hypothalamic region by transplanting pituitaries into the sella turcica of immature and adult rats. In females bearing transplants, typical cycles were established even when pituitaries from immature rats were

transplanted to the cella turcica of mature animals. Both ovulation and the formation of corpora lutea were observed.

The importance of the median eminence region was emphasized by the work of Harris and Jacobsohn (1952). Their results indicated that a pituitary transplanted under the temporal lobe did not show normal function, but when placed under the median eminence, portal connections were re-established and the pituitary became highly functional. Immature pituitaries transplanted under the median eminence were stimulated in their development. Desclin (1956) found that pituitaries transplanted to the kidney capsule produced very little gonadotropins but substantial amounts of prolactin. Goldberg and Knobil (1957) transplanted fetal pituitaries into the anterior chamber of the eye of hypophysectomized rats, and observed a basal level of gonadotropin release in the male hosts. Spermatogenesis was maintained at a low level but only very slight stimulation of interstitial tissue was noted.

Probably the most conclusive pituitary transplant work was reported by Nikitovitch-Winer and Everett (1957). They found that pituitaries transplanted to the kidney capsule lost their ability to secrete gonadotropins, but when these same transplants were re-transplanted under the median eminence, they resumed a normal rate of gonadotropin secretion despite considerable injury in the transplantation procedure. The same type of transplantation produced even

more convincing results when re-transplanted pituitaries were shown to produce normal cycles in their hosts and even established pregnancies became possible in these animals (Nikitovitch-Winer, 1958). Pituitary transplants under the kidney capsule lost their differentiation very rapidly. When they were re-transplanted under the median eminence, they re-differentiated from chromophobes to both acidophilic and basophilic cells. Transplantation under the temporal lobe gave neither a resumption of gonadotropin release, nor a differentiation of the chromophobes (Nikitovitch-Winer, 1959).

Direct intra-testicular transplants into the hypophysectomized guinea pig were shown to be without any effect in stimulating the function of the surrounding testicular tissue (Aron and Petrovic, 1958). Contrary to these findings, Herlant et al. (1959) demonstrated normal gonadal activity in the male with hypophysial transplants. They also found excessive mammary development. The discrepancy between the works of Aron and Petrovic (1958) and Herlant et al. (1959) could very well be due to the site of transplantation. Intratesticular transplants could be subject to direct inhibition by androgens in relatively high quantities.

Interesting work on pituitary hypothalamic dependency was reported by Moszkowska (1959). She found that transplantation of a male pituitary into the ovary of an immature female rat produced follicular growth but no corpus

luteum formation. If, however, a piece of hypothalamic tissue was transplanted next to the pituitary transplant, corpora lutea were formed. Presumably this demonstrated the dependency of pituitary LH release on a hypothalamic neurohormone. It is doubtful, however, that hypothalamic tissue can survive when transplanted (Montemurro, 1963) and these results need to be confirmed. Even if the hypothalamic tissue remained alive, it is doubtful whether it retained its usual functional properties in the absence of connections to other parts of the brain. The number of animals employed in Moszkowska's (1959) experiment was also too small to give meaningful results.

A very precise demonstration on hypothalamic dependency by the pituitary was observed by Halasz et al. (1962) when they implanted pituitary tissue into various parts of the hypothalamus of hypophysectomized rats. Implants into the ventral hypothalamus were capable of maintaining gonadotropic function. Implants in the higher centers were ineffective. They termed the effective region the "hypophysiotrophic area". This area was defined by a much more sophisticated technique developed by Halasz and Pupp (1965). By using a miniature knife, they separated the "hypophysiotrophic area" completely from other parts of the brain. Despite this separation, they found maintenance of several hypophysial functions.

Implantation of neonatal pituitaries into the supra-optico-hypophysial tract of hypophysectomized rats resulted in active testicular maintenance (Knigge, 1962). Nearly normal testicular activity was demonstrated with kidney capsule transplants by Ahren and Hjalmarson (1962).

D. Evidence from Hypothalamic Extracts

The most direct proof for the existence of hypothalamic neurohormones comes from demonstration of releasing activity in extracts of hypothalamic tissue, especially from the median eminence. The work in this area perhaps goes back to 1941 when it was reported that an acetylcholine-like substance from the hypothalamus could produce pseudopregnancy when applied directly to the pituitary (Taubenhaus and Soskin, 1941). The effects of oxytocin on gonadotropin secretion was studied by Shibusawa and Saito (1955) in rats, dogs, and humans. They reported an increase in urinary excretion of gonadotropins following oxytocin injections. The purity of their oxytocin preparations was questionable however, and could have been contaminated with other hypothalamic neurohumors. A rise in the urinary excretion of gonadotropins was also claimed following intravenous infusions of posterior hypophysial hormones (Martini et al., 1959). These results were obtained by the use of the "mouse uterine weight" assay, which is sensitive but of questionable specificity and precision. The results with posterior pituitary hormones have not been confirmed (McCann, 1962),

and it is doubtful that posterior pituitary hormones have anything to do with gonadotropin release.

Release of luteinizing hormone following intravenous infusions of acid extract of rat median eminence tissue was probably demonstrated first by McCann et al. (1960) through the use of the Parlow bioassay. They found that the active substance in the extracts was different from histamine, serotonin, substance P, epinephrine, vasopressin, or oxytocin. These results were confirmed a year later by Courrier et al. (1961). They found that hypothalamic extracts from both rats and sheep can induce release of LH from the pituitary. This extract was active even in the nembutal anesthetized rat. Extracts of cerebral cortex were found to be inactive. McCann and Taleisnik (1961) found that the plasma LH activity of estrogenized-ovariectomized rats could be elevated in 10 minutes by an intravenous infusion of acid extracts of rat median eminence.

Direct intrapituitary infusions of median eminence extracts were studied in the rabbit by Campbell et al. (1961). A significant increase in the rate of ovulation was found compared to the control group infused only with the extracting solution. In similar infusion experiments in pentobarbital blocked rats, Nikitovitch-Winer (1962) found both bovine and rat extracts to be active in very minute amounts. Extracts of cerebral cortex tissue were inactive.

The effectiveness of hypothalamic extracts was demonstrated in estrogen-progesterone blocked rats by McCann (1962a), but these were found to be ineffective in ovariectomized rats since the plasma levels of LH were already very high. It is also possible, however, that the indirect assay for LRF used by McCann (1962a) was not sensitive enough to detect small increases in LRF activity. One must remember that hypothalamic extracts infused intraveneously are diluted considerably by the plasma before reaching the pituitary. The active substance in these extracts was found to be relatively heat stable, only partially inactivated by pepsin, but completely inactivated by trypsin digestion. Ramirez and McCann (1963) found LH releasing activity in median eminence extracts of both mature and immature rats. Gellert et al. (1964) found precocious vaginal opening in young female rats following injections of extracts of steer median eminence and pars tuberalis, indicating that this neurohumor plays an important role in the process of sexual maturation. Similar results were observed in the immature male rat by Johnson (1965).

Partial purification of hypothalamic extracts by sephadex filtration was attempted by Guillemin et al. (1963). The LH releasing activity was found in the polypeptide region, preceding arginine vasopressin. Further and extensive studies on the purification of LRF were reported by Schally and Bowers (1964). They found LRF activity in

extracts of sheep and beef hypothalami that were subjected to purification through sephadex columns and chromotography. LH release was demonstrated by *in vivo* and *in vitro* techniques. Further studies by Schally and Bowers proved LRF to be chemically different from vasopressin and to have a molecular weight of 1200-1600. The probable number of amino acid residues were also determined with the molar ratios of amino acids.

III. Site of Feedback Action of Gonadal Steroids

A. Estrogen

More than thirty years ago, Evans and Simpson (1929) showed that transplanted pituitaries from adult female rats had the ability to stimulate the gonads of immature rats. The amount of stimulation increased if the donor animals were ovariectomized for various periods of time prior to transplantation. This indicated that ovariectomy removed some inhibitory influence on gonadotropin synthesis.

By somewhat different methods Biddulph et al. (1940) showed the influence of ovarian hormones on gonadotropin release. Using parabiotic rats, they observed that estradiol, estriol and progesterone all had the ability to prevent the increased release of gonadotropins after castration. Estradiol was found to be the most potent. After 25 years, these results are still valid. Meyer et al. (1946) went one step further in their parabiotic studies, and showed that not only were the ovaries of the parabiotic partner

stimulated intensively by castration, but pituitary gonadotropin activity was reduced.

These early studies led to the extensive studies of Greep and Jones (1950). By a comprehensive study on parabiotic animals, they clearly demonstrated the negative feedback action of steroids on pituitary gonadotropin release. Their studies raised the question of whether this feedback was directly on the pituitary or possibly on some hypothalamic control mechanism. The same year studies by Desclin (1950) indicated that estrogen can inhibit the pituitary directly. Desclin did not, however, rule out the possibilities of an indirect feedback mechanism.

Interesting studies on parabiotic animals were conducted by Miyake (1961). These studies showed that the gonadotropic potency of the male is larger in both the intact and the castrate animal. These studies also revealed, that the amount of steroid needed to inhibit post-castration increases in gonadotropin release was greater in the male than in the female. The LH content of immature males and females was found to be about the same, both being low (Hoogstra and Paesi, 1955), indicating that in the immature animals the feedback mechanisms in the two sexes, if at all existent, do not differ. In the adult, the LH content increases, being higher in the male than in the female.

The increase in pituitary FSH after ovariectomy has been found to be greater than after orchidectomy (Paesi et al.,

1955). Estrogen has been found to be without effect in the intact female rat, but is an effective block for FSH synthesis in the castrate. Similar results have been observed on the serum LH content of adult castrates, with the exception that the content in the castrate male was higher than in the female (Gans, 1959). Estradiol and testosterone were equally effective in depressing serum LH in the castrate male rat, but estradiol was more effective in the castrate female. The serum levels in intact animals could not be depressed by steroids.

The development of the ovarian ascorbic acid depletion assay by Parlow (1961) provided a much more sensitive and specific bioassay for LH than previously used methods. Using the Parlow method, McCann and Taleisnik (1961a) reported detectable amounts of LH in the plasma of castrate female rats. A single injection of estradiol inhibited LH release in the ovariectomized rat in one to three days, depending on the dose administered. Estradiol was also shown to depress pituitary LH content after ovariectomy, clearly indicating that estrogen acted on the synthesis of LH, since both plasma and pituitary LH contents decreased.

These results were essentially confirmed by Hallbaum et al. (1961). Post-ovariectomy increases in both FSH and LH were found in the pituitary and plasma. Administration of estradiol for 45 days depressed both FSH and LH content. Hellbaum's studies also included histological evaluations

of the pituitary. These showed an increase in gonadotrophe cells after ovariectomy and a decrease in response to estrogen treatment. These results are especially important since they show simultaneous changes in the LH content of the pituitary and the plasma, coupled with changes in the pituitary histology. However, they still failed to differentiate between direct and indirect feedback mechanisms. More extensive studies by Ramirez and McCann (1963) revealed that castration can increase release of LH from the pituitary of females regardless of sexual maturity, but will not do so in the immature male. This indicates a basic difference in the LH releasing mechanism of the male and the female.

One must always be careful in extrapolating from one species to the other. This was shown by Parlow (1964). He found that FSH content of the pituitary increases in the mouse after ovariectomy but the LH content remains unchanged. This is in sharp contrast to the rat. A definitive answer to the effects of estrogen on pituitary LH content after castration can be found in the comprehensive work of Parlow (1964a). He studied the effects of estrogen in ovariectomized rats over a wide range of doses and found a progressive depression in pituitary LH content with increasing doses of estradiol benzoate. Again, the question regarding site of inhibition remained unanswered. Depression of pituitary LH stores below normal levels was shown by Saunders (1964) with a wide variety of estrogens.

In vivo studies did little to clear up the controversies regarding the site of action of estrogen. Most of these studies were conducted by implanting estrogen pellets into various sites of the hypothalamus or pituitary. This method provides very high local concentrations but poor distribution of the implanted steroid. Initial indications regarding the actual existence of a hypothalamic center controlling LH release were reported by Flerko (1957). He found that a daily dose of 1 ug of estradiol could completely prevent corpus luteum formation in the rat. If, however, treated animals were subjected to a hypothalamic lesion between 45 and 49 days of life, the inhibiting effects of estrogen were completely abolished. This gave a good indication of the existence of an estrogen sensitive center at the site of the lesions. Such lesions were found to be most effective when located just below the paraventrioular nucleus.

Implantation of ovarian grafts into the hypothalamus produced reduction of uterine weights in the host animals (Flerko and Szentagothai, 1957). The most sensitive portion of the hypothalamus was found to be the region of the paraventricular nucleus, but the implants in the mammillary region also gave some reduction in uterine weights. Intrapituitary grafts of ovarian tissue were without any effect. Placement of lesions and implantation of estrogen into a large number of hypthalamic sites were studied by Lisk (1960).

Destruction of the arcuate nucleus was found to result in complete gonadal atrophy in both the male and female. Implantation of estrogen produced atrophy when located in the arcuate nucleus or the mammillary body. The studies of Lisk (1960) and Flerko (1957) indicate the uncertainty regarding the exact location of the estrogen sensitive centers in the hypothalamus.

Subsequent studies by Flerko and Bardos (1961) showed a good reciprocal relationship between the degree of luteinization and the amount of functional ovarian tissue in lesioned rats exhibiting constant estrus. This showed that the degree of LH release is inversely proportional to the amount of circulating estrogen. Simultaneous studies on gonadal atrophy and hypothalamic histology were conducted by Lisk and Newlon (1963). For the first time it was shown that hypothalamic implants of estrogen cause cytological changes in the hypothalamic nuclei. Decreased nucleolar size in the arcuate nucleus was always accompanied by gonadal atrophy.

Implants of estradiol into the mammillary complex were shown to be highly effective in preventing compensatory hypertrophy in hemicastrate female rats (Littlejohn and de Groot, 1963). These same investigators also found that implants in the anterior and antero-medial amygdala produced increased hypertrophy, indicating that extrahypothalamic sites may also be involved in the feedback mechanism of

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gonadal steroids. Ramirez et al. (1963 and 1964) showed that hypothalamic implants inhibited the post-ovariectomy rise in pituitary LH content. Most of the effective implants were located in the basal tuberal region. However, these studies also indicated that direct pituitary implants inhibited LH release, although this was not as effective as hypothalamic implants.

The results of Ramirez et al. (1963 and 1964) and those of his own investigations prompted Bogdanove (1963) to suggest direct pituitary inhibition by estrogen. His explanation for inhibition of LH release by intra-hypothalamic implants was that these implants merely released the steroid into the portal circulation, and were in fact more effective than intra-hypophysial implants since the estrogen was more efficiently distributed to the pituitary tissue than in the case of localized hypophysial implants. He supported his case by histological studies on pituitary implants. These studies showed a localized but distinct reduction in the size and number of castration cells around the implanted estrogen pellet, indicating a definite but localized effect.

In direct contradiction to the above findings are those of Kanematsu and Sawyer (1964), who found inhibition of LH release in the ovariectomized rabbit following estrogen implantation into the hypothalamus, and an increase in plasma LH rather than a decrease following direct hypophysial implantation. Their observations are not the only ones found

in the literature which indicate that estrogen can be stimulatory to LH release under some conditions. Bradbury (1947) studied the effects of estrogen in immature rats. He found that administration of estrogen was followed by increases in ovarian and pituitary weight. At the same time he found a decrease in the pituitary gonadotropin content. This confirmed the early observations of Hohlweg (1932) who showed that large doses of estrogen could induce ovulation.

Sawyer et al. (1949a) also reported evidence that the stimulatory action of estrogen occurs at the CNS level. When they injected estrogen into the pregnant rat they obtained ovulation within 48 hours. They obtained similar results with LH injections. Administration of dibenamine or atropine blocked the effect of estrogen but not that of LH. Their results did not, however, rule out the possibility of direct action at the pituitary level. Further evidence for the action of estrogen at the CNS level came from the studies of Presl (1961). Using hitochemical techniques, he showed the ability of estrogen to induce cholesterolization of the ovaries 48 hours after injection. These techniques, however, lacked adequate quantitation. Phenobarbitol injections could block ovulation up to 32 hours after estrogen administration.

The numerous evidence for the ovulation-inducing effect of estrogen was reviewed by Everett (1961b). Results obtained by Gans and van Rees (1962) complicate the matter.

Their data suggest that estrogen has a differential threshold for the stimulation of the synthesis and release of LH. However they found stimulation at lower doses and inhibition at high doses.

In a review on the subject of estrogen action on LH release, McCann (1963 and 1964) concluded that estrogen can act on the hypothalamus to inhibit LH release. He does not rule out the possibility of direct pituitary action under some conditions. The studies of Ramirez and Sawyer (1965a) indicate that variations in the hypothalamic LRF content occur during the estrous cycle. They found LRF activity at its lowest level in late proestrus and early estrus. These results complement the work of Ramirez and McCann (1964) on the plasma content of LH during different phases of the cycle. They found the highest peak of plasma LH activity during the afternoon of the day of proestrus.

Evidence for the direct action of estrogen on the hypothalamus comes from the studies of Michael (1962 and 1962a) and of Attramadal (1964). Both found an affinity of certain hypothalamic neurons for estrogen. An accumulation of labeled steroid could be found after systemic injection and direct hypothalamic implantation.

These radioisotopic studies are supported by the electrophysiological studies of Sawyer and Kawakami (1961). They found that certain steroids block ovulation by elevating the EEG afteraction threshold after coitus in the rabbit.

It is hard to imagine how this would affect a spontaneous ovulator such as the rat. One of the latest suggestions regarding the control of LH release comes from the work of Szontagh and Uhlarik (1964). Administering PMS to ovariectomized rats, they found a reduction in pituitary LH content. Based on their results they suggested a theory of internal feedback, in which the pituitary gonadotrophes feed back on the hypothalamus to inhibit the release of their respective releasing factors. A thorough and well organized review of the actions of steroids on the control of LH release was recently written by Bogdanove (1964a).

B. Testosterone

The studies on the function of testosterone in the male are far less numerous than those on estrogen in the female. The control of LH release in the male appears to be clearer than in the female, but this is not true for the release of FSH. Early studies by Biddulph et al. (1940) established that in the male castration causes a rise in gonadotropin secretion as indicated by gonadal hypertrophy of the parabiotic partner. Administration of testosterone can prevent such hypertrophy in both the male and the female. Testosterone was found to be less potent than estrogen in this respect. Hellbaum and Greep (1943) studied the pituitary and serum FSH and LH content in both intact and castrate rats. They found that the normal male pituitary contains primarily FSH while the serum contains a higher LH

than FSH activity. Castration was found to increase both the FSH and the LH content of the pituitary. At the serum level, castration was found to decrease LH activity but not to affect FSH activity significantly. Administration of testosterone propionate decreased the pituitary LH content in the castrate animal and caused an increase in the serum LH activity. No significant changes in pituitary FSH stores could be detected but the serum FSH decreased. These results tended to indicate that LH release from the pituitary is dependent on normal testicular function or testosterone administration. On the other hand, the secretion of FSH appears to be inhibited by the presence of testosterone.

In a somewhat later study, Greep and Jones (1950) found inhibition of gonadotropin release following testosterone administration, but they did not find testosterone to inhibit gonadotropin synthesis. Wijnans (1953) found compensatory hypertrophy in hemicastrate male rats, but attributed it to a reflex function through direct innervation of the testes, since injection of procaine into the scrotum abolished this compensatory response.

In contrast to the findings on LH, testosterone was found to increase the pituitary FSH content in both normal and castrate male and female rats (Hoogstra and Paesi, 1957). Estradiol did not interfere with the action of testosterone but decreased FSH when given alone. Using the ventral prostrate weight assay for LH, Gans (1959) found small amounts

of LH in the plasma of both intact adult males and females. Castration increased the plasma level of LH in both sexes, the male rat having a higher content than the female. In ovariectomized females 2 μg estradiol was found to be more effective in depressing plasma LH than 100 μg of testosterone. In the intact male rat, both steroids were found to be ineffective in depressing the plasma levels.

Davidson et al. (1960a) found a 16-fold increase in FSH content and a 2-fold increase in LH content of the pituitaries of castrate dogs. All of these investigations indicate that at least systemic testosterone treatment is inhibitory to LH release and probably also to its synthesis. The results on FSH release are somewhat confusing and sometimes contradictory.

A limited amount of information is available on the implantation of testosterone into the hypothalamus. Davidson and Sawyer (1961) implanted small amounts of testosterone into the posterior median eminence and found testicular and prostatic atrophy. Lisk (1962) tried implantation of testosterone in both the male and the female rat. It was followed by seminal vesicle and ovarian atrophy respectively. These results suggest that the inhibitory effect of testosterone on LH release is mediated through the hypothalamus. Simultaneous studies on pituitary gonadotropin content and pituitary histology show good correlation (Hellbaum et al., 1961). Castration in both sexes resulted

in an increase in the pituitary FSH and LH contents and the frequency of red and purple gonadotropic cells. Administration of testosterone to such animals reduced the pituitary LH content and the frequency of red gonadotrophes. The FSH content remained unchanged and no reduction of purple gonadotrophes occurred.

Parlow (1964b) demonstrated a considerable increase in the LH content of the orchidectomized rat. Contrary to previous findings, however, he found no increase in the pituitary FSH content after castration. His work pointed out the importance of specific quantitative bioassays in evaluating pituitary changes. Gay et al. (1964) found no significant change in the pituitary LH content of castrate male rats when treated with testosterone for only 7 days. They did, however, obtain a significant drop in the serum LH, indicating that an inhibition of LH release is not necessarily accompanied by a reduction in the pituitary content. Bogdanove (1964) confirmed these results, when he showed that the pituitary LH content decreased only after a three-week treatment period. Plasma LH on the other hand decreased after only one week of treatment.

Perhaps one of the most important roles of testosterone is played very early in life. This involves the sexual differentiation of the hypothalamus in the first five days of the post-parturition period. The early work of Pfeiffer (1936) demonstrated that the presence or absence of

functional testicular tissue determined the future of a bipotential pituitary gland. Transplantation of testicular tissue to the newborn female had a total masculinizing effect on the pattern of gonadotropin release. Transplantation of ovarian tissue to the post-partum male did not influence the future pattern of gonadotropin release. Castration of the male at this early age, however, did prevent the development of the male type tonic discharge of gonadotropins in adult life.

Later studies by Harris and Levine (1962) indicated that Pfeiffer's (1936) studies were correct in regard to the necessity for functional testicular tissue, but differentiation occurred at the hypothalamic level and not at the pituitary. If the pituitaries from androgen sterilized females were transplanted to normal females, they resumed a cyclic pattern of gonadotropin release. Similarly, if the pituitary of castrate male was transplanted to a normal male, the gonadotropin release was of the normal male pattern. More detailed studied by Gorski and Barraclough (1962) showed that the pattern of FSH secretion in androgen sterilized females is essentially comparable to the normal female. Hemicastration in such animals also resulted in compensatory hypertrophy which is similar to the normal female. Hypothalamic lesions curtailed FSH release. Since such animals exhibit constant estrus, it can be assumed that the abnormality occurs in the LH releasing mechanism.

Further convincing evidence that the action of testosterone is on the hypothalamus rather than the pituitary comes from the studies of Johnson (1963). Administration of extracts of sheep hypothalamus to androgen sterilized females caused ovulation. This indicates that the pituitary of such a masculinized female is capable of responding to the stimulus of the normal hypothalamus. The differentiation of the hypothalamus in response to the presence of androgenic hormones lends support to the view that testosterone probably acts on the hypothalamus rather than on the pituitary in regulating LH release from the pituitary. The literature is void of any attempt to determine changes in hypothalamic LRF activity following testosterone administration.

C. Progesterone

Early observations by Everett (1940) showed that in the persistent estrous rat, progesterone administration could induce ovulation and corpus luteum formation. Withdrawal of the hormone caused a resumption of continuous estrus. In the same work, Everett (1940) also found that progesterone could prevent the effects of constant light in young cyclic rats. Mardones et al. (1951) showed that progesterone could prevent ovulation in intrasplenic ovarian autografts and that the quantity necessary to accomplish this was less than that produced in the ovary during the luteal phase. These two papers seem to contradict each other. An explanation can perhaps be found in the work of

Sawyer and Everett (1959). These workers found that in the rabbit, progesterone first facilitated the induction of ovulation by electrical stimulation of the hypothalamus. Such facilitation lasted from one to four hours after progesterone administration. After a longer time interval of 24 hours, progesterone became highly inhibitory to induced ovulation. Confirmation of this work came from Kawakami and Sawyer (1959) who found that in the estrogen-primed rabbit, progesterone first lowered and then elevated the EEG arousal threshold and the after reaction threshold after coitus.

Study of changes in the pituitary gonadotropin content after progesterone administration by van Rees (1959) showed that one mg of progesterone was ineffective in reducing pituitary ICSH content in the male and the female rat. This was true in both the intact and the gonadectomized animal. They found, however, that this dose was enough to reduce the plasma LH of the gonadectomized animal. Somewhat contrary to these results, Beyer and Potts (1962) found that in the male rat, progesterone counteracted the depressing effects of estrogen on pituitary LH secretion.

Gorski and Barraclough (1962a) reported that the pituitary LH content of the persistent estrous rat is only about 1/3 of that of a normal proestrous animal. Progesterone priming in such a persistent estrous animal caused a 75% increase in the pituitary LH content, and ovulation was induced by hypothalamic stimulation.

McCann (1962a) reported interesting results on the effects of progesterone on release of LH from the hypophysis. He found that progesterone alone was incapable of reducing plasma LH after ovariectomy except when given in large pharmacological doses (25 mg/day). If, however, the animals were primed with estrogen, physiological doses of progesterone blocked LH release completely. In later work, Ramirez and McCann (1963a) reported that in an estrogen-progesterone blocked animal, hypothalamic extracts were capable of releasing LH from the pituitary. This indicated that the estrogen-progesterone block occurred at the hypothalamic level.

Saunders (1964) indicated that some progestational compounds reduced pituitary gonadotropins only in doses which were much higher than those required to prevent fertility. This would leave their actions partially unexplained, although pituitary content is not always a good indicator of the rate of release from the pituitary. Littlejohn and de Groot (1963) found progesterone implants ineffective in the anterior hypothalamus. In general, the results reported on the effects of progesterone on LH release are far from clear and are much less voluminous than those on estrogen.

IV. External Environmental Factors

A. Nutrition

Marrian and Parkes (1929) demonstrated that continued reduced dietary intake can result in ovarian atrophy in

the rat. They showed, furthermore, that anterior pituitary preparations can reactivate the ovaries of such animals. Jackson (1932) found little effect of starvation in the male rat. While the loss of bodyweight was extensive, the testes remained relatively unchanged. Further confirmation of the effects of reduced caloric intake on the female reproductive system came from Mulinos and Pomerantz (1940). They found an absence of maturing, graffian follicles and an increase in the number of primary follicles. Mulinos and Pomerantz (1941) also found an increase in the weight of the reproductive organs with administration of gonadotrophins. These results indicate that the effect of reduced food intake is not a direct one on the gonads, but is the result of a reduction in gonadotropic stimulation. The work of Meites and Reed (1949) and others, however, indicates that this effect is probably not on pituitary gonadotropin synthesis, since the gonadotropin content of underfed rats remains the same or may even increase if related to a pituitary weight basis. This indicates that some failure occurs in the mechanism normally controlling gonadotropin release.

Srebnik and Nelson (1958) fed protein deficient diets and noted a "pseudohypophysectomy" condition in rats. They found pituitary gonadotropins to be effective in the same doses as in the surgically hypophysectomized rat. Pregnancy gonadotrophins were somewhat more potent.

Absence of dietary protein was shown to result in a slight increase in the FSH content of the female pituitary but in no change in LH content (Srebnik et al., 1961). Ovariectomy resulted in an elevation of both, showing that despite the protein deficiency, the pituitary is still capable of responding to a stimulus. In the male rat, a protein deficient diet was shown to result in marked accessory atrophy but only in limited reduction in testicular weight (Srebnik and Nelson, 1962). In the adult male there is a moderate decrease in the pituitary FSH and LH content, while in the young male, the decrease is considerable. Extensive reviews on the effects of nutrition on hormone release have been written by Leathem (1959 and 1961). There is some reason to believe that the effects of nutritional deficiencies are exerted at the hypothalamic level, but little direct evidence is yet available on this subject. Recently Meites and Fiel (1965) reported that starvation in rats reduced hypothalamic content of growth hormone releasing factor.

B. Light

The great amount of work on the effects of visible radiation on gonadotropic function indicates that light is one of the most potent influences regulating reproductive function and behaviour in many species. In fact, it appears to be the most potent external environmental factor in some species (Critchlow, 1963). The early work of Bissonette and Bailey (1936) showed that ferrets can be made to breed

"off season" if they are exposed to increased amounts of light per day. Hemmingsen and Krarup (1937) studied the effects of light on the estrous cycle of the rat. They demonstrated a complete reversal of the cyclic pattern as well as in the behavioural pattern if the hours of light and dark were reversed. In a more detailed study, Fiske (1939) obtained precocious maturation in the rat when the immature animal was exposed to continuous illumination. Correspondingly, a complete absence of light proved to be a delaying influence on sexual maturation. Her studies indicated, furthermore, that the pituitaries from light-exposed animals contained primarily follicle stimulating activity, while those from animals in continuous darkness contained increased amounts of ICSH activity as indicated by stimulation of seminal vesicle development. Species differences in response to light were demonstrated by Chase (1941) in an anophthalmic strain of mice. He showed that congenitally blind mice developed their first estrus at an earlier age than for normal mice.

More detailed work by Fiske (1941) in the rat showed that the estrous phase is extended by continuous illumination. In continuous darkeness, the metestrous phase seems to be the most extended one. Through augmentation tests with HCG and FSH, she demonstrated that under continuous illumination, secretion of FSH is favored, while in continuous darkness, LH is secreted in larger amounts.

Maqsood and Parsons (1954) found no effect in the rabbit from continuous lighting, again indicating the importance of species differences. In a reivew of the effects of visible radiation, Hammond (1954) presented evidence for both initial stimulatory effects and a subsequent depressing influence of light.

In the avian class, light appears to be an even more important environmental factor than in mammals. Bissonnette and Wadlund (1932) showed the importance of wave length, duration, intensity, and the rate of change of duration in light on the testis of the starling Sturnus vulgaris.

Perhaps the most extensive work on the relation of light to gonadotropin function in the bird was reported by Benoit and Assenmacher (1959). Not only did they demonstrate the necessity of proper illumination for normal testicular development and function, but they also found that this effect is a direct one on the hypothalamic gonadotropin releasing mechanism. When the special gonadotropic zone of the hypothalamic area was severed from the portal circulation, the photosexual response was completely abolished. Their studies clearly indicated that the mediation of the response is a neurohumoral one. Benoit (1962) presented further evidence for the control of gonadotropic function in the bird by light. Sectioning of the hypothalamo-hypophysial stalk completely abolished the photosexual response. The inductive effects of light in the bird seem

to be much stronger than in other vertebrates. Direct evidence for hypothalamic involvement also comes from the works of Hirano et al. (1962), who found an increase in the amount of neurosecretory material in the median eminence following increased illumination.

Decreased testicular and prostatic weights were found in the male rat kept in permanent darkness. This was also accompanied by reduced spermatogenesis (Itoh et al., 1962).

Histological and histochemical studies played an important role in defining the mechanism of action of visible radiation in stimulating gonadotropic function. Fiske and Greep (1959) studied the histochemical changes in various parts of the hypothalamus during various lighting conditions. They found that the amount of Gomori positive material was most abundant during continuous illumination and scarcest in continuous darkness. It appeared to be concentrated in the cells of the hypothalamo-hypophysial tract. The paraventricular nucleus appeared to be unaffected.

Further studies by Flament-Durand and Desclin (1960), indicated that the nucleoli of the neurons of the supraoptic tract swell in response to permanent illumination, while those of the paraventricular neurons remain unchanged. These results suggest possible involvement of the supraoptic nucleus. Ortavant et al. (1964) presented evidence for a photoperiodic response in domestic animals. They demonstrated significant differences between species, and

showed that in some species there are optimal as well as refractory periods of illumination.

Extremely interesting work by Ganong et al. (1963) showed that light can penetrate the hypothalamus directly since external light could be detected by micro-photocells implanted into the hypothalamus. This would indicate that the retina may not be an essential part of the "photosexual" response.

An interesting interpretation of the photoperiodic responses involves the pineal gland. Kitay and Altschule (1954) reviewed the early literature on the role of the pineal in sexual maturation. Perhaps the most conclusive evidence came from the clinical observations on children with precocious sexual development, many of whom were observed to have pineal tumors. More basic evidence for the involvement of the pineal in sexual maturation comes from the studies of Reiss et al. (1963), who found that in the immature rat, aqueous extracts of pineal stimulated the gonads, while in the adult such extracts proved to be inhibitory.

Wurtman et al. (1963) studied the effects of melatonin (a product of the pineal) on the estrous cycle of the rat. It was found that microgram quantities of this compound could decrease the incidence of estrus in adult cycling rats. It was also found that melatonin counteracted the stimulatory effects of constant light. Chu et al. (1964) showed that none of the precursors of melatonin demonstrated

such a depressing action during the estrous cycle. Wurtman et al. (1964) found that exposure of the rat to continuous illumination caused a decrease in melatonin synthesis in the pineal, and at the same time stimulated the gonads and the uterus. Removal of the eyes and the superior cervical ganglia abolished both of these effects of light.

A number of other external environmental factors have also been shown to influence the release of gonadotropins. Wells and Zalesky (1940) found that a constant environmental temperature of $4^{\circ}C$ transforms the testes of the ground squirrel to a condition resembling that in the breeding season. At the same time pituitary stores of gonadotropins remain high. Browman (1943) found that in the absence of cyclic illumination, fluctuations in the environmental temperature can still influence the rhythmicity of the estrous cycle.

Audiogenic stimuli have been shown to accelerate the development of the gonads of the immature rabbit and to increase the size of the ovaries in the adult (Zondek and Tamari, 1960). It was also demonstrated by the same workers, that strong audiogenic stimuli will interfere with the establishment of pregnancy. Further studies by Zondek and Tamari (1964) suggest that such stimuli can induce infertility and may even terminate existing pregnancies. This was found in both the rat and the rabbit.

Somewhat similar results were shown with odoriferous stimuli by Parkes and Bruce (1961). They demonstrated that presence of the odor of strange male mice will prevent the establishment of pregnancy or pseudopregnancy in the female. It appeared that such a stimulus interfered with the development of corpora lutea, probably by interfering with the release of prolactin. It was also shown, however, that release of large amounts of FSH occurs. It is apparent from the literature that external environmental factors can profoundly influence the secretion of pituitary gonadotropins, but the mechanisms of mediation remain to be established.

MATERIALS AND METHODS

I. Animals

A. Experimental Animals

All rats used were of the Sprague-Dawley strain obtained from the following sources: Spartan Research Animals, Inc. (Haslett, Michigan), and Holtzman Company (Madison, Wisconsin). Hypophysectomized female and male rats averaged 200 and 300 g, respectively, at the beginning of each experiment. Hypophysectomized rats were obtained from Hormone Assay Labs., Chicago, Illinois.

Female rats were housed 10 animals to a cage, while males were placed eight to a cage. Hypophysectomized rats were housed four to a cage. All animals with the exception of hypophysectomized rats and those on nutritional experiments were maintained on a diet of Wayne Lab Blox (Allied Mills, Chicago, Illinois) and tap water *ad libitum*. The diet of hypophysectomized rats was supplemented with whole wheat bread, fresh oranges, carrots, and sugar tablets.

The lighting schedule consisted of 14 hours of light and 10 hours of darkness (7:00 a.m. to 9:00 p.m.). Exceptions in lighting schedule will be described. The temperature of the animal room was maintained at 25±1.5°C. Hypophysectomized rats were kept at 27°C.

B. Animals for Bioassay

Animals for LH and FSH bioassays were of the Sprague-Dawley strain obtained from the following sources: Spartan Research Animals (Haslett, Michigan), Holtzman Company (Madison, Wisconsin) and Sprague-Dawley, Inc. (Madison, Wisconsin). Animals used for the bioassay of FSH were delivered at 21 days of age and used at 22 days. Rats used for LH assays were delivered at the age of 23 days and used at 25 days. Assay animals were maintained on the same diet as experimental animals, with occasional supplements of fresh oranges. Assay rats were housed 10 to a cage and exposed to the same lighting schedule and room temperatures as all other animals.

Animals used for LH assays received 50 IU of pregnant mare serum (Upjohn Company, Kalamazoo, Michigan and Ayerst Labs., Inc., New York) at 25 days of age, and 25 IU of human chorionic gonadotropin (Nutritional Biochemical Company, Cleveland, Ohio) 60±4 hours later. They were used for assay on the seventh day after the HCG injection.

II. Incubation Techniques

A. Preparation of Acid Extracts of Hypothalamus

Animals serving as hypothalamic donors were killed by decapitation. The hypothalami were removed immediately, placed in cold 0.1 N HCl, quickly frozen and stored at -30°C. On the day of incubation the hypothalami were thawed, homogenized in a ground glass homogenizer with cold 0.1 N HCl,

boiled for five minutes and centrifuged at 30,000 g for 45 minutes at 4°C. Immediately after use, the extracts were adjusted to pH of 7.2 by adding a drop at a time of 5.6% $NaHCO_3$ and testing with a glass electrode. The extract was then diluted to the desired volume and made isotonic with synthetic medium 199 (10X, Difco Lab., Detroit, Michigan).

B. Preparation of Pituitaries for Incubation (LRF assay)

Adult male rats served as pituitary donors and were killed by decapitation. The anterior pituitaries were removed and placed into one ml of synthetic medium 199 (Difco Lab., Detroit, Michigan) in 25 ml Erlenmeyer flasks. Each anterior pituitary was hemisected, one-half being added to the control flask and the other half to the experimental flask. A total of two glands were added to each flask and placed in a Dubnoff shaker pre-heated to 37°C. One ml of the neutralized hypothalamic extract was then added to each flask, so that the final volume in each flask was 2.0 ml. The pituitaries were then incubated for one hour in an atmosphere of 95% O_2-5% CO_2.

C. Preparation of Medium 199

Synthetic medium 199 (10X, Difco Labs., Detroit, Michigan), was used as a stock solution. The stock solution was used in a proportion of 10 ml per 100 ml of medium made. The stock solution was drawn through a sterilized needle and placed into a 100 ml volumetric flask. Six ml of 5.6%

$NaHCO_3$ were added and the solution was diluted to volume with triple glass distilled water. The pH of the fresh medium was 7.4 as measured by a glass electrode on a Beckmann Zeromatic pH meter. The prepared medium was pre-heated to 37°C before use.

D. Collection and Storage of Incubation Medium

After the one hour incubation period, the medium was collected, centrifuged at 2,300 g and stored for bioassay. If the storage period was longer than one week, the frozen medium was lyophilized and stored at -30°C. For storage periods of less than one week, the medium was frozen and stored at -30°C. The incubated pituitaries were weighed, frozen and stored at the same temperature as the medium.

III. Bioassays

A. Follicle Stimulating Hormone (FSH)

Pituitary FSH content was assayed by the method of Steelman and Pohley (1953) as modified by Parlow and Reichert (1963). Pituitaries were ground in a glass homogenizer and extracted with physiological saline solution. The solutions were centrifuged and stored at 4°C during the assay.

B. Luteinizing Hormone (LH)

LH assays were done by the ovarian ascorbic acid depletion method of Parlow (1961). This method was used for both the incubation media and pituitary extracts. All materials were assayed at two dilutions with a four-fold

difference in concentration between them. The media were assayed in doses equivalent to 2.5 and 10.0 mg of incubated pituitary tissue, respectively. Pituitary LH content was assayed by injecting 1.0 and 4.0 mg of pituitary tissue, respectively.

C. Reference Standards

The reference standard hormone used for FSH assays was NIH-FSH-S2. The standard doses used were 100 and 200 ug, respectively. The reference standard used for LH assays was NIH-LH-S5. These two hormones were supplied by the Endocrinology Study Section, National Institutes of Health. The standard LH doses used were 5 and 20 ug. Standards used for the bioassay of pituitary extracts were dissolved in physiological saline solution. Standards used for the bioassay of incubation media were dissolved in medium 199.

IV. Histological Preparations

Tissues were fixed in Bouin's solution, sectioned at 4-6 microns for routine examination, stained in eosin-hematoxylin solution and examined microscopically. Mammary tissues were fixed in Bouin's solution, stained in Mayer's hematoxylin, and examined as whole mounts.

V. Statistical Analyses

Statistical comparisons were made according to the Student's "t" test (Steel and Torrie, 1960). Bioassays were analyzed according to Bliss (1952). All comparisons were made with a two-sided 't' test.

EXPERIMENTAL

I. PRELIMINARY EXPERIMENTS ON INCUBATION PROCEDURES

A. Bioassay Experiments

1. Comparison of two methods of assay for LH by ovarian ascorbic acid depletion

The most sensitive method for the assay of luteinizing hormone at the present time is that developed by Parlow (1961). This method is based on the ability of LH to deplete the ascorbic acid content of the ovary. This property is unique to LH and is not shared by any of the other anterior pituitary hormones. Two modifications of this assay have been employed by various investigators. Experiments were designed to determine the more suitable of the two methods for studying the concentrations of LH in pituitary tissue and in incubation media.

2. Comparison of ascorbic acid content of left and right ovaries

Ten immature female rats, previously treated with PMS and HCG, were examined for possible differences in ascorbic acid content between the left and right ovaries. The results in Table 1 indicate that if both ovaries are removed at the same time, no significant differences can be detected in the ascorbic acid content of the two ovaries, provided they are expressed on a mg % basis.

Table 1. Comparison of Ascorbic Acid Content of Left and Right Ovaries

No. of Animal	Side of Animal (L or R)	Ovarian Weight in mg	mg % Ascorbic Acid
1	R	77	68.0
	L	82	67.5
2	R	87	65.0
	L	80	70.2
3	R	131	76.1
	L	135	74.2
4	R	123	74.7
	L	127	78.0
5	R	102	73.6
	L	94	65.6
6	R	98	67.0
	L	106	65.6
7	R	72	73.6
	L	83	70.1
8	R	74	68.1
	L	83	62.2
9	R	116	69.3
	L	90	65.7
10	R	87	86.0
	L	94	80.8

Mean Ovarian Weight: 97.4±6.1
96.7±6.6

Mean Ascorbic Acid Content:
Left = 67.0±1.89
Right = 72.1±1.94

3. Dose-response relationships with removal of one ovary

This experiment illustrates the modification of the ascorbic acid depletion method used by several investigators, but is not the original method devised by Parlow (1961). In this method, immature rats previously treated with PMS and HCG, are anesthetized lightly with ether. One ovary is removed and analyzed for ascorbic acid content. Immediately after removal of the ovary, the animal is injected intravenously with the test material. Four hours later, the second ovary is removed and analyzed for ascorbic acid. The difference in the ascorbic acid content is used as an indicator of the presence of LH in the test material. The mean values are compared by the student's 't' test.

The results in Table 2 suggest that this method is not a very accurate indicator of LH. It was found that groups injected only with Ringer's solution also showed a significant depletion in ascorbic acid content after four hours. Furthermore, the response to NIH-LH-S5 was poor. No linear relationship could be found between the ascorbic acid depletion and the logarithm of the dose of standard LH injected.

4. Dose-response relationship without removal of one ovary

This experiment was conducted according to the original description of Parlow. Animals pre-treated with PMS and HCG were lightly anesthetized with ether and injected

Table 2. Dose-response Relationship With and Without Previous Surgery on Rats

Dose of LH in ug	With Previous Surgery mg % Ascorbic Acid		Without Previous Survery mg % Ascorbic Acid	
	Left Ov.	Right Ov.	Left Ov.	Right Ov.
0.0	131.6	120.1	91.7	90.7
1.2	126.2	116.2	74.1	74.2
4.8	129.0	86.4	51.4	51.3
19.2	---	---	38.2	35.8

Number of Assay Animals per Group:

1. With surgery = 10 per dose level.
2. Without surgery = 8 per dose level.

intravenously with the test material. In this case, the response was checked with NIH-LH-S5. The ovaries were removed four hours later and analyzed for ascorbic acid. Table 2 shows the results of the experiment. No significant differences could be detected between the two ovaries. The response to NIH-LH-S5 showed a good linear relationship to the logarithm of the dose of the standard hormone. By this method the potency of unknown materials is estimated by the statistical analysis of Bliss (1952). On the basis of these results it was decided to use the original method of Parlow (1961) for the determination of LH in subsequent experiments. Apparently, previous surgery influences the response of the animals. The original method of Parlow also appeared to be faster and simpler to perform.

5. Standard lines

Table 3 and Figure 1 indicate the variability of the assay animals in their response to the same standard dose of NIH-LH-S5. These results were obtained during a two and one-half year period. It appears that seasonal variations occur in the ascorbic acid content of the assay rats as well as in their response to LH. This is indicated by the great differences in the elevations, slopes, and lambda values of the various standard lines. These results point out the dangers in the use of a standard line which was not obtained at the same time as the results on an unknown sample. It is difficult to explain these seasonal variations under controlled environmental conditions.

Table 3. Variations in the Slope, Mean Ascorbic Acid Content, and Lambda Values of Standard Lines for LH Assay

Year	Month	Slope	Mean Ascorbic Acid Content (mg %)	Lambda
1963	July	-31.9	43.6	0.162
1963	September	-34.2	60.4	0.278
1963	December	-38.5	67.0	0.178
1964	March	-39.2	62.6	0.100
1964	April	-24.7	61.1	0.216
1964	June	-17.6	37.4	0.282
1964	August	-20.6	32.9	0.363
1964	September	-37.3	34.5	0.183
1964	October	-36.9	31.6	0.268
1964	October	-32.3	35.0	0.241
1964	November	-31.7	36.0	0.085
1964	December	-29.3	38.1	0.212
1965	March	-34.1	42.4	0.189
1965	April	-25.2	47.6	0.146
1965	April	-21.1	44.8	0.212
1965	May	-26.0	46.1	0.309
1965	June	-17.6	47.4	0.282
1965	June	-17.5	45.9	0.335
1965	July	-26.0	55.9	0.167
1965	September	-26.4	49.8	0.240
1965	October	-25.2	39.7	0.146
1965	October	-26.3	52.4	0.172
1965	November	-38.8	50.7	0.175

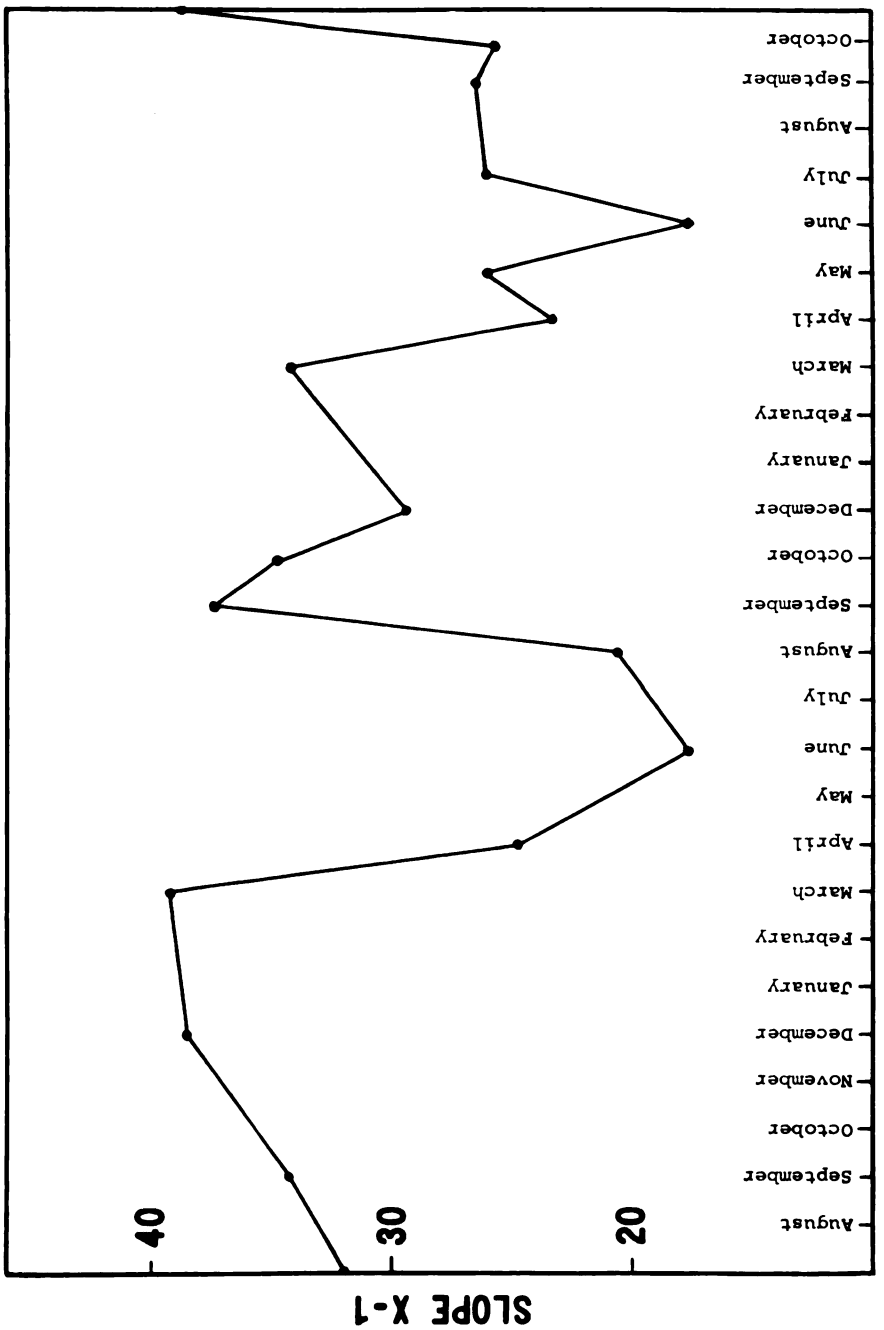

Figure 1. Seasonal changes in the slopes of standard lines.

6. Determination of ability to measure differences in LH content of pituitary tissue

An attempt was made to use the Parlow assay for the determination of LH in crude pituitary extracts rather than in purified preparations. Twenty adult female rats were ovariectomized and twenty adult females served as controls. Three weeks after ovariectomy, the animals were killed and the pituitaries were collected. The glands were homogenized with ice cold saline and appropriate dilutions were made. The material was then assayed for LH. The results are summarized in Table 4. It is apparent that ovariectomy caused a significant increase in the LH content of the pituitaries as was reported earlier (McCann, 1963). These results also indicate that the bioassay is sensitive and precise enough to detect changes in LH content induced by various physiological conditions. The presence of other pituitary hormones in the extract did not influence the slope of the unknown lines. No deviation from parallelism could be found when the unknowns were compared with the standard line through the method of Bliss (1952). Considerable differences in relative potency existed between the two groups of experimental animals. These differences could have been caused by a number of variables. First of all, they could have been inherent in the pituitaries of the donor rats. Secondly they could have been caused by inactivation of the medium due to storage. Finally, the differences in the

Table 4. Pituitary Luteinizing Hormone Content of Intact and Ovariectomized Female Rats

Treatment	Group Number	Relative Potency ug/mg	95% Confidence Limit	Lambda
Intact	1	2.67	1.73-3.03	0.202
	2	0.67	.36-1.25	0.275
Ovariec-tomized	1	7.01	5.14-9.55	0.206
	2	3.52	3.52-6.16	0.260

results could have been caused by variations in the response of two different groups of assay rats. Such differences between assays have been reported by other investigators (Parlow, 1964a).

B. Demonstration of LH Release In Vitro

1. Release of LH by anterior pituitary in relation to incubation time

Hypothalami from adult female rats were extracted and added to the incubation medium with adult male pituitaries. An incubation period of three hours was chosen arbitrarily. Two hypothalamic equivalents per incubated pituitary were added to the medium. The medium was assayed for LH content. The results of the experiment are summarized in Table 5. The data suggest that the three hour period was not an optimum time for the demonstration of a stimulating effect by hypothalamic extract. No significant stimulation of LH release was found when the experimental values were compared with the control data.

On the basis of these results it was decided that two other time intervals should be tried for the in vitro stimulation of LH release by hypothalamic extracts. Subsequently, a shorter and a longer incubation time was attempted. Adult male pituitaries were incubated with three hypothalamic equivalents per gland for periods of one and five hours. The results (Table 6) indicate that the difference between the hypothalamic and cortical extract stimulated systems

Table 5. Effect of Hypothalamic and Cortical Extracts on the Release of LH from Pituitaries in a 3 Hour Incubation Period

Treatment	Group Number	Relative Potency ug/mg	95% Confidence Limit	Lambda
Hypothalamic Extract	1	3.88	3.18-4.74	0.132
	2	1.70	1.00-2.90	0.204
	3	4.94	1.98-12.24	0.286
Cortical Extract	1	3.60	1.54-8.36	0.272
	2	3.80	2.10-6.80	0.216

Table 6. Stimulation of LH Release *in vitro* by Hypothalamic Extract During Periods of 1 and 5 Hours

Duration of Incubation (Hours)	Treatment	Relative Potency ug/ml	95% Confidence Limit	Lambda
1	Cortical Extract	3.24	1.78-5.92	0.180
1	Hypothalamic Extract	67.86	36.68-122.6	0.178
5	Cortical Extract	12.42	9.58-18.10	0.107
5	Hypothalamic Extract	26.80	17.70-40.54	0.140

was the highest at one hour after the beginning of incubation. At five hours, the LH content of the medium containing hypothalamic extract was still high, but the medium containing cortical extract also had a high LH content, which was not significantly different from the medium containing hypothalamic extract when expressed on a per mg tissue equivalent basis.

Apparently, the incubated pituitary showed a slow, tonic release of LH even without hypothalamic extract. This could be due to leakage found only during incubation or to an inherent releasing activity which is augmented by the hypothalamic releasing factor. On the basis of these experiments, it was decided that a one hour incubation period would be used for all subsequent experiments.

2. Inactivation studies

The results in Table 6 suggest the possibility of inactivation of LH in the medium. It is apparent from the results of these incubations, that the medium from the five hour incubation was significantly less potent than from a one hour incubation period. In order to study the possibility of inactivation, NIH-LH-S5 as well as rat pituitary extracts were incubated for various lengths of time. The purified NIH-LH was incubated in a concentration of 40 ug per ml of medium. Rat pituitary extracts were incubated in a concentration of 10 mg pituitary tissue equivalent per ml. The media were incubated for 0, 1, 3 and 5 hours.

The results on NIH-LH-S5 show that the standard hormone at this concentration was highly susceptible to inactivation. Inactivation appeared to be greatest during the first hour of incubation. The LH activity of the rat pituitary extract on the other hand remained constant, except for a slight decrease at five hours. The results are summarized in Table 7. The relative potencies for the three and five hour incubated samples could not be calculated since their slopes were significantly different from the slope of the non-incubated sample, which served as the standard. This change in slope was due to the fact that the more dilute points of these samples were off the sensitive portion of the assay.

3. LH Synthesis during short term incubation

It was also of interest to determine whether hypothalamic extracts stimulated synthesis of LH as well as release. Three hypothalamic equivalents per incubated pituitary were used to study the effects on LH synthesis. Cerebral cortical extract was used in weight equivalent to 3 hypothalami per pituitary. At the end of the 5 hour incubation period, the medium was homogenized with the incubated pituitary tissue and assayed for LH.

No significant differences were found in the combined total LH content of incubated pituitary tissue and incubation medium when the hypothalamic extract treated samples were compared with the samples treated with cortical

Table 7. Inactivation of NIH-LH-S5 and Rat Pituitary LH in Incubation Medium

Type of LH	Duration of Incubation	Slope	Relative Potency ug/ml	95% Confidence Limit	Lambda
NIH-LH-S5 (Ovine)	0 Hours	-37.0	20.00	-	0.277
	1 Hour	-30.3	3.76	0.260-13.42	0.357
	3 Hours	-18.4	-	-	0.303
	5 Hours	- 9.3	-	-	0.915
Rat Pituitary Extract	0 Hours	-37.8	9.00	5.72-14.16	0.190
	1 Hour	-34.1	8.04	4.32-14.92	0.266
	3 Hours	-35.8	9.28	5.12-16.86	0.221
	5 Hours	-37.1	5.42	3.98- 7.32	0.158

extract (Table 8). These results indicated that no detectable synthesis of LH occurred during the five hour period. One can only conclude this about the net synthesis in the system.

C. Dose-Response Relationships

It was necessary to determine whether the releasing activity in the hypothalamic extracts was due to a specific releasing factor or whether this was due to non-specific release caused by possible toxic substances or other agents in the extract. In order to prove the specificity of the release mechanism, it was decided that a dose-response relationship should be demonstrable between the amount of hypothalamic extract in the medium and the amount of LH released by pituitary tissue. This was considered necessary in order to assess variations in the releasing activity of hypothalami from animals under different experimental conditions.

A total of 90 adult female rats served as hypothalamic donors. Adult male pituitaries were incubated with 0.75, 1.50, and 3.00 hypothalamic equivalents per gland. Cerebral cortical tissue, in doses equivalent to the weight of three hypothalami was used as a control. In this experiment the incubated pituitaries were cut into quarters and distributed equally among the four groups. Incubation of the pituitaries with hypothalamic extracts confirmed the belief that the rat hypothalamus contains a luteinizing hormone releasing

Table 8. Total Combined LH Content of Incubated Pituitary Tissue and Incubation Medium

Treatment	Group Number	Relative Potency ug/ml	95% Confidence Limit	Lambda
Cortical Extract	1	34.7	13.3-120.3	0.284
	2	14.1	4.7- 42.4	0.212
Hypothalamic Extract	1	44.2	13.1-256.0	0.335
	2	18.2	6.9- 81.1	0.264

factor (LRF). The results (Table 9) also indicated that this factor shows a definite dose-response relationship. A linear relationship was demonstrated between the amount of LH release and the logarithm of the dose of hypothalamic extract used (Figure 2). Cerebral cortical extract at a level equivalent to the highest dose of hypothalamic extract used, had a lower releasing activity than the lowest dose of hypothalamic extract. This small amount of activity was attributed to non-specific leakage due to cutting of the pituitaries into quarters rather than halves. Such leakage was not observed when pituitaries were cut into halves.

Approximately a three and one-half-fold difference in releasing activity was found between the lowest and highest doses of hypothalamic extract. No significant deviation from parallelism was observed with the various doses of hypothalamic extract. When medium 199 and hypothalamic extract were incubated without pituitary tissue, there was no significant slope. On the basis of the dose-response relationship, it was decided to use 2.0 hypothalamic equivalents per incubated pituitary. Assay of the LH content of the incubated pituitary tissue revealed an inverse relationship to LRF content in the incubation medium (Table 10 and Figure 3). This complemented the results of the bioassay on the LH activity of the medium. Calculation of the amount of LH left in the pituitary explants indicates that

Table 9. Dose-response Relationship Between Hypothalamic LRF Content and Release of Pituitary LH

Treatment	Relative Potency (Equiv. NIH-LH) ug/ml	95% Confidence Limit	Lambda	Mean Ascqrbic Acid Concentration, mg %
0.75 Hypothalamic Equivalents	10.92	2.48-17.92	0.302	58.5
1.50 Hypothalamic Equivalents	24.96	19.10-34.04	0.125	50.3
3.00 Hypothalamic Equivalents	39.30	28.58-66.92	0.162	45.8
Cerebral Cortex	8.54	3.32-10.66	0.247	60.9
Medium 199	-	-	---	93.8
3 Hypothalamic Equivalents Incubated without Pituitary	-	-	---	85.5

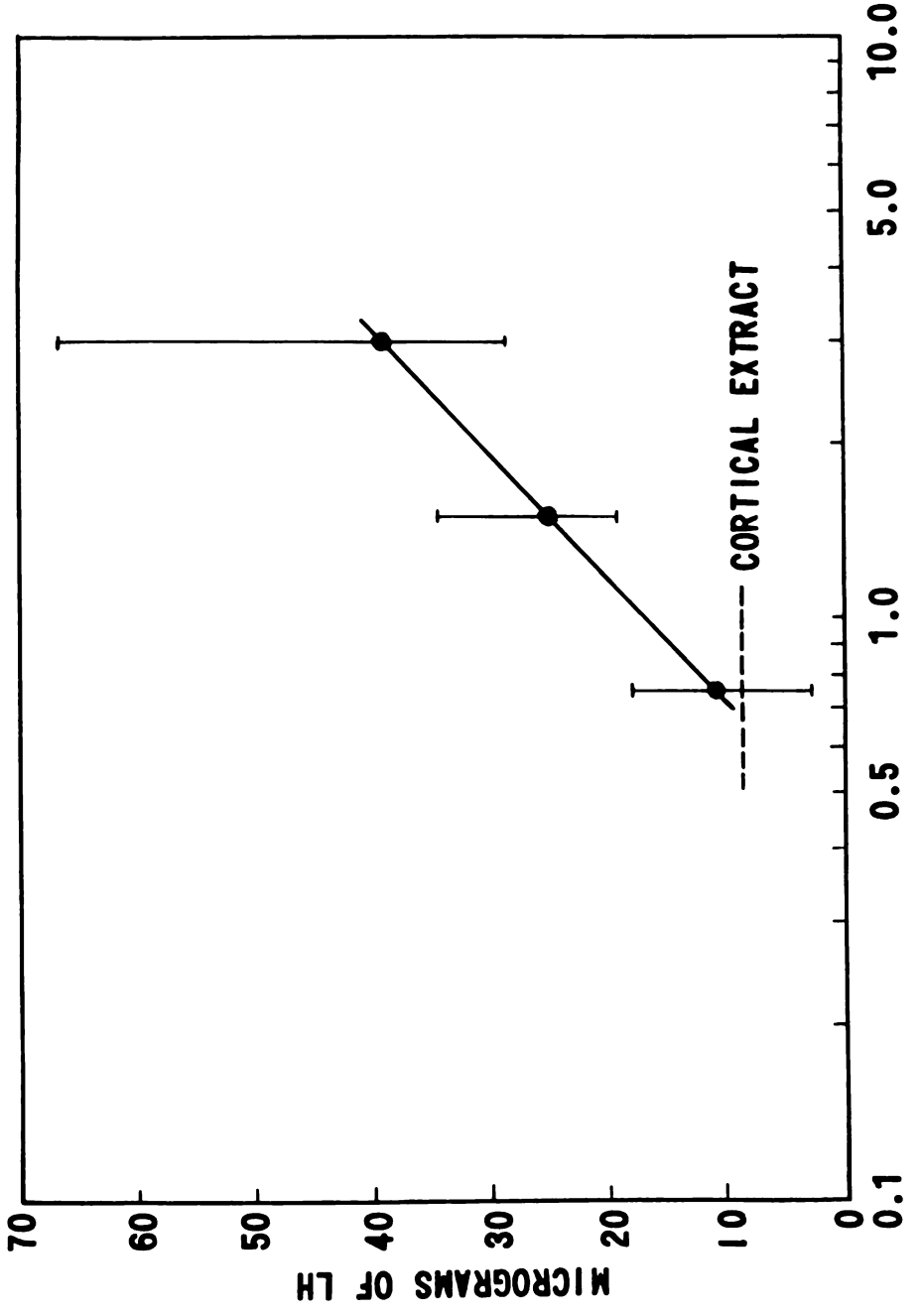

Figure 2. Dose-response relationship between hypothalamic LRF content and LH potency of incubation medium (relative potencies and 95% confidence limits).

Table 10. Dose-response Relationship Between Hypothalamic LRF Content and LH Content of Incubated Pituitaries

Treatment	Relative Potency (Equiv. NIH-LH) ug/mg	95% Confidence Limit	Lambda	Mean Ascorbic Acid Concentration, mg %
0.75 Hypothalamic Equivalents	37.50	27.35-73.06	0.172	57.5
3.00 Hypothalamic Equivalents	14.54	8.93-21.16	0.181	66.3
Cerebral Cortex	72.00	50.59-147.76	0.138	51.5
Medium 199	-	-	-	101.2

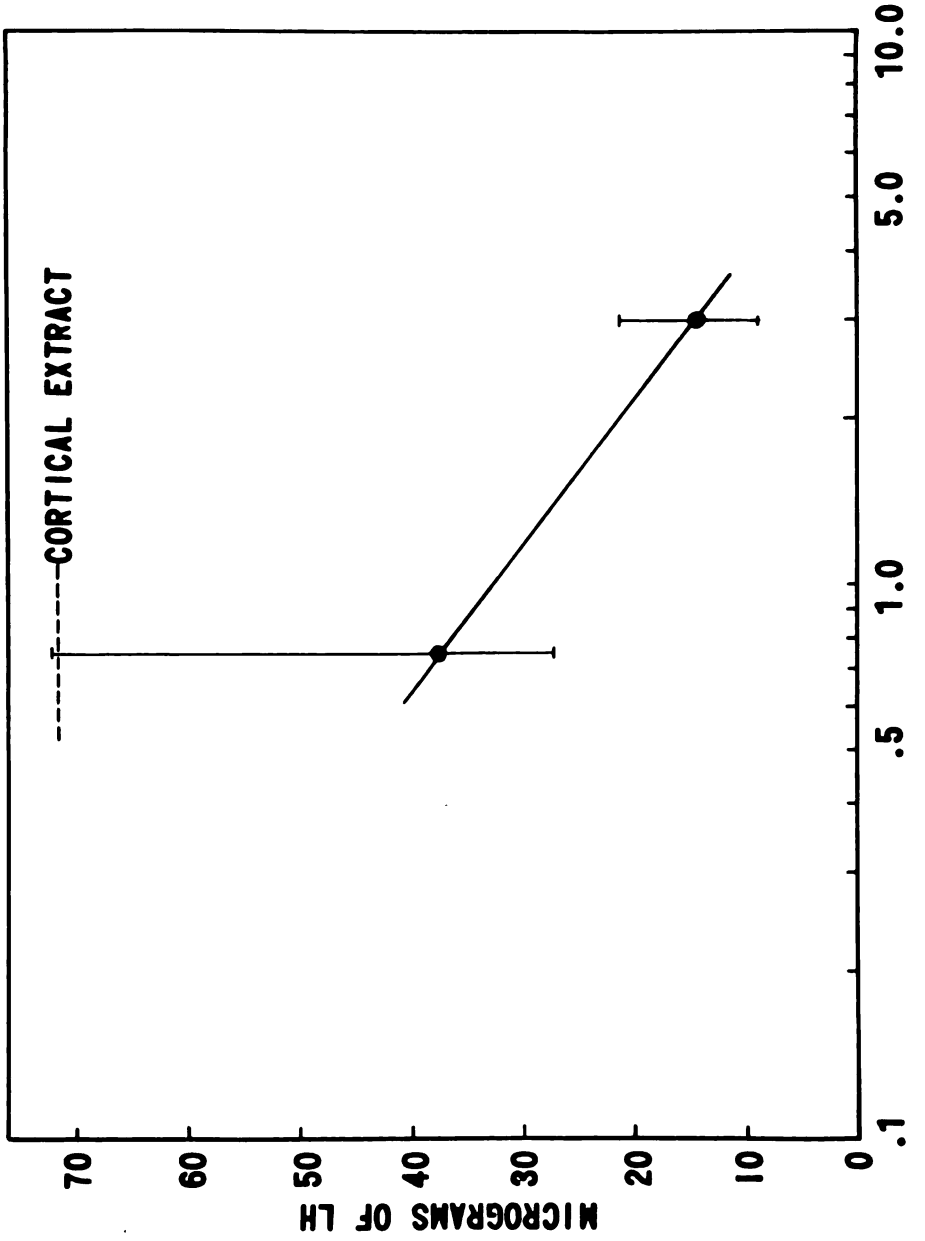

Figure 3. Dose-response relationship between hypothalamic LRF content and LH content of incubated pituitary tissue (relative potencies and 95% confidence limits).

somewhat more LH was released than the amount recovered in the medium, suggesting the possibility of some inactivation in the medium or loss during assay. There was approximately two and one-half times as much LH left in the pituitary explants incubated with 0.75 hypothalamic equivalents as in the explants incubated with 3.0 hypothalamic equivalents. There was an almost five-fold difference between the LH contents of the pituitary explants incubated with cerebral cortex as when incubated with three hypothalamic equivalents. This further confirmed the presence of a specific LH releasing factor in hypothalamic tissue.

These results on the dose-response relationship indicated that LRF activity in the rat hypothalamus can be demonstrated by in vitro methods, and that variations in activity can be detected with the above procedure. Histological examination of the pituitary explants incubated for a period of five hours indicated no appreciable necrosis of the tissue (Figure 4). It can therefore be assumed that in a one hour period, the incubated pituitary tissue remained viable.

Figure 4. Photomicrograph of pituitary tissue incubated for 5 hours with hypothalamic extract (420 X)

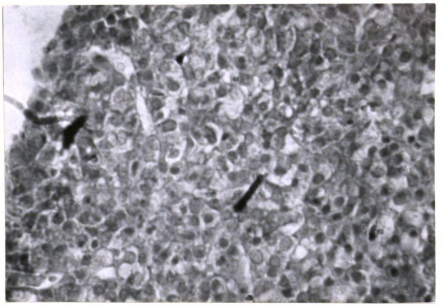

Figure 4

D. Discussion

1. Bioassay Experiments

The selection of a suitable bioassay appeared to be essential, since low amounts of LH which might be expected in an *in vitro* releasing system require a highly sensitive method. It was also important that the bioassay be very precise, since the dose-response relationship between LRF and LH release was unknown, and it was necessary to measure possible differences in hypothalamic LRF content as a result of hormonal treatments.

The ovarian ascorbic acid depletion method of Parlow (1961) appeared to have the required sensitivity; however, several modifications were found in the literature and it was necessary to determine which method provided the best degree of precision. It was apparent from the results that removal of one ovary for a control before the injection of the test material did not provide a precise method. The differences in ascorbic acid content four hours after injection of the test material were just as great in the saline injected controls as they were in the rats given the lower dose of NIH-LH standard. That this was not due to the selection of the left ovary as control and the right ovary for the experimental treatment was indicated by the results on the analysis of the ascorbic acid content of the left and right ovaries in a group of assay rats. These ovaries were removed at the same time and showed no significant difference in ascorbic acid content.

The assay of NIH standard LH, without removal of one ovary as a control, gave an excellent linear relationship between the logarithm of the dose of LH administered and the ascorbic acid content of the ovaries. There was also a very good correlation between the left and the right ovaries of these animals. From these results, it appears that prior surgery for the removal of control ovaries influenced the response of the test animals during the four hour period which followed. On the basis of these results, it was decided that the most accurate and sensitive procedure did not require removal of one ovary.

The importance of running two standard doses with each individual assay became apparent after a number of assays were run during a period of several months. Significant differences in the slope, as well as in elevation of the various standard lines were found. The data suggested that there may be seasonal variations in the ascorbic acid content and in sensitivity of the animals, both being the lowest during the period between December and March. The data obtained in these studies, however, is not sufficient to prove a definite periodicity. It should be noted that the animals were kept under controlled lighting and temperature.

An attempt to determine differences in the LH concentration of crude pituitary extracts was successful. Ovariectomy produced an increase in pituitary LH

concentration. This increase ranged from a two and one-half-fold to a five fold difference. It was apparent from the results that a minimum of a two-fold difference in LH content was necessary to show a significant difference. The results of ovariectomy were confirmed in subsequent experiments.

2. Demonstration of LH release in vitro

Previous results by McCann (1962) and Schally (1964) indicated that the mammalian hypothalamus contained a specific neurohumor which stimulates the release of LH from the rat pituitary. McCann's (1962) work was done with rat hypothalamic extract in vivo while that of Schally and co-workers (1964) was with ovine hypothalamus, and their work was done both in vitro and in vivo. Since there was no previous work on in vitro techniques for demonstrating stimulation of LH release from the pituitary by rat hypothalamic extracts, it was necessary to devise a suitable method for this purpose.

An in vitro technique was chosen for several reasons. First, previous in vivo work indicated that injection of hypothalamic extracts into the venous circulation of assay animals caused the extract to be diluted abnormally. When one considers the anatomical arrangement of the portal circulation to the pituitary, it is obvious that any neurohumor released into the portal vessels will reach the anterior hypophysis directly and not through the systemic circulation. An in vitro method, with only one ml of medium

per pituitary gland and the equivalent of three hypothalami, might approximate more closely the normal levels of the neurohumor in the portal circulation that would act on the pituitary. Of course, this can not be proven until the levels of neurohormones in the portal circulation can be measured.

Second, there is a good possibility that a specific neurohumor given systemically will be metabolized or inactivated in the general circulation. This possibility exists since it has since been shown (Schally, 1964; Nikitovitch-Winer, 1964) that LRF is a polypeptide of approximately 2000 molecular weight. It could therefore be inactivated by specific and non-specific peptidases. Third, the in vitro method was chosen because it provided a good method for separating the hypothalamus from the pituitary of an animal, so that the site of action of gonadal steroids could be studied.

No significant differences were detected in the relative potencies of the incubation media from the control (cortical extract) and experimental (hypothalamic extract) groups when the incubation was carried on for a 3 hour period. Two possible explanations could account for this lack of a stimulatory response by the hypothalamic extract in the experimental medium. First, it is possible that the time interval was too short for any appreciable release. Second, it is possible that the time interval was too long, and the control medium

released the same amount during this period by non-specific leakage.

Results of the 5 hour incubation with hypothalamic extract indicated no significant stimulation of LH release when compared with the control group containing cortical extract, although both media were relatively much more potent than the 3 hour incubation media. Incubation of the pituitaries for a period of only 1 hour resulted in the largest difference between LH release by the pituitaries incubated with cortical extract as compared to those incubated with hypothalamic extract. It appears from these results that release of LH by the pituitary is a rapid phenomenon, involving a definite quota of LH release in response to the dose of LRF given. There also appears to be a slow tonic rate of LH release present even in the groups incubated with cortical extracts. The in vitro method does not differentiate between non-specific leakage due to the cutting of the pituitaries and a slow active release rate. In vivo work suggests that a slow tonic LH release may be present throughout the estrous cycle of the rat, with an ovulatory surge of a large quantity of LH released in the relatively short period of time on the afternoon of proestrous (Everett et al., 1949; Lawton and Schwarz, 1965). This latter surge in LH release could be LRF activated, while the tonic release might be independent of LRF. At the present, no evidence exists for this hypothesis.

Two other points regarding the incubation needed further study. It was necessary to investigate the possibilities of inactivation of released LH in the medium. Since LH appears to be released early in the course of incubation in response to LRF in the hypothalamic extract, one must consider the possibilities of appreciable loss in potency during the remainder of the incubation period. Incubation of known quantities of NIH-LH-S5 standard hormone indicated that a considerable amount of LH was inactivated during the first hour of incubation. This inactivation reduced the potency of the medium to approximately 20% of its value at zero time. By the end of a 5 hour incubation period, the amount of LH remaining in the medium was insufficient to produce a significant slope in the bioassay, when assayed at two dilutions of four-fold difference. Incubation of the rat pituitary extract did not produce a similar phenomenon. No significant inactivation was found, except for a slight loss of potency at the end of a 5 hour incubation period.

There could be several explanations for the inactivation, as well as for the difference between the response of ovine purified LH and crude rat pituitary extract. One cause of inactivation could have been the incubation temperature. The temperature of the incubator varied from 36.5 to 37.5 degrees. This is a relatively high temperature for proteins in solution. There is also the possibility of the presence of proteinases in the incubation medium from the

purified ovine LH or rat pituitary. Such a possibility should have favored increased inactivation in the non-purified rat pituitary extract. Finally, since proteins have a tendency to be more unstable in dilute than in more concentrated solutions, it is possible that the standard LH was inactivated because of the low protein concentration in the medium. One cannot, however, exclude the possibility of a species difference between the two LH sources with regard to the stability of the hormone.

Another point that needed investigation was the possibility of synthesis during the incubation period. It was of interest to find out whether the increased potency of the medium containing hypothalamic extract was due to release only or if LRF also stimulated synthesis of LH in the incubated pituitary. Since the total LH content of the incubated medium and the incubated explants did not differ between the control (cortical extract) and the experimental medium (hypothalamic extract) after a 5 hour incubation period, it was assumed that in a 5 hour or shorter time interval, no significant stimulation of LH synthesis occurred. With a slow tonic infusion of hypothalamic extract for a longer period of time, one might be able to stimulate LH synthesis. It was concluded, therefore, that the increased potency of the medium containing hypothalamic extract was due only to increased release of LH.

3. Dose-response relationships

The studies on dose-response relationships indicate a linear regression between the amount of LH release and the dose of hypothalamic extract added to the rat pituitary during incubation. This complements the studies of McCann (1962) who found a similar relationship in his *in vivo* studies. Schally and Bowers (1964) have also reported that an increased amount of hypothalamic extract can increase release of LH, but they did not analyze their results statistically.

Since hypothalamic extract incubated without pituitary tissue did not produce a significant depletion of ovarian ascorbic acid in the assay animals, it can be assumed that the LH activity detected in the dose-response studies was released by the incubated pituitaries and not by the assay animals own pituitary. These results also suggest that hypothalamic LRF is rapidly inactivated in the incubation medium. The lack of response by the assay animals to incubated LRF also eliminates the possibility of LH in the hypothalamic tissue.

Cerebral cortex tissue appeared to be a suitable control tissue, since the amount of LH released by the pituitaries incubated with cortical tissue equivalent to 3 hypothalami was less than that released by the pituitaries incubated with 0.75 hypothalami. This small amount of LH release was probably due to a non-specific leakage from the pituitaries

which in this experiment were cut into quarters rather than halves. Such leakage was not present in latter experiments where the pituitaries were cut into halves.

A dose-response relationship was also evident in the LH content of the pituitary at the end of incubation. Approximately 2.5 times more LH was left in the pituitary incubated with only 0.75 hypothalamic equivalents than in pituitary incubated with 3 hypothalamic equivalents. The explants incubated with cortical extract contained 5 times as much LH as did those incubated with equivalent amount of hypothalamic tissue extract. The results indicate that the 95% confidence intervals get broader as the amount of LH in the incubation medium or in the pituitary increases. This appears to be a limitation of the assay procedure. The variance tends to increase as one approaches the maximum ascorbic acid depletion. The 95% confidence intervals are also exaggerated by the logarithmic scale. In conclusion it can be said that the rat hypothalamus contains a specific LH releasing factor and that this factor can be detected by *in vitro* techniques in a quantitative manner.

II. EFFECTS OF CASTRATION AND GONADAL HORMONES ON HYPOTHALAMIC CONTENT OF LUTEINIZING HORMONE RELEASING FACTOR (LRF)

A. Systemic Effects of Estrogen and Ovariectomy

1. Objective

Since estrogen has been shown to be a potent inhibitor of LH release, it was of interest to find out whether this

inhibition is a direct one on the anterior pituitary or whether it is indirect through the hypothalamus. Experiments were designed to determine if estrogen could alter the hypothalamic content of LRF.

2. Methods and results

A total of 210 female rats was used in three separate experiments to study the effects of ovariectomy and estrogen treatment. Ninety control rats were ovariectomized and received 0.1 ml corn oil only for a period of 21 days. Sixty castrate rats were injected once daily with 0.8 ug of estradiol benzoate in 0.1 ml corn oil for 21 days, and 60 intact rats received 0.1 ml corn oil only for 21 days. At the end of the 21 day period, the animals were killed and the hypothalami were tested for LRF content. The pituitaries were removed and assayed for LH content.

The results of the assays for pituitary LH content are summarized in Tables 11 and 12. Ovariectomy elicited approximately 1 4-fold increase in the LH content of the pituitaries in the 21 day post-ovariectomy period. This increase was almost completely prevented by the administration of 0.8 ug estradiol benzoate per day.

The results of Experiment I indicate that in the ovariectomized females, hypothalamic LRF activity was significantly reduced during the 21 day post-castration period (Tables 13 and 14). The decrease in activity was more than 3-fold. In Experiment II, the hypothalamic LRF activity was

Table 11. Pituitary LH Content of Intact, Ovariectomized and Estradiol Benzoate-Treated Rats

Treatment	ug NIH-LH Equiv. per mg Wet Tissue ug/mg	95% Confidence Limit	Lambda
Intact	10.69	6.20-23.20	0.273
Ovariectomized	38.84	27.82-69.00	0.217
Ovariectomized + 0.8 ug estradiol benzoate	17.50	11.98-26.88	0.198

Table 12. Pituitary LH Content of Intact, Ovariectomized and Estrodiol Benzoate-Treated Rats

(Analysis Using Ovariectomized Rats as Control)

Treatment	Relative Potency	95% Confidence Limit	Lambda
Intact	0.243	0.003-0.410	0.341
Ovariectomized	1.000	-	-
Ovariectomized + 0.8 ug estradiol benzoate	0.479	0.169-0.942	0.260

Table 13. Effects of Ovariectomy and Estrogen on Hypothalamic LRF Activity

Exp. No.	Assay Number	Treatment	Relative Potency* ug/ml	95% Confidence Limit	Lambda
I	1	Intact	13.38	7.64-22.26	0.231
	1	Ovariectomized	4.12	0.76- 5.66	0.279
II	1	Intact	15.86	12.92-19.44	0.204
	1	Ovariectomized	7.62	6.18- 9.42	0.209
	1	Ovariectomized + 0.8 ug estradiol benzoate	5.32	4.78- 5.92	0.140
	2	Ovariectomized	15.62	10.42-21.76	0.155
	2	Ovariectomized + 0.8 ug estradiol benzoate	6.54	2.58- 9.44	0.202
III	1	Ovariectomized	19.60	13.40-25.62	0.189
	1	Ovariectomized + 0.8 ug estradiol benzoate	7.02	2.02-12.82	0.212

*Expressed as NIH-LH-S5 equivalent

Table 14. Effects of Ovariectomy and Estrogen on Hypothalamic LRF Activity (Analysis Using Ovariectomized Rats as Control)

Exp. No.	Assay Number	Treatment	Relative Potency	95% Confidence Limit	Lambda
I	1	Ovariectomized	1.000	-	-
	1	Intact	3.317	1.626-6.766	0.190
II	1	Ovariectomized	1.000	-	-
	1	Intact	2.081	1.700-2.553	0.206
	1	Ovariectomized + 0.8 ug estradiol benzoate	0.697	0.626-0.776	0.174
	2	Ovariectomized	1.000	-	-
	2	Ovariectomized + 0.8 ug estradiol benzoate	0.418	0.308-0.572	0.178
III	1	Ovariectomized	1.000	-	-
	1	Ovariectomized + 0.8 ug estradiol benzoate	0.358	0.103-0.654	0.200

compared in intact, ovariectomized, and ovariectomized-estrogen treated rats. The results again indicate that hypothalamic LRF activity decreased as a result of ovariectomy. An additional decline in LRF activity was observed when the ovariectomized rats were given estradiol benzoate and compared with the ovariectomized controls. This estrogen-induced decrease was significant but was not as large as observed after ovariectomy. It ranged from a 1.5 to a 2-fold decrease in activity.

In Experiment III, estradiol benzoate again produced a decline in the LRF content of the ovariectomized rat. In this case the decrease was somewhat under a 3-fold difference. These results definitely indicate that ovariectomy results in a decline in the hypothalamic LRF content, and that administration of estrogen causes a further decrease in LRF content.

B. Direct Effects of Estrogen on Pituitary LH Release

The results of the above experiments necessitated a study of the direct action of estrogen on the incubated pituitary. This was especially important, since Michael (1962) reported the H^3 labeled estradiol accumulated in the hypothalamic areas. This means that the hypothalamic extracts used for the incubation probably contained some residual estrogen. Since this residual estrogen could have inhibited the release of LH directly, it was necessary to

study the effects of estradiol when added directly to the medium.

Thirty female rats were used in Experiment I to study the direct effects of estradiol when added to the incubation medium together with hypothalamic extract and pituitary tissue. In Experiment II the effects of estradiol on pituitary LH release were studied without hypothalamic extract. In both Experiments, estradiol (Nutritional Biochemicals) was used. In Experiment I it was used in a dose of 0.1 ug per ml of medium. In Experiment II, the doses were 0.025 and 0.10 ug per ml of medium. The control medium contained equivalent amounts of solvent (0.001 ml of absolute alcohol/ml).

The results (Table 15) show that estradiol in the medium did not reduce the releasing activity of the hypothalamic LRF. On the contrary, a small but statistically insignificant increase in the releasing activity was observed. The results of Experiment II indicate that in the absence of hypothalamic extract, estrogen caused a statistically significant increase in the release of LH from the incubated pituitary. These results suggest that the reduced LH release with hypothalamic extract from estrogen-treated rats was due to reduced LRF activity, and inhibition by estrogen at the hypothalamic level.

Table 15. Direct Effects of Estradiol on Pituitary LH Release

Exp. No.	Assay Number	Treatment	Relative Potency ug/ml	95% Confidence Limit	Lambda
I	1	0.1 ug Estradiol + hypothalamic extract in medium with AP	8.34	4.00-17.46	0.244
	1	Control medium + hypothalamic extract with AP	3.44	2.26- 5.26	0.217
II	1	0.025 ug Estradiol with AP	22.80	15.88-33.08	0.181
	1	0.1 ug Estradiol with AP	19.06	14.38-25.22	0.174
	1	Control medium with AP	0.154	0.88- 2.68	0.230

C. Effects of Castration, Progesterone, and Combined Estrogen-Progesterone on Pituitary LH Release

Since ovariectomy also removes the major source of progesterone in the female rat, it was of interest to study the effects of progesterone and combined estrogen-progesterone treatment in the ovariectomized rat. Sixty ovariectomized rats were injected with 0.1 ml of corn oil per day for 21 days; 30 rats received 4.0 mg progesterone daily for 21 days; and another 30 rats were given a combination of 0.8 ug estradiol benzoate and 4.0 mg progesterone per day for 21 days. At the end of the treatment period the animals were killed, the hypothalami were removed and tested for LRF activity.

The results indicate that progesterone alone did not alter hypothalamic LRF activity after ovariectomy, whereas combined treatment with estradiol benzoate and progesterone prevented the previously shown depressing effect of estrogen and produced a two-fold rise in LRF activity as compared with the ovariectomized controls. Due to the low precision of this particular assay, however, this rise was not significant at the 95% confidence level. The results of both experiments are summarized in Tables 16 and 17.

D. Effects of Castration and Testosterone on Pituitary LH Release in Male Rats

The pattern of pituitary LH release appears to be different in the male than in the female. No apparent cyclicity has been observed in the male, analogous to the estrous cycle.

Table 16. Effects of Progesterone and of Combined Estrogen-Progesterone Treatment on Hypothalamic LRF Activity

Exp. No.	Treatment	Relative Potency	95% Confidence Limit	Lambda
I	Ovarietomy	4.10	0.50- 9.22	0.255
	Ovariectomy + 4 mg progesterone	4.32	0.54- 9.52	0.257
II	Ovariectomy	2.56	0.56- 7.14	0.213
	Ovariectomy + 0.8 ug estradiol benzoate + 4 mg progesterone	5.32	0.98-11.10	0.250

Table 17. Effects of Progesterone and of Combined Estrogen-Progesterone Treatment on Hypothalamic LRF Activity

(Analysis Using Ovariectomized Rats as Control)

Exp. No.	Treatment	Relative Potency	95% Confidence Limit	Lambda
I	Ovariectomy	1.000	-	-
	Ovariectomy + 4 mg progesterone	1.052	0.496-1.750	2.243
II	Ovariectomy	1.000	-	-
	Ovariectomy + 0.8 ug estradiol benzoate + 4 mg progesterone	2.055	1.413-5.016	0.193

It was important to find out whether a similar negative feedback mechanism exists in the male between the gonads and the hypothalamic releasing mechanism. A total of 150 adult male rats was used in two separate experiments to study the effects of castration and testosterone administration on the hypothalamic content of LRF. Thirty intact controls and 60 orchidectomized controls received corn oil only (0.1 ml/day) for 21 days. Another 60 orchidectomized rats were injected with 1.0 mg testosterone propionate daily for 21 days. At the end of the 21 day period the rats were killed and the hypothalami were tested for their LH releasing activity.

The results of the experiment (Tables 18 and 19) indicate that castration elicited a significant increase in the hypothalamic LRF activity. The results of the two assays in Experiment I showed that the measured relative potencies were homogeneous, indicating that the values were an accurate indicator of the amount of LH released.

The increase produced by castration was completely abolished by the administration of 1.0 mg testosterone propionate daily. This was also confirmed in Experiment II. The amount of LH released during the 1 hour incubation period varied considerably from one experiment to the other, but in each case the amount released by the pituitaries incubated with hypothalamic extract from the testosterone propionate treated group was significantly less than the amount released

Table 18. Effects of Orchidectomy and Testosterone on Hypothalamic LRF Activity

Exp. No.	Assay Number	Treatment	Relative Potency	95% Confidence Limit	Lambda
I	1	Intact	3.84	2.48- 5.94	0.192
	1	Castrate	11.42	9.88-13.20	0.167
	1	Castrate + 1 mg testosterone propionate	4.20	2.62- 6.68	0.194
	2	Intact	3.14	0.82- 4.88	0.235
	2	Castrate	8.16	5.84-13.30	0.253
	2	Castrate + 1 mg testosterone propionate	3.00	0.76- 5.64	0.239
II	1	Castration	27.86	19.52-43.90	-.168
	1	Castration + 1 mg testosterone propionate	10.74	4.84-18.18	0.171

Table 19. Effects of Orchidectomy and Testosterone on Hypothalamic LRF Activity (Analysis Using Castrated Rats as Control)

Exp. No.	Assay Number	Treatment	Relative Potency	95% Confidence Limit	Lambda
I	1	Castrate	1.000	-	-
	1	Intact	0.337	0.218-0.521	0.179
	1	Castrate + 1 mg testosterone propionate	0.368	0.231-0.586	0.193
	2	Castrate	1.000	-	-
	2	Intact	0.417	0.164-0.613	0.197
	2	Castrate + 1 mg testosterone propionate	0.432	0.148-0.630	0.201
II	1	Castrate	1.000	-	-
	1	Castrate + 1 mg testosterone propionate	0.412	0.131-0.664	0.214

by the pituitaries incubated with hypothalamic extract from the castrate controls.

E. Discussion

1. Effects of ovariectomy and estrogen on pituitary LH release

The changes in pituitary LH content following castration and estrogen administration in the female rat confirm those previously reported by McCann and Taleisnik (1961a) and Parlow (1964). Ovariectomy resulted in a four-fold increase in pituitary LH content, while administration of estrogen almost completely prevented this increase. This suggests, therefore, that some direct or indirect inhibitory influence on LH synthesis is removed by ovariectomy. One must also take into consideration the possibility of decreased release which would cause an accumulation of LH in the pituitary. This possibility is, however, excluded by the work of Ramirez and McCann (1963) and Parlow (1964a). They showed that plasma LH activity increases after castration in the female.

Since administration of estrogen inhibited the increase in pituitary LH after castration, it must be concluded that estrogen is at least one substance that can act as an inhibitory agent on pituitary LH synthesis. The work of McCann and Taleisnik (1961a) tends to rule out the possibility that estrogen treatment stimulated LH release, since they found a depression of plasma LH following intravenous estrogen

infusions. Decreased pituitary content was, therefore, due to reduced synthesis.

The present study also indicates that estrogen depressed LRF activity in the hypothalamus, which may be responsible for the decrease in pituitary LH synthesis and release. These observations could explain the reduced plasma LH activity after estrogen administration. Reduced synthesis of LRF could result in an eventual depletion of this substance in the hypothalamus and subsequently in reduced stimulation of LH secretion by the pituitary.

The possibility that residual estrogen in the hypothalamic extract inhibited the release of LH by a direct action on the incubated pituitary must also be considered, since the presence of labeled estrogen in the hypothalamus has been reported (Michael, 1962), as well as inhibition of LH release by intra-pituitary estrogen implants (Ramirez et al., 1964). However, the results of this study on incubation of pituitary tissue in the presence of estradiol appears to refute this possibility, since no decrease in pituitary LH release was observed when estrogen was added to the medium with hypothalamic extract. On the contrary, stimulation of LH release was observed. When pituitary tissue was incubated with estrogen in the absence of hypothalamic extract, estrogen stimulated release of LH to a significant degree when compared with the control pituitary tissue not treated with estrogen. Conceivably, different

concentrations of estrogen in the hypothalamus or pituitary may have different effects on pituitary LH release.

These results suggest that estrogen has a 2-fold action on LH release, i.e., it reduces LRF activity in the hypothalamus but increases pituitary LH release directly. This is in good agreement with the findings of Kanematsu and Sawyer (1964) in the rabbit who found increased plasma LH activity in the animals bearing direct pituitary implants of estrogen, but decreased plasma activity when estrogen implants were placed in the hypothalamus. The observations that large doses of estrogen, acutely administered, can produce LH release and elicit ovulation (Everett, 1961) may be explained by a direct stimulatory action of estrogen on the pituitary. The report by Ramirez and McCann (1963) that direct pituitary implantation of estrogen inhibited LH release in the rat may be related to the dose of estrogen employed.

2. Effects of castration, progesterone and combined estrogen-progesterone treatment on pituitary LH release

Progesterone alone failed to modify hypothalamic LRF activity, but combined treatment with estrogen and progesterone not only prevented the depressing effect of estrogen on hypothalamic LRF but resulted in a slight elevation in hypothalamic LRF activity. The observations by McCann (1962) that progesterone alone in physiological doses was ineffective in preventing the post-ovariectomy increase in

plasma LH activity, may be due to its lack of effect on hypothalamic LRF. High doses of progesterone may inhibit the release of LRF from its hypothalamic storage site whereas low doses of progesterone may favor LRF release. The latter is indicated by the work of Everett (1940), who found that small doses of progesterone induced ovulation in the persistent estrous rat. One must also consider the possibility that different ratios of progesterone to estrogen could have different effects on LH release.

In the ovariectomized animal, both synthesis and release of LRF may be increased, with the increase in release rate being greater than that of synthesis. This could result in a decrease of LRF in the hypothalamus, but in an increase in pituitary and plasma LH content. Administration of both estrogen and progesterone, on the other hand, could result in a reduction in pituitary and plasma LH (McCann, 1962) but in an increase in hypothalamic LRF stores, as the results of this study suggest. It is possible, therefore, that estrogen primarily controls the synthesis of LRF, while progesterone in the presence of estrogen controls its release from its hypothalamic storage site.

3. Effects of orchidectomy and testosterone on pituitary LH release

In the male rat, orchidectomy increased hypothalamic LRF activity, suggesting removal of an inhibitory influence on the hypothalamus. Increased LRF apparently stimulates

synthesis and release of pituitary LH, as indicated by the increase in LH content of the pituitary and plasma observed in castrate male rats (Parlow, 1964; Ramirez and McCann, 1963). Administration of 1 mg per day of testosterone propionate prevented the post-castration elevation in LRF activity, suggesting that this dose of testosterone inhibited synthesis and release of LRF.

Testosterone administration has been reported to result in reduced pituitary and plasma LH content (Bogdanove, 1964), which can be explained on the basis of reduced LRF stimulation. The present results in the male rat suggest that testosterone inhibits release and synthesis of LH through a feedback action on the hypothalamus. They do not rule out the possibility that testosterone may also act directly on the pituitary.

The effects of castration in the male are different from those in the female, insofar as hypothalamic LRF content is concerned. One must remember, however, that in the male animals, the pattern of LH release is not cyclic as in the female. There appears to be a definite difference in the control of LH release between the two sexes. Hypothalamic differentiation has been reported to take place early in neonatal life and appears to depend on the presence or absence of circulating androgens (Harris and Levine, 1962). It is possible that in the female, there are two separate LH releasing mechanisms, one controlling the tonic discharge of

LH and the other controlling the neurogenic trigger responsible for the ovulatory burst of LH. Some support for this hypophysis comes from the observation that in persistent estrous rats, estrogen secretion apparently continues but the cyclic ovulatory burst of LH is missing.

It is difficult to reconcile these observations on the ability of gonadectomy and gonadal steroids to alter hypothalamic LRF content with the negative results obtained with castration and gonadal steroids (except testosterone) by Chowers and McCann (1965). It is possible that the discrepancy is a result of the differences in experimental procedures employed, including the different doses of steroids employed. It is likely that elimination of endogenous gonadal steroids in castrated animals is a better method for studying the effect of gonadal steroids on LRF than in the intact animal, since the feedback mechanism is probably more sensitive in the castrate rat. The in vitro procedure probably permitted stimulation of the incubated pituitary by higher levels of LRF than is possible in vivo, where injected hypothalamic extracts are diluted many fold by the circulatory fluid volume. In conclusion, these results definitely indicate that castration or administration of gonadal steriods can modify the LRF activity of the hypothalamus.

III. EFFECTS OF REDUCED FOOD INTAKE, CONTINUOUS LIGHT, EPINEPHRINE AND ACETYLCHOLINE ON GONADOTROPIC FUNCTION

A. Objective

This study was initiated in order to determine whether continuous light, epinephrine or acetylcholine could re-initiate gonadal function in female rats after such function had ceased as a result of reduced food intake. The mechanisms responsible for such changes in gonadal function were also studied.

B. Procedure

Adult female rats were used to determine the effects of underfeeding, and of continuous light, epinephrine and acetylcholine in underfed rats. A total of 120 rats was used in two separate experiments. The animals were obtained from Spartan Research Animals (Haslett, Michigan). Upon arrival, they were allowed 1 week to adjust to their new environment. After the 1 week adjustment period, the estrous cycle of each rat was followed by daily vaginal smears for a minimum of 3 normal cycles. They were then divided into groups of 10 animals each. Daily food consumption was estimated during a period of 6 days. At the end of the 6 day period, the daily food ration was reduced to 50% of the average daily intake estimated previously. Twenty rats remained on normal food intake and served as controls. All animals were smeared daily. When all animals on reduced food intake showed a minimum of 7 days of diestrous or anestrous type smears, the

group on reduced food intake was divided into 4 subgroups. Group 1 received 0.1 ml of corn oil twice daily. Group 2 received 0.25 mg epinephrine in 0.1 ml of corn oil twice daily. Group 3 was placed under continuous illumination by placing a gooseneck lamp with 100 watt bulbs in front of each cage of 10 rats. Group 4 received 2.5 mg acetylcholine in 0.2 ml of physiological saline twice daily.

All treatments were continued for a period of 10 days. At the end of this period, all animals were killed by decapitation. The hypothalami were removed and tested for their LRF content. The pituitaries were removed and assayed for FSH and LH content. The ovaries and uteri, and in some cases the adrenals, were removed and fixed in Bouin's solution for histological examination. All pituitaries, ovaries, uteri, and adrenals were weighed before fixation. The mammary glands were fixed in Bouin's solution and stained in Mayer's hematoxylin for histological examination.

C. Results

1. Effects of reduced food intake

The daily vaginal smears indicated that all rats on reduced food intake stopped cycling completely and exhibited an anestrous or diestrous type smear (Table 20). The cessation of cycling occurred between the 25th and 30th days. There was a significant reduction in ovarian and uterine weights (Tables 23 and 24). The pituitary weights were also reduced (Table 25). Histological examination of the ovaries

(Figure 5) revealed almost complete atrophy. There was a complete absence of mature follicles and of recently formed corpora lutea. Numerous primary and secondary follicles could be found. The interstitial tissue was reduced in quantity and atrophic in appearance.

The uteri (Figure 6) showed an almost complete degeneration of the endometrial layer, indicating lack of circulating estrogen. Despite the fact that both the ovaries and the uteri demonstrated a complete lack of gonadotropin secretion, the anterior pituitaries of the starved animals contained nearly the same level of FSH as that of the normal controls (Table 27). The LH content of the starved rats, however, was lower than that of the controls (Table 28).

Assays of the hypothalamic LRF content of the half-starved rats indicated that the level of this neurohumor was sharply reduced as a result of the reduced food intake (Table 29). In Experiment I the LRF content of the hypothalami was reduced to only 25% of the control value. In Experiment II the LRF content of the starved rat hypothalami was so low that no significant LH release could be detected. The medium from the incubation with the hypothalami from half-fed rats did not show a significant slope. The mammary glands were examined under low magnification. Duct development was moderate but some alveolar tissue was observed.

2. Effects of reduced food intake and continuous illumination

Reduced food intake caused the animals to stop cycling in this case just as it did to the control group. Most of

the rats exhibited a continuous diestrous type smear after the 21st day of reduced food intake. Exposure to continuous light caused all animals to come into late proestrous or estrous between the sixth and the tenth day of light exposure (Table 21).

A highly significant increase in ovarian weight was observed at the time of autopsy, 10 days after the rats were placed on constant light (Table 23). The ovarian weight was equal to or above that found in the control rats on normal food intake. The uterine weights were also increased to a highly significant degree (Table 24). There was approximately a 100% increase in the uterine weight, both on an absolute and on a per cent body weight basis.

Microscopic examination of the ovaries (Figure 7) indicated a high degree of follicular development. Large numbers of mature and cystic follicles were the predominant features of the ovaries of all animals under continuous illumination. There was a lack of functional or recently formed corpora lutea. The few corpora found were degenerate and non-functional in appearance. The uteri showed a distinct increase in development of the endometrial layer in comparison to the rats on reduced food intake without continuous illumination (Figure 8).

Exposure to continuous illumination also produced a highly significant increase in pituitary weight (Table 25). Despite this increase, no change was found in the pituitary

FSH content of the rats under continuous illumination (Table 28). All these values are expressed on a per mg of tissue basis. The total gonadotropin content of these pituitaries was higher than that of the animals on reduced food intake without any light treatment.

No change in the adrenal weights was found as a result of constant illumination (Table 26). The mammary glands exhibited primarily ductal development with an almost complete lack of alveolar growth. No change was found in the hypothalamic LRF content of these animals (Table 29).

3. Effects of reduced food intake and epinephrine

These animals also stopped cycling, as indicated by daily smears, by the third week after the beginning of the half-feeding period (Table 22). About the third or the fourth day after initiation of epinephrine administration, the smears exhibited an increase in the number of vaginal epithelial cells but did not show any cornification or reduction in the number of leukocytic cells.

The ovarian weights of the animals were significantly increased at the time of the autopsy (Table 23). The uterine weights (Table 24) showed a very slight increase, but this was not significant at the 95% confidence level. The characteristic feature of the ovarian histology was a predominance of large, recently formed functional corpora lutea (Figure 9). The ovarian tissue was so heavily luteinized that almost the entire gland consisted of corpora lutea.

An occasional mature follicle could be found. Examination of the uterus revealed moderate repair of the endometrial layer (Figure 10). There was a marked increase in the number and size of the secretory glands.

No significant increase in pituitary weight was found (Table 25). The FSH content of the pituitary did not differ from the normal level or from the non-treated rats on reduced food intake (Table 27). No change was found in the LH content of the pituitaries when the content was expressed on a per mg of tissue basis (Table 28). The LRF content of the hypothalamus showed a slight increase, but this was not significantly different from the controls (Table 29). The mammary glands showed an increase in both the ductal and alveolar development. Examination of the adrenals showed a significant increase in weight when compared with the controls on reduced food intake (Table 26).

4. Effects of reduced food intake and acetylcholine

The results of acetylcholine administration appeared to be negative as judged by all the parameters used. No changes in ovarian or uterine weight were found (Tables 23 and 24). No significant change was obtained in pituitary weight (Table 25). Ovarian histology was essentially the same as that of the non-treated animal on reduced food intake.

Table 20. Vaginal Smears of Female Rats on Half-Normal Food Intake

Number of Rat	Daily Smear Readings
1	DEDPDDEDDDPEDDEDDDEPPDPDEDDDDDDDDDDDDDDDDD
2	MPEDDPDDDPMDDDDDEDDDEPDDDDDDDDDDDDDDDDDDDD
3	MDEEMDPEDDPEDDEDDDPDPDEDDDDPDDDDDDDDDDDDDD
4	DDDPEMPPEDDEEDDPEDDEDDDDDDDDDDDDDDDDDDDDDD
5	EDDPDDPDDDDPEMDPMDDPEDPEEDDDDDDDDDDDDDDDDD
6	PEPMDEEMDDDDDDDDDDDDDPEDEEDEDDDDDDDDDDDDDD
7	DPPEMDPEDDPEDDEDDDDDEDDPEDDPDDDDDDDDDDDDDD
8	DPEDDPEDDEEDDPDDPEDDDPDDDDDDDDDDDDDDDDDDDD
9	DDPEDDDPDEEDDDDDPDEDDDDDDDDDDDDDDDDDDDDDD
10	DDEEEDDDEDDDDDDDDDDDPEDDDDDDDDDDDDDDDDDDD
1	PEPEEEDPEMDDDEDDDPEDDPEDDDDDDDDDDDDDDDDDD
2	DPEPDDEMDDPEDDDDEPDDDDDPDDDDDDDDDDDDDDDDD
3	EEDDPEEDDPEEDDPEDPDDEMDDDDDDDDDDDDDDDDDDD
4	MDPEDDEEDDEEDDEEDDDDPDDDDDDDDDDDDDDDDDDDD
5	PDPPDDPEDDPEDDPEDDDDDDPPDDDDDDDDDDDDDDDDD
6	EDDPEMDPEDDPEDDPPDDPEDDDDPDDDDDDDDDDDDDDD
7	EEPMDPEMDPEDDPEDDPEEDDDEEDDDDDDDDDDDDDDDD
8	DDEMDDPEEMDPEMDDPDPDDPPDDDDDDDDDDDDDDDDDD
9	EMDPEDDEEDDEDDDPDDDDEDDDDDDDDDDDDDDDDDDD
10	DPEDDPEDDPEDDPEDDPEPDEMDDDDDDDDDDDDDDDDDD

P = proestrus
E = estrus
M = metestrus
D = diestrus

Table 21. Vaginal Smears of Female Rats on Half-Normal Food Intake and Continuous Illumination

Number of Rat	Daily Smear Readings
1	PDDPEEDDDEEDDPEDDDDDDDDDPDDDDDDDDDDDDDDDDPE
2	PMDDPEDDEEEDDDMDDDDDEMDDDDDDDDDDDDDDDDDDPEE
3	DDEDDPEDDDEEDMDEDDDEEDDDDDDDDDPDDDDDDDDDPEED
4	PPDDDDEEPEPEEDEDEDDDEDDDDDDDDDDDDDDDDDDEEMDP
5	DDMPMDPPDDDPEDDMEMDDEEEDDDDDDDDDDDDDDDDDEED
6	DPEDDPEDDPEDDPDDPDEDDDDDDDDDDDDDDDDDDDDDPEE
7	DPEDDPEDDPEDDPDDPDEDDDDDDDDDDDDDDDDDDDDDPEEM
8	DPEDDPEMDPMDDEDDEMDDEEDDPDDDDDDDDDDDDDDDDPEE
9	DPEDDPEMDPMDDEDDEMDDEEDDDDDEDDDDDDDDDDDDDPEM
10	EMPEDDPEDDDPDDEDDDEDDDEDDDDDDDDDDDDDDDDDDDP
1	DEMDDPMEEPEDDPDDPDEDDDEEDDDDDDDDDDDDDDDDDPP
2	EDDPEDDPEDDPEDEDDDPEDDDDDPPDDDDDDDDDDDDDDEM
3	EDDPEEDDPEEDDDDDEEEDDPDDDDDDDDDDDDDDDDDDPEED
4	DPEPDDPEDDPEEDDDEDDDPEDDDDDDDDDDDDDDDDDDPEEDDP
5	DPEPDDEMDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDPE
6	PPEPDDEDDDPEMDPEDDDPDDDDDDDDDDDDDDDDDDDDPEEE
7	DDEEDPEDDEEDDDDDDPDDDPDDDDDDDEDDDDDDDDDDDPEM
8	DDDPDDPEEDDPEMDPEDDDPEDDDDEDDDDDDDDDDDDDPEED
9	DPDPPMDPEEDDDDEDDDDPDDDDDDDDDDDDDDDDDDDDPEMP
10	EEDDDDEDDDEPDDEDDDDEDDDDDDDDDDDDDDDDDDDDDDP

↑ Beginning of continuous illumination

Table 22. Vaginal Smears of Female Rats on Half-Normal Food Intake and Epinephrine

Number of Rat	Daily Smear Readings
1	PMDDDDMDDEEDDDEDDDPDDMDDEDDDDDDDDDDDDDDDDD
2	MEDDDDDDDDDDDDDDEDDDEDEDDDPDDDDDDDDDDDDDDD
3	DDPMPEDDDPEDDPEDDDPDDDEDDDDDDDDDDDDDDDDDDD
4	DPEDDEEMDDPDDDDDDDDDEPDDDDDDDDDDDDDDDDDDDD
5	MPEDDPPDDEEDDEDDPEDDEEDDDDDDDDDDDDDDDDDDDD
6	EPDDDPDDDDMPEPDDDDDDDDDDPEDDDDDDDDDDDDDDDD
7	DDEMPPEEDPEEEEDDPEMDDDDEDDDDDDDDDDDDDDDDDD
8	DDDDDDEDDDEPEDDEDDDDEDDDDDDDDDDDDDDDDDDDD
9	MPEEDDPEDDPEDDEEDPEDDPEDDDDDDDDDDDDDDDDDD
10	EDPEMDPEMDDDDDDEEDDPPDDDEDDDDDDDDDDDDDDDD
	↑
1	PEMDPDDDPEDDDDDPDDEEDPDDDDDDDDDDDDDDDDDDD
2	DDPEEEMMMDMDDDDDDEEDDDDDDDDDDDDDDDDDDDDD
3	EEMDPEEDDPEDDDDDPEDDDEEDDDDDDPDDDDDDDDDDD
4	EEDDDDEDDPEDDDDDDPEDDEEDDDDDDDDDDDDDDDDD
5	EDDDPEMDEEDDPEDDDEMMPDDDEDDDDDDDDDDDDDDDD
6	DPEDDPEDDDEMDDEDDDMEEEDDDDDDDDDDDDDDDDDD
7	PEDDPEDDDEEDDDPDDDEDDDDDDDDDDDDDDDDDDDDD
8	EDPEEEDDDEEDDDPEDDEEDDPEPDDDDDDDDDDDDDDD
9	EDDPEEDEDPEMDDDDPEPDDDPDDDDDDDDDDDDDDDDD
10	DDPEDDPPDDDEDDDDDDDDDDDDDDDDDDDDDDDDDDDD
	↑

↑ Beginning of Epinephrine Administration

Table 23. Effects of Continuous Light, Epinephrine, and Acetylcholine on the Ovarian Weight of Partially Starved Rats

Treatment	Experiment Number	Number of Rats Used	Mean Body Weight	Mean Ovarian Weight + S.E. Absolute	mg/100 g B.W.
Full Diet	1	20	230	52.8±5.3	23.0±1.76
50% Normal food intake	1	10	180	34.0±3.3	18.9±1.04
	2	16	182	34.8±1.1	19.2±0.65
50% Normal food intake + continuous illumination	1	10	206	64.2±3.4	31.1±1.13
	2	20	211	53.4±1.7	25.2±1.02
50% Normal food intake + 0.50 mg epinephrine	1	10	179	46.2±3.9	25.8±1.30
	2	22	191	47.8±2.4	25.1±1.40
50% Normal food intake + 5.0 mg acetylcholine	1	10	175	40.6±5.3	23.2±1.78

Table 24. Effects of Continuous Light, Epinephrine, and Acetylcholine on the Uterine Weight of Partially Starved Rats

Treatment	Experiment Number	Number of Rats Used	Mean Body Weight	Mean Uterine Weight + S.E. Absolute	mg/100 g B.W.
Full Diet	1	20	230	518.1±16.8	225.3± 7.4
50% Normal food intake	1	10	180	229.3±17.3	127.4±10.0
	2	16	182	124.1± 7.4	68.1±16.6
50% Normal food intake + continuous illumination	1	10	206	412.8±42.7	199.4±20.8
	2	20	211	401.1±25.8	186.6±10.6
50% Normal food intake + 0.50 mg epinephrine	1	10	179	275.0±36.0	154.0±20.1
	2	22	191	170.4±21.7	90.9± 4.0
50% Normal food intake + 5.0 mg acetylcholine	1	10	175	252.4±24.9	144.2±11.9

Table 25. Effects of Continuous Light, Epinephrine, and Acetylcholine on the Pituitary Weight of Partially Starved Rats

Treatment	Experiment Number	Number of Rats Used	Mean Body Weight	Mean Pituitary Weight + S.E. Absolute	mg/100 g B.W.
Full Diet	1	20	230	12.2±0.41	5.30±0.17
50% Normal food intake	1	10	180	7.4±0.28	4.11±0.08
	2	16	182	7.2±0.46	4.03±0.11
50% Normal food intake + continuous illumination	1	10	206	9.1±0.21	4.51±0.10
	2	20	211	10.7±0.11	5.05±0.14
50% Normal food intake + 0.50 mg epinephrine	1	10	179	7.6±0.34	4.24±0.14
	2	22	191	7.9±0.25	4.20±0.13
50% Normal food intake + 5.0 mg acetylcholine	1	10	175	8.1±0.42	4.60±0.15

Table 26. Effects of Continuous Light and Epinephrine on the Adrenal Weight of Partially Starved Rats

Treatment	Number of Rats Used	Mean Body Weight	Mean Adrenal Weight + S.E. Absolute	mg/100 g B.W.
50% Normal food intake	16	182	45.1±1.4	24.8±0.89
50% Normal food intake + continuous illumination	20	206	47.4±1.6	22.8±1.19
50% Normal food intake + 0.50 mg epinephrine	22	191	52.4±1.8	28.1±1.25

Table 27. Follicle Stimulating Hormone (FSH) Content of the Pituitaries of Starved Rats Treated with Continuous Light or Epinephrine

Treatment	Relative Potency ug NIH-FSH-S2 Equiv. per 10 mg Tissue	95% Confidence Limits	Lambda
Full Diet	77	63-100	0.113
50% Normal food intake	85	58-117	0.141
50% Normal food intake + continuous illumination	76	67- 93	0.110
50% Normal food intake + 0.50 mg epinephrine	89	64-119	0.133

Table 28. Effects of Continuous Light and Epinephrine on the Pituitary LH Content of Partially Starved Rats

Treatment	Relative Potency ug NIH-FSH-S5 Equiv. per 10 mg Tissue	95% Confidence Limit	Lambda
Full Diet	113.6	72.0-129.9	0.157
50% Normal food intake	34.4	16.5- 65.8	0.181
50% Normal food intake + continuous illumination	48.0	29.3- 59.0	0.115
50% Normal food intake + 0.50 mg epinephrine	33.1	10.9- 79.0	0.227

Table 29. Effects of Continuous Light or Epinephrine Treatment on the Hypothalamic Luteinizing Hormone Releasing Activity (LRF) of Starved Rats

Treatment	Experiment Number	Relative Potency ug NIH-LH-S5 Equiv. per ml.	95% Confidence Limit	Lambda
Full Diet	1	10.04	7.88-17.18	0.131
	2	6.48	3.20-10.46	0.164
50% Normal food intake	1	2.64	1.84- 3.98	0.162
	2	Not detectable*	-	-
50% Normal food intake + continuous illumination	1	2.52	1.70- 3.36	0.146
50% Normal food intake + 0.50 mg epinephrine	1	3.66	1.80- 7.22	0.196

* No significant slope.

Figure 5. Photomicrograph of ovary from rat on reduced food intake (32 X)

Figure 6. Photomicrograph of uterus from rat on reduced food intake (100 X)

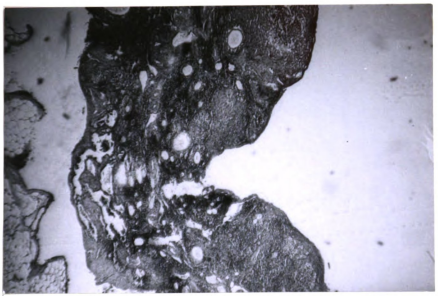

Figure 5

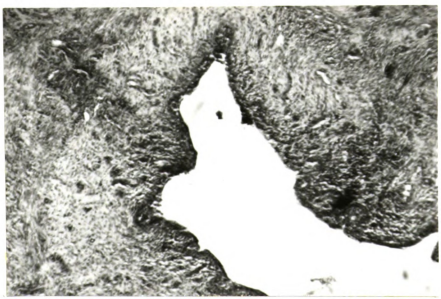

Figure 6

Figure 7. Photomicrograph of ovary from rat on reduced food intake and continuous light (32 X)

Figure 8. Photomicrograph of uterus from rat on reduced food intake and continuous light (100 X)

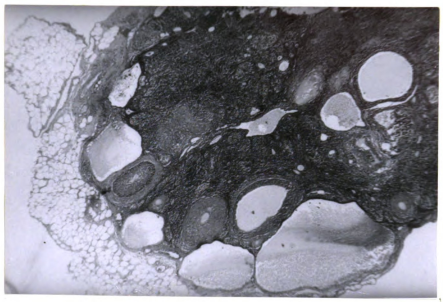

Figure 7

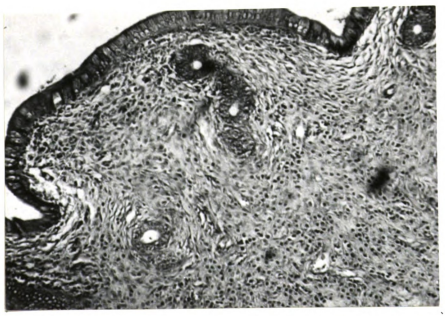

Figure 8

Figure 9. Photomicrograph of ovary from rat on reduced food intake and epinephrine (32 X)

Figure 10. Photomicrograph of uterus from rat on reduced food intake and epinephrine (100 X)

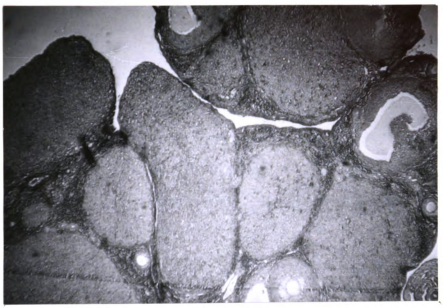

Figure 9

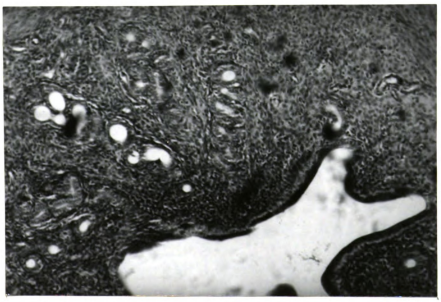

Figure 10

D. Discussion

These experiments were done for several reasons. First, it was of interest to investigate whether reduced food intake causes a cessation of reproductive function through a direct action on the gonads, by an effect on the pituitary, or by reducing hypothalamic stimulation to the hypophysis. Second, an attempt was made to determine whether the inhibitory action of underfeeding on gonadal function could be counteracted by different types of stimuli.

The present results of the effects of chronic underfeeding confirm those of earlier workers (Marrian and Parkes, 1929; Mulinos and Pomerantz, 1940). Ovarian weights of the underfed rats were reduced, and microscopic examination of the ovaries revealed pronounced degeneration of the ovarian tissue. The complete absence of developing follicles indicated a lack of FSH release. There was also a lack of functional interstitial tissue, suggesting greatly reduced LH secretion. Correlated with this are the findings on uterine histology, which showed an almost complete lack of estrogen stimulation by the ovaries. The uterus showed a typical diestrous picture, although the loss of endometrial epithelium was even more pronounced than during the normal cycle.

The work of Mulinos and Pomerantz (1941) indicated that atrophic ovaries and uteri elicited by underfeeding are capable of reactivation by administration of exogenous gonadotropins. This suggests that the effects of underfeeding

are manifested on other sites than the ovary, such as on the pituitary and the hypothalamus. The results of this study indicate that there is no change in the pituitary FSH content of rats on reduced food intake. This is in agreement with the work of Meites and Reed (1949), who found no decrease in the total gonadotropin content of the pituitary in rats on reduced caloric intake. However, the pituitary LH content of the rats on reduced food intake did show a significant drop in this experiment. These results suggest that the pituitary may maintain some ability to synthesize FSH, but there is a failure in FSH release. The drop in LH content could be due to reduced LH synthesis as well as decreased release by the pituitary. The reduction in hypothalamic LRF content parallels the reduction in pituitary LH content. Thus the reduced pituitary LH content was probably the result of reduced LRF stimulation.

Since the present observations suggest that the effects of chronic starvation are exerted on the hypothalamic mechanism, an attempt was made to reactivate gonadotropin release by stimuli previously shown to stimulate gonadotropin release through the central nervous system (CNS). Visible light was observed to be a very potent stimulator of FSH release (Critchlow, 1963) and was, therefore, one of the stimuli used on the chronically underfed rats.

The results of exposure to continuous illumination were striking. In only 10 days, all animals came into estrus or

proestrus. The increase in ovarian weight indicated a definite release of FSH. This was confirmed by histological examination of the ovaries. Since the increase in ovarian weight was due primarily to follicular development, the exposure to continuous light did not appear to have an appreciable stimulating effect on LH release. Some LH secretion, however, must have been present. This is indicated by the finding of a definite and significant increase in the uterine weight of the animals as well as the presence of cornified vaginal cells. This is indicative of elevated estrogen output by the ovaries, which presumably is not possible without some LH release (Parlow, 1964). Histological examination of the uteri confirmed the presence of increased estrogenic activity. Enhanced thickness of the endometrium, glandular development and more prominent endometrial epithelium, all suggested the presence of increased levels of circulating estrogen.

Further evidence of increased estrogen output by the ovaries is indicated by the development of the mammary ducts and the increased size of the pituitary. A few animals in Experiment 2 came into estrus earlier than the majority of the group. These animals appeared to go into diestrus after one or two days of estrus. Examination of the ovaries revealed some freshly formed corpora lutea, indicating that ovulation did occur. The number of corpora, however, was much smaller than under normal conditions.

The results of these experiments indicate that reduced FSH release produced by chronic underfeeding is most likely a consequence of reduced stimulation of the pituitary by the hypothalamus, since it could be counteracted by constant light which is mediated through the CNS. Study of the hypothalamic changes in FSH-RF content upon reduced food intake and continuous illumination would further elucidate this problem.

Epinephrine and acetylcholine have been reported to induce ovulation in the rabbit, and dibenamine and atropine have been shown to block ovulation, suggesting the presence of both an adrenergic and cholinergic component in this process (Sawyer et al., 1946). On the basis of these studies, as well as studied on the effects of these drugs on prolactin release (Meites et al., 1959, 1960), it was decided that epinephrine and acetylcholine should be tried in an attempt to stimulate LH release. Acetylcholine treatment did not appear to stimulate the release of gonadotropins. This was indicated by the lack of stimulation of ovarian and uterine weights, as well as lack of an increase in follicular size in the ovaries.

The results of the epinephrine treatment are interesting but somewhat difficult to explain. Although the epinephrine-treated rats on reduced food intake never came into estrus, an increase in the number of epithelial cells was noted in the vaginal smears. No noticeable cornification, however,

could be observed in any of the smears. Examination of the ovaries indicated a definite increase in ovarian weight which was due primarily to an increase in the number and size of the corpora lutea of the ovaries. There was also an increase in follicular development, since several normal appearing mature follicles were found. The uterine weight did not show a significant increase but did show some repair in the endometrial epithelium, and an increase in the vascularization of the endometrium.

Since prolactin is the luteotropic hormone in the rat, the enhanced development of luteal tissue strongly indicates release of this hormone as a result of epinephrine treatment. This confirms the work of Meites et al. (1959,1963), who showed that epinephrine stimulates the release of prolactin in rats and rabbits and can induce lactation. It is also possible that prolactin secretion was increased as a result of starvation, but this could not be detected in the starved control rats because of lack of functional ovarian tissue. The fact that some follicles were found, and that the corpora lutea were recently formed and appeared highly functional, indicates that epinephrine administration stimulated release of gonadotropins, especially of LH, and induced ovulation and corpus luteum formation. Increased progesterone secretion was also suggested by the development of the mammary glands which exhibited not only ductal growth but also alveolar development.

It is probable that at the time of autopsy, most of the animals were pseudopregnant. This is supported by the appearance of the uterine histology, ovarian histology and mammary development. No significant changes could be found in total pituitary gonadotropin content or in hypothalamic LRF content, although a slight increase in the latter was noted. Regrettably, FSH-RF could not be measured because of lack of sufficient hypothalamic tissue.

These studies on the reinitiation of gonadotropic function in rats on reduced caloric intake, indicate that such a treatment affects the hypothalamic production of gonadotropic releasing neurohumors rather than the ability of the pituitary to respond to CNS stimulation, or the ability of gonads to respond to pituitary gonadotropic stimulation. Stimuli, such as light and epinephrine, which have been shown to stimulate the release of gonadotropins through the CNS, can elicit increased gonadal stimulation in rats on reduced food intake.

In conclusion, the probable explanation of the effects of chronic starvation on gonadal function is reduced synthesis of hypothalamic neurohumors, which results in a depletion of these substances in the hypothalamus and subsequently in reduced secretion of pituitary gonadotropins. Further studies of the effects of nutrition, light, epinephrine and other agents on LRF should prove interesting. It would also be of interest to study the combined effects of light and

epinephrine on gonadal, pituitary and hypothalamic function in starved rats.

IV. EFFECTS OF CONTINUOUS LIGHT ON GONADOTROPIC FUNCTION OF TRANSPLANTED PITUITARIES IN HYPOPHYSECTOMIZED FEMALE RATS

A. Objective

The purpose of this experiment was to determine whether gonadal function in adult hypophysectomized female rats could be reinitiated to any extent by subcutaneous pituitary transplantation and continuous light. The mechanisms underlying such possible changes were also studied.

B. Procedure

Adult female rats, hypophysectomized at 2 months of age, were used for this experiment. They were shipped 10 days after surgery from Hormone Assay Labs., Chicago, Illinois. Intact adult female rats of the same age and strain were also ordered, and 7 days after their arrival they were killed by decapitation and pituitaries from every 2 such animals were transplanted into 1 hypophysectomized rat under ether anesthesia. Both pituitaries were implanted subcutaneously, one into each inguinal region. All transplanted rats received 45,000 units of procaine penicillin G at the time of the surgery. On the day after transplantation, the rats were divided into 2 groups. Group 1 remained on a controlled light schedule of 14 hours of light and 10 hours of darkness. These animals served as a control group. Group 2 was placed

under constant illumination (24 hours/day). Three weeks after the transplantation, the experiment was terminated. In Experiment I, the animals were killed; their ovaries, uteri, adrenals, and thyroids were removed, weighed and fixed for histological examination. The pituitary transplants were removed and examined histologically in Experiment I.

In Experiments II and III, the animals received 20 IU of HCG for 3 or 5 days, respectively, for augmentation prior to killing. In Experiment III two groups of non-transplanted, hypophysectomized rats were also included. One of these groups was kept under controlled illumination, while the other was placed under constant lighting. Twenty-four hours after the last injection of HCG, the animals were killed and their ovaries and uteri were removed for weighing and histological examination.

In all groups, the mammary glands were also removed, fixed and examined under low power magnification. The sella turcica were examined for residual pituitary tissue.

C. Results

The body weights of the hypophysectomized animals indicated that secretion of growth hormone from the transplants was adequate to cause a significant increase in body weight. Rats were hypophysectomized at an average weight of 180 grams. At the time of the transplantation none of the rats weighed more than 150 g. After 21 days post-transplantation,

animals in all three experiments returned very close to their pre-hypophysectomy weights. Rats in groups 3 and 4 in Experiment III, which did not receive a transplant, did not exhibit an increase in body weight. Curiously, in Experiment II, there was a significant decrease in body weight of the light-exposed as compared to the control group.

In Experiment I (without HCG augmentation), the rats exposed to continuous light had a significantly higher mean ovarian weight than the control group maintained on controlled illumination (14 hours light + 10 hours darkness; Table 30). This was true on both an absolute and on a mg per cent body weight basis. In Experiment II the 3-day augmentation with HCG increased the ovarian weights of both the control and constant light groups. However, the difference in ovarian weights was highly significant, the controls again averaging less than those of the rats under continuous illumination. In Experiment III (5 days of HCG augmentation and pituitary transplants) the ovarian weights were significantly larger in the continuously illuminated group than in the control group when compared on the basis of mg per cent body weight. No significant difference in ovarian weights was found between Groups 3 and 4 in Experiment II. Neither of these two groups had pituitary transplants, but both were augmented with HCG for 5 days. Group 4 was also exposed to constant light. The ovarian weights

Table 30. Effects of Continuous Light on Ovarian Weight of Hypophysectomized Rats with Pituitary Transplants

Exp. No.	Group Number	Number of Animals Used	Treatment	Final Body Weight Mean + S.E.	Ovarian Weight mg Mean + S.E.	Ovarian Weight mg/100 g Mean + S.E.
I	1	10	Hypophysectomized with transplant	179.2±3.72	7.80±0.64	4.40±0.43
	2	10	Hypophysectomized with transplant + continuous light	179.0±2.92	11.76±0.67	6.46±0.36
II	1	15	Hypophysectomized with transplant + 20 IU HCG/day for 3 days	180.8±1.75	16.90±1.02	9.38±0.55
	2	14	Hypophysectomized with transplant + continuous light + 20 IU HCG/day for 3 days	167.3±2.89	27.42±1.44	16.50±1.02
III	1	10	Hypophysectomized with transplant + 20 IU HCG/day for 3 days	173.7±4.78	36.30±3.87	21.30±1.13
	2	10	Hypophysectomized with transplant + continuous light + 20 IU HCG/day for 3 days	172.3±2.18	49.31±4.22	29.42±2.23
	3	7	Hypophysectomized + 20 IU HCG/day for 3 days	147.6±1.27	45.81±1.23	31.00±2.29
	4	10	Hypophysectomized + continuous light + HCG	153.6±4.39	48.40±1.63	31.60±1.21

of the rats in Groups 3 and 4 were not significantly different from those in Group 2 (pituitary transplants, 5 days of HCG augmentation and constant light).

The ovarian histology of the animals in Experiment I, Groups 1 and 2 (Figure 11 and 12), indicated that exposure to continuous light resulted in no substantial increase in the interstitial tissue of the ovaries. Some increase in the follicular growth was observed in the rats with pituitary transplants under continuous light (Figure 12). The increase in the ovarian weights was apparently due to an increase in the follicular mass. Examination of the ovaries from Experiment II (3 days HCG augmented) showed excessive luteinization of the follicular tissue. Luteinization occurred in the primary and secondary follicular phase. There was an increase in the number of mature follicles in Group 2, although these follicles were smaller than normal. Examination of 10 ovaries in each group revealed 17 tertiary or mature follicles in the continuously light-exposed group compared with only 2 in the control group (Figures 13 and 14). Histological examination of the ovaries in Experiment III (5 day HCG augmented) indicated that rats from Group 2 (with transplant and constant light) exhibited distinct follicular ovaries (Figure 15), while those in Group 4 (no transplants and constant light) showed a complete lack of follicular growth (Figure 16). This was interesting since there was no significant difference between the ovarian weights of these two groups.

Figure 11. Photomicrograph of ovary from hypophysectomized rat bearing pituitary transplant (100 X)

Figure 12. Photomicrograph of ovary from hypophysectomized rat bearing pituitary transplant and exposed to constant light (100 X)

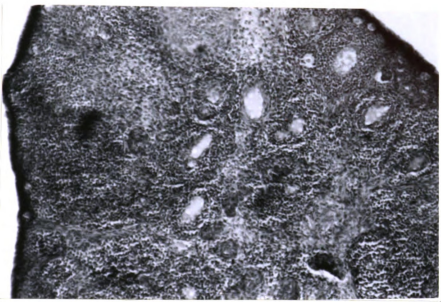

Figure 11

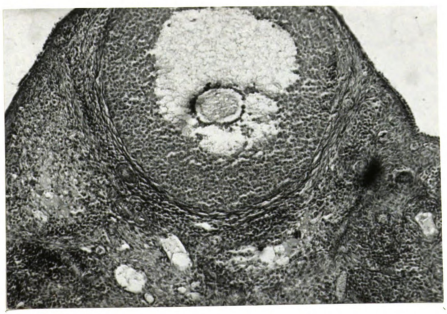

Figure 12

Figure 13. Photomicrograph of ovary from hypophysectomized rat bearing pituitary transplant (HCG augmented for 3 days) (100 X)

Figure 14. Photomicrograph of ovary from hypophysectomized rat bearing pituitary transplant and exposed to constant light (HCG augmented for 3 days) (100 X)

Figure 13

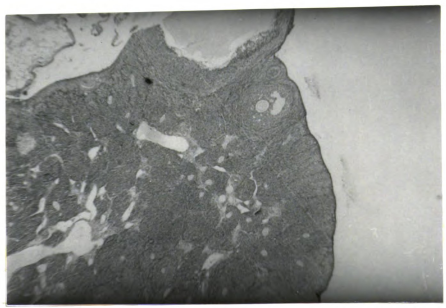

Figure 14

Figure 15. Photomicrograph of ovary from hypophysectomized rat bearing pituitary transplant and exposed to constant light (HCG augmented for 5 days) (32 X)

Figure 16. Photomicrograph of ovary from hypophysectomized rat without pituitary transplant and exposed to constant light (HCG augmented for 5 days) (32 X)

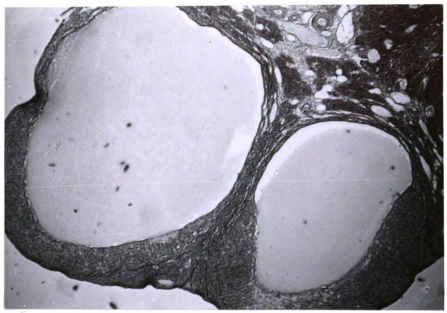

Figure 15

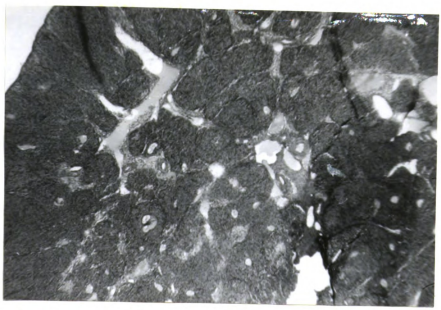

Figure 16

Histological examination of the pituitary transplants showed several consistent features in the groups exposed to continuous light (Figure 18) when compared to those from control groups (14 hours light, 10 hours darkness; Figure 17). The most prominent feature was an increased amount of viable tissue and less necrosis in comparison to the control groups (14 hours light, 10 hours darkness). Other outstanding features in the transplants of the animals exposed to constant light were an increased uptake of stain (especially hematoxylin), more prominent and normal looking nuclei and an increase in total size. These transplants also showed an increased peripheral vascularization in comparison to the controls (14 hours light, 10 hours darkness).

The uterine weights of the continuously light-exposed rats (Group 2) were found to be significantly heavier than the controls (Group 1), when the weights were expressed on a mg per cent basis (Table 31). In Experiment II, a highly significant difference was found in the uterine weights between the control and the continuously light-exposed groups. This was true on both an absolute and mg per cent weight basis. The same was also true in experiment III. There was a significant difference between the uterine weights of the two non-transplanted groups given HCG, but this difference was not significant if the weights were expressed on a mg per cent basis. The transplanted control group had significantly lower uterine weights than all other groups in Experiment III.

Figure 17. Photomicrograph of pituitary transplant from hypophysectomized rat under controlled light (14 hours light, 10 hours darkness) (950X)

Figure 18. Photomicrograph of pituitary transplant from hypophysectomized rat under constant light (950 X)

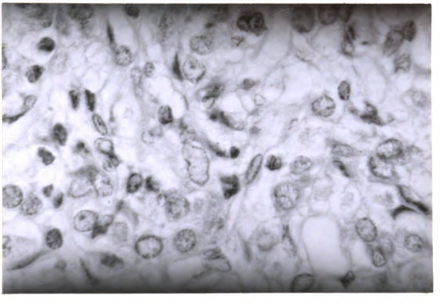

Figure 17

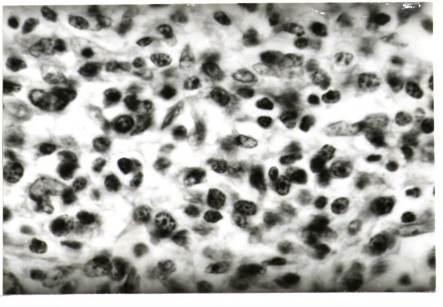

Figure 18

Table 31. Effects of Continuous Light on Uterine Weight of Hypophysectomized Rats with Pituitary Transplants

Exp. No.	Group Number	Number of Animals Used	Treatment	Final Body Weight Mean + S.E.	Uterine Weight mg Mean + S.E.	Uterine Weight mg/100 g Mean + S.E.
I	1	10	Hypophysectomized with transplant	179.2±3.72	81.0±3.2	45.5±2.01
	2	10	Hypophysectomized with transplant + continuous light	179.0±2.92	96.2±3.9	53.1±1.71
II	1	15	Hypophysectomized with transplant + 20 IU HCG/day	180.8±1.75	72.6±2.5	40.2±1.42
	2	14	Hypophysectomized with transplant + continuous light + 20 IU HCG/day	167.3±2.89	144.0±16.7	87.4±11.5
III	1	10	Hypophysectomized with transplant + 20 IU HCG/day	173.7±4.78	109.9±7.12	63.4±4.41
	2	10	Hypophysectomized with transplant + continuous light + 20 IU HCG/day	172.3±2.18	195.0±7.68	117.4±6.24
	3	7	Hypophysectomized + 20 IU HCG/day	147.6±1.27	159.0±2.69	108.1±6.14
	4	10	Hypophysectomized + continuous light + 20 IU HCG/day	153.6±4.39	199.7±5.71	130.6±4.87

The uterine histology of the rats in Experiment II (3 days HCG augmented) indicated that the endometrial layer of the continuous light-exposed group was thicker in general and more defined than that of the control group. It also appeared to show increased glandular development. The lumen of the continuously exposed animals appeared larger than that of the controls (Figure 19 and 20).

No difference was found in the thyroid and the adrenal weights of the continuously exposed animals when they were compared to the controls. These organs were weighed only in Experiment I (Table 32). Examination of the mammary glands did not reveal a consistent picture in either the control or the continuously light-exposed groups. The results were highly variable and ranged from complete atrophy in some glands to considerable ductal growth and alveolar development in others. Interestingly, in some glands, development was localized in only a restricted region of the gland, the rest of the gland exhibiting a complete lack of development.

D. Discussion

If the hypothesis that light increases FSH-RF production is a correct one, then it is conceivable that continued stimulation by light could increase the circulating FSH-RF content to such a level that a pituitary transplant at a point removed from the hypothalamus could be stimulated. The present experiments indicate that continued stimulation by external light did indeed show the effects of FSH

Figure 19. Photomicrograph of uterus from hypophysectomized rat bearing pituitary implant (HCG augmented for 3 days) (100X)

Figure 20. Photomicrograph of uterus from hypophysectomized rat bearing pituitary implant and exposed to constant light (HCG augmented for 3 days) (100 X)

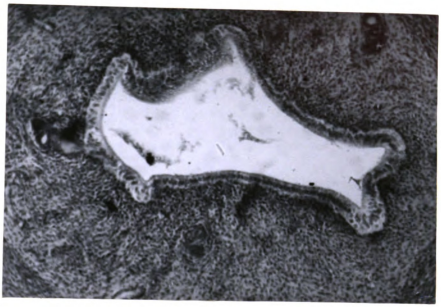

Figure 19

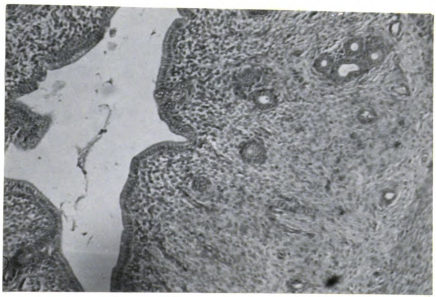

Figure 20

Table 32. Effects of Continuous Light on Thyroid and Adrenal Weight of Hypophysectomized Rats with Pituitary Transplants

Exp. No.	Number of Animals Used	Treatment	Thyroid Weight mg Mean + S.E.	Thyroid Weight mg/100 g Mean + S.E.	Adrenal Weight mg Mean + S.E.	Adrenal Weight mg/100 g Mean + S.E.
I	10	Hypophysectomized with transplant	8.90±0.68	5.07±0.16	14.0±0.36	7.85±0.20
	10	Hypophysectomized with transplant + continuous light	9.40±0.67	5.21±0.39	14.7±0.47	8.23±0.34

augmentation in hypophysectomized rats bearing hypophysial transplants.

The ovarian weight and the ovarian histology definitely indicated increased ovarian stimulation by FSH. Although the ovarian picture in the rats exposed to continuous light was not that of an intact cycling rat, the presence of mature follicles indicated that at least a moderate increase in FSH secretion by the transplants occurred. Some gonadotropin secretion by transplanted pituitaries has been reported previously by Hertz (1960). However, Hertz (1960) used 4 pituitary transplants in the kidney capsule to show stimulation of ovarian weight. By administering HCG to the transplanted animals, Hertz (1960) was able to augment the response of the ovaries to endogenous FSH. He obtained an increase in the absolute weights of the ovaries in both the transplanted and the non-transplanted control groups, as well as differences between the mean ovarian weights of the two groups.

Based on Hertz' (1960) study, some of the rats in this study were also given HCG. The results with HCG were in good agreement with those obtained without augmentation. A significant increase in the ovarian weights was obtained as a result of exposure to continuous illumination. The ovarian histology of these animals was difficult to interpret, because administration of large doses of HCG caused massive luteinization of the ovaries. It appeared that this luteinization included the granulosa tissue of the secondary

follicles as well. There was, however, an increase in the number of mature follicles as a result of exposure to continuous light.

The increased amount of luteinization in the HCG treated rats was probably also due to release of prolactin by the transplanted pituitaries. The effects of the removal of the pituitary from hypothalamic influence on pituitary prolactin release have been clearly demonstrated by the studies of Meites et al. (1962) and by Nikitovitch-Wiener and Everett (1958, 1960).

The ovarian weights of the non-transplanted animals given HCG were just as great as those from the transplanted and constant light exposed groups. However, it must be considered that all groups received the same dose of HCG regardless of body weight. If one examines the body weights of the various groups, it becomes obvious that the non-transplanted animals were significantly smaller and, therefore received a relatively larger dose of HCG than did the transplanted ones. This increase in body weight also indicated that some somatotropin was released by the transplanted pituitary gland without hypothalamic stimulation. This is in agreement with the work of Meites and Kragt (1964), who reported a significant gain in body weight of young hypophysectomized rats with a single pituitary transplant. Changes in the histology of pituitary transplants supported the hypothesis that light increased the amount of circulating

neurohumors. Increased cell population and uptake of stain indicated that they probably contained an increased number of chromophil cells as a result of exposure to constant light. Positive identification of these cells could not be made, however, since differential staining was not done.

Changes in uterine weight appear to parallel those of the ovaries. Exposure to continuous illumination always produced a significant increase in the uterine weight. The uterine histology showed increased stimulation by estrogen, as evidenced by increased size, glandular development and increased epithelial layers of the endometrium, and an increase in size of the lumen. Examination of the endometrial tissue under higher magnification also suggested some progesterone stimulation in the animals exposed to continuous illumination. This may be due to increased estrogen priming rather than to a difference in the progesterone secretion rate of the luteinized tissue in the ovaries of the two groups. The uterine weights of the non-transplanted groups given HCG in Experiment 2 were equal to those of the transplanted rats given HCG, but as in the case of the ovaries, no significant difference could be found between those under a normal light schedule and those under constant illumination.

The observations on mammary development are difficult to interpret, since they were extremely variable. This

variability was probably due to the fact that some of the transplants were located in the area of the mammary gland and produced stimulation of mammary tissue by local action.

The present observations are believed to constitute strong evidence that continuous illumination in hypophysectomized female rats can stimulate release of sufficient hypothalamic gonadotropin releasing factors into the systemic circulation to evoke gonadotropin secretion by transplanted pituitaries. This suggests that other strong environmental stimuli may also elicit release of hypothalamic neurohumors into the general circulation. Demonstration of the presence of hypothalamic neurohumors in the general circulation would confirm the present findings.

GENERAL DISCUSSION

In this thesis an attempt was made to determine how environmental factors, the central nervous system, the pituitary and the gonads interact to control gonadotropin secretion. In order to demonstrate that hypothalamic neurohumors have a physiological function, it was necessary to show that a definite dose-response relationship exists between the amount of neurohumor given *in vivo* or *in vitro* and the amount of anterior pituitary hormone released. Second, it had to be demonstrated that stimuli from the internal and external environment could induce changes in the hypothalamic content of these neurohumors.

In this study, special emphasis was placed on hypothalamic LRF and the control of luteinizing hormone release by the pituitary, although several experiments involved a study of FSH release as well. Since the collection of hypophysial portal blood is technically extremely difficult in the rat, it was decided to use an *in vitro* method to demonstrate the presence of and measure the changes in LRF content of the hypothalamus. The results show that a dose-response relationship exists between the amount of LRF in the incubation medium and the amount of LH released by the pituitary. This indicates that LH release is responsive to hypothalamic

stimulation. It cannot be concluded from this study that the amount of LRF reaching the pituitary is the sole factor determining the rate of LH release, since other agents may also act directly or indirectly on the pituitary.

The role of LRF in LH synthesis requires further clarification. The experiments conducted in this work only prove that in a period of 5 hours, no net synthesis occurred in response to LRF contained in the hypothalamic extract. It is possible however, that some synthesis did occur, but the rate of inactivation in the medium and/or in the incubated gland, was great enough to offset the results of any possible synthesis. It is also possible that a slow infusion of hypothalamic extract would have been more effective than the addition of a single large dose at the beginning of the incubation period, since LRF could have been rapidly inactivated during incubation. A study of the incorporation of radioactive amino acids into pituitary LH could yield further information on this problem. Even if such a study should prove that hypothalamic extracts stimulate LH synthesis, it would still be necessary to prove that only one chemical entity was responsible for stimulation of both release and synthesis. It can be concluded from this study only that the hypothalamic extract used contained a specific humoral substance (LRF) which stimulated the release of LH from the pituitary.

In order to further elucidate the physiological role of LRF, an attempt was made to induce changes in the hypothalamic content of this neurohumor by altering the steroid levels of the experimental animals. Since in vivo studies indicated the possibility of indirect feedback for both estrogen and androgen, it was of interest to investigate the possibility that such feedback was mediated by depressing synthesis of LRF by the hypothalamus. Estradiol benzoate and testosterone propionate were found to depress hypothalamic LRF content, but the results obtained on castration pointed to a basic difference in the feedback mechanism between the two sexes. In the male orchidectomy produced a significant elevation of LRF content, while in the female ovariectomy produced a significant decrease in the hypothalamic LRF activity. This dichotomy between the two sexes remains to be clarified. One must recall, however, that in the female, a cyclic mechanism exists for acute LH release which is lacking in the male. Also estrogen is not the only steroid involved in the feedback mechanism in the female. Progesterone in the presence of estrogen has been shown to be a very effective blocking agent for LH release and in small amounts may stimulate LH release.

The results of the present study suggest that both estrogen and progesterone are involved in the feedback mechanism controlling LH release in the female. The specific action of each of these two steroid hormones, however,

appears to be different. Although both can inhibit (or stimulate) LH release, estrogen appears to depress the synthesis of LRF resulting in less LRF going to the anterior pituitary via the portal circulation. Physiological doses of progesterone, on the other hand, appear to counteract the inhibitory action of estrogen on LRF synthesis by the hypothalamus, and may actually favor LRF synthesis. It is also possible that larger doses of progesterone block the release of LRF from its hypothalamic storage site.

Even if the above explanations are correct, one can not completely explain the feedback mechanisms operating during the estrous cycle. It is paradoxical that at the time when the "neurogenic" trigger for ovulation is activated, the estrogen titer is almost at its highest level during the estrous cycle. This suggests the possibility of a positive feedback mechanism. The results of this study indicate that estrogen can directly stimulate the pituitary release of LH, and may sensitize it to the action of LRF. The <u>in vitro</u> incubation studies showed that estrogen in the incubation medium increased the amount of LH released into the medium in response to hypothalamic extract. It is possible therefore, that the action of estrogen is twofold. At the hypothalamic level it could be inhibitory to LH release, but at the pituitary level it could be stimulatory to LH release. This may depend to a considerable extent on the level of estrogen present in the blood. The level of progesterone

is also important, since low doses of progesterone have been shown to stimulate LH release whereas high doses will inhibit LH release. The single dose of progesterone and estrogen used in the present experiments is inadequate to draw any definite general conclusions.

Changes in the steroid titers are not, however, the only factors regulating gonadotropin release. External environmental factors can influence the rate of synthesis and release of FSH and LH by the pituitary. An attempt was made to define the site of action of nutrition and light, since both of these factors have been shown to alter gonadotropin release. An attempt was also made to study the effects of these two environmental influences simultaneously.

The present studies indicate that the effects of constant light are primarily on the release of FSH rather than on LH. The influence of light appears to be mediated through the hypothalamus, presumably by increasing the synthesis of follicle stimulating hormone releasing factor (FSH-RF). Apparently the stimulus produced by constant illumination was great enough to activate pituitary transplants at a distant site in hypophysectomized rats. Constant illumination also counteracted the depressing action of reduced food intake on FSH output by the <u>in situ</u> pituitary. These environmental influences were apparently superimposed on the equilibrium normally present between the target organs

and the hypothalamo-pituitary axis. The function of negative feedback (direct or indirect) by the gonadal steroids may be to keep the hypothalamo-pituitary-gonadal axis at such a level of activity that it is within an optimal range of sensitivity. A somewhat similar view has recently been expressed by Bogdanove (Seminar, MSU, Jan. 18, 1966) to explain the function of negative feedback of estrogen. His explanation was, however, based entirely on a direct feedback. The present studies suggest that this feedback is probably mediated through the hypothalamus, and that it is the hypothalamus which is kept at an optimum level of sensitivity.

Although the results obtained in these experiments indicate that light primarily affects the FSH releasing mechanism, it is also probably involved in the release of LH. This was indicated by the observations on the uterine weights and histology in the underfed rats exposed to constant light. Estrogen production would not have been possible without some LH release. This hypothesis is further supported by observations that the time of ovulation in the rat can be changed by altering the daily lighting schedule. This could possibly be explained on the basis of a positive feedback mechanism between the ovaries and the pituitary. Stimulation by light could result in increased FSH release, which in turn would produce an elevation in estrogen output. This could ultimately trigger the release of LH by the pituitary.

Such a scheme, however, would not explain the observation that ovulation can be blocked by Nembutal during a critical period of less than two hours on the day of proestrus. If the positive feedback would be directly on the pituitary nembutal would not block ovulation. This suggests therefore that if there is a positive feedback, it is through the CNS. No evidence is available at the present time, however, to indicate such a positive feedback through the hypothalamus.

Light appeared to be such a potent stimulator of FSH release that even in the animal on reduced food intake (pseudohypophysectomized) it produced ovaries full of mature follicles. The control animals on reduced food intake showed a complete lack of FSH release, as indicated by the pronounced ovarian atrophy. Since light, a neurogenic stimulus, counteracted the effects of reduced food intake, it appears probable that the effects of reduced nutrition were also exerted at the hypothalamic level. This was supported by the finding that in the underfed rats pituitary LH content was reduced significantly, along with the hypothalamic LRF content. Although the FSH content was not significantly changed, there was no indication that it was released in the half-fed rats. Reduced food intake could have resulted in a decreased availability of amino acids for the synthesis of LRF and FSH-RF, which are believed to be polypeptides.

The study with epinephrine in the rat on reduced food intake further supports the view that the nutritional deficiency influenced gonadotropin release through the hypothalamus. Although histological examination of the ovaries of the epinephrine-treated animals indicated primarily prolactin release, the formation of such a large number of corpora lutea would not have been possible without previous release of FSH and LH. The size of the corpora lutea indicated that they were formed from mature follicles. Since epinephrine has been shown to be active at the hypothalamic level rather than on the pituitary, it can be assumed that the blockage of gonadotropin release caused by underfeeding occurred at the hypothalamus.

The most convincing proof for hypothalamic involvement in the effects of reduced food intake comes from the finding that hypothalamic LRF content was reduced to almost undetectable levels as a result of decreased food intake. Since this reduced LRF content was coupled with reduced LH release, it can be assumed that it was a result of both reduced synthesis and release of LRF.

In all the preceding studies, hypothalamic content was emphasized. It is recognized here, as in other gland content studies, that only the difference between the amount synthesized and the amount released is being measured. However, when hypothalamic content of LRF or FSH-RF can be related to the amount of LH or FSH produced and released by the

pituitary, or to changes in gonadal activity, it becomes more significant. Regretably, it was not always possible to study all these parameters simultaneously. However, there can be no question that the ability of gonadal steroids or environmental factors to elicit changes in content of LRF in the hypothalamus is itself of physiological significance. In general, most of the data reported here support the view that external and internal environmental influences on pituitary gonadotropin secretion are exerted mainly through the hypothalamus.

In conclusion, the data in this thesis indicate that the control of gonadotropin release by the pituitary is much more complicated than previously indicated by early investigators. A simple negative feedback between the gonads and the pituitary cannot explain gonadotropin regulation completely. Even a feedback through the hypothalamus does not explain all the phenomena occurring during the estrous cycle. The possibility of a positive feedback by estrogen must be considered, as well as the variable effects of different doses of estrogen or progesterone. There is also a possibility that different ratios of estrogen to progesterone may selectively stimulate or inhibit the hypothalamus. Superimposed on any equilibrium established by the gonadal-pituitary-hypothalamic axis, one has to consider the large number of environmental agents which can influence gonadotropin

secretion. It should also be considered that other areas of the CNS, in addition to the hypothalamus, are probably involved in the regulation of gonadotropin secretion.

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APPENDIX

LIST OF STATISTICAL FORMULAS

1. Relative Potency

$$M' = \frac{\bar{y}u - \bar{y}s}{bc}$$

2. Confidence Limits

$$X'_L = \bar{x}_s - \bar{x}_u - \frac{c^2 (\bar{y}s - \bar{y}u)}{bc} \pm tCs_M$$

3. $C^2 = \frac{B^2}{B^2 - s^2t^2}$ = correction term for relat. potency

4. $b_C = \frac{\Sigma[xy]}{\Sigma]x^2]}$ = combined slope

5. $s_M = \lambda_c \sqrt{\frac{1}{Nu} + \frac{1}{Ns} + \frac{(\bar{y}u - \bar{y}s)^2}{B_C^2 - s^2t^2}}$ = standard error of potency

6. $B_C^2 = \frac{(\Sigma [xy])^2}{\Sigma[x^2]}$ = combined B^2

7. $B^2 = \frac{[xy]^2}{[x^2]}$

8. $\lambda_C = \frac{Sc}{bc}$ = combined index of precision

9. $Sc = \frac{\Sigma[y^2] - \Sigma B^2}{n_s + n_u}$

10. $[y^2] = \Sigma y^2 - (\Sigma y)^2/N$; $[x^2] = f\ [\Sigma x^2 - (\Sigma x)^2/k]$

11. $n = N - 2$; $N = f \times k$; k = no. of dose levels of x
f = no. of responses at each dose level

12. $[xy] = \Sigma x(\Sigma y_i) - (f \cdot \Sigma x \cdot Ey)/N$

u - unknown

s - standard