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IN THE LIMBIC SYSTEM

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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The Ohio State University
1969

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CHAPTER I
INTRODUCTION

The regulation of pituitary-gonadal function by the central nervous system has been the subject of many studies, most of which deal with the hypothalamus. Suggestions that the nervous system might play a role in the regulation of anterior pituitary function came from observations that environmental stimuli can alter gonadotropin secretion (Austin and Braden, 1954). Alterations in illumination (Critchlow, 1963; Ganong et al., 1963) have important effects on the estrous cycle of some mammals, and in some species, such as the rabbit, ovulation is normally triggered by copulation. It has also been demonstrated (D'Angelo, 1963), again in lower mammalian forms, that environmental temperature changes are mirrored by alterations of thyroid function mediated via hypophyseal thyrotropic hormone (TSH). It is also clear that a variety of injurious stimuli increase the secretion of adrenocorticotropic hormones (ACTH) in man and animals (McDermott et al., 1951; Farrell and McCann, 1952). In the 1920's, alterations in pituitary-gonadal function were induced by lesions in the hypothalamus causing gonadal atrophy in the dog (Bailey and Bremer, 1921): however, investigators were reluctant to assign the nervous system any more than a limited role in hypophyseal regulation.
Progress in the understanding of the hypothalamic control of hypophyseal function was accelerated when Harris (1943) published the first comprehensive review of the evidence for hypothalamic control of the pituitary.

In general, reproductive processes in female mammals are characterized by cyclic alterations in the genital tract and in sexual receptivity. The recurrent reproductive period of mature female mammals depends upon the rhythmic secretion of hormones by the adenohypophysis and ovary. However, the rhythm is not intrinsic to these glands but is imposed by the central nervous system, especially by the hypothalamus and related structures of the brain. Pfeiffer (1936), investigated the effects of gonadectomy and gonadal transplantation in newborn rats on the rhythmic secretion of gonadotropic hormones when these animals became adult. Pfeiffer concluded that pituitary function is plastic and its pattern of secretion is determined by the presence of functional gonads. Martinez and Bittner (1956) showed that castrated adult male mice, with transplanted ovarian tissue and pituitaries grafted from either male or female donors, did not display cyclic pituitary function. Martinez and Bittner concluded that Pfeiffer's explanation was not adequate to explain their results and suggested that the cyclic or non-cyclic character of the female or male pituitary was dependent upon a sexual differentiation of neural tissue which develops early in life. Everett et al. (1949) and Harris (1955) expressed the view that the sexual differentiation of the nervous system must occur in fetal or neonatal
life under the action of testosterone in the male. Barraclough and Leathem (1954) and Barraclough (1961) found that the injection of testosterone in the early neonatal period of the female rat would masculinize the central nervous system, resulting in the acyclic release of gonadotropin from the pituitary when the animal was an adult. The presence or absence of testosterone in the neonatal period of development determined how the central nervous system controlled the pituitary in the adult animal. Harris and Levine (1962) stated that the pituitary of neonatal androgenized female rats responded to injections of estrogen, indicating that the pituitary was not masculinized, and Harris (1964) concluded that the trigger that normally causes the ovulatory dose of gonadotropins resulting in ovulation was no longer able to function and that this trigger came from the central nervous system.

Due to the work of Moore and Price (1932) stating that the adenohypophysis and ovary were linked together in a self-contained system of hormonal interactions, it was believed in the early thirties that the peripheral hormonal milieu was able to influence the hypophyseal tropic hormone secretion exclusively by direct action on the pituitary. The results of other experiments performed at about the same time (Bailey and Bremer, 1921) on the effects of central nervous system lesions on the reproductive functions showed that the central nervous system had an influence on the secretion of the different gonadotropic hormones. Intensive work on the central nervous system, and especially the hypothalamus were undertaken to show the influence of the hypothalamus on the
control of gonadotropin secretion. Sectioning of the pituitary stalk in both male and female rats was reported to result in varying periods of gonadal atrophy (Brolin, 1945; Westman and Jacobsohn, 1938). The hypothalamohypophyseal portal vessels were found to be capable of rapid regeneration after simple stalk section (Harris, 1949; 1950) and when portal vein regeneration was prevented by the insertion of a barrier between the median eminence and the pituitary gland, ovarian atrophy occurred invariably (Westman and Jacobsohn, 1938; Harris, 1950). Autotransplantation of the pituitary to a heterotropic site (Harris and Jacobsohn, 1952) resulted in cessation of the estrous cycle and atrophy of the gonads. The gonadotropic function of the pituitary was restored if it was reimplanted into its normal place and its connections with the median eminence permitted to regenerate (Greep, 1936). Following reimplantation of the pituitary back to its normal location, not only were the weight and function of the target organs restored, as was shown in the experiments by Nikitovitch-Winer and Everett (1959), but the normal histological structure of the anterior lobe, which had become highly dedifferentiated during its transplantation to the heterotropic site, was completely restored. The explanation seems to be that grafts under the median eminence acquire their blood supply from regenerated hypophyseal portal vessels, restoring the neurovascular link with the hypothalamus. It is believed that the principal blood supply of the anterior lobe of the pituitary is by portal vessels, and the direction of blood flow is from the median eminence towards the pars distalis of the anterior lobe. A
review on this subject has been prepared by Landsmeer (1963). The
nerve supply of the adenohypophysis has been the subject of contro­
versy for many years. There is little doubt that vegetative fibers
(vasomotor) reach the hypophysis along its arterial supply. These
fibers do not innervate the glandular cells of the adenohypophysis
(Szentagothai, 1962). True hypothalamic innervation of the adeno­
hypophyseal tissue is slight in the pars tuberalis and pars distalis.
Nerve fibers ending on the median eminence capillary loops, which
drain into the pituitary portal vessels, have been described repeat­
edly. A good description of the nerve endings on the capillary loops
has been given by Szentagothai (1962), and Szentagothai and Halasz,
(1964). They described the termination of the tuberoinfundibular
tract at two preferential sites. One corresponds to the nerve termin­
als surrounding the capillary loops that drain toward the pituitary
portal system. The other endings, grapelike in shape, are in a super­
ficial zone of the median eminence and proximal part of the stalk.
The tuberoinfundibular tract originates chiefly from small nerve cells
of the anterior periventricular nucleus (Szentagothai and Halasz, 1964).
The functional significance of the nerve endings of this tract on the
capillary loops draining into the portal vessels is taken by Flerko
(1966) to mean that the endings liberate pituitary-regulating substan­
ces, produced in the hypothalamus, into the portal circulation. The
following conclusions were drawn by Flerko (1966): (a) the gonadotro­
pic function of the hypophysis is under the direct control of the
central nervous system, and especially under the control of the dien­
cephalon; (b) this diencephalic control upon the adenohypophysis is mediated via the pituitary stalk, especially through the hypothalamo-hypophyseal portal vessels.

If the view that nerve fibers entering the anterior pituitary are too few for secretomotor regulation and that the flow of blood in the hypothalamo-hypophyseal portal vessels is mainly toward the adenohypophysis, the chemotransmitter hypothesis brought forth by Harris (1955) is a plausible explanation of the experimental data. Slusher and Roberts (1954), Guillemin and Rosenberg (1955) and Saffran et al. (1955) demonstrated that hypothalamic extracts stimulated adrenocorticotropic hormone (ACTH) secretion, and thus furnished evidence for the existence of corticotropin-releasing factor (CRF). At about the same time, the existence of a hypothalamic factor stimulating thyrotropic stimulating hormone (TSH) was demonstrated (Shibusawa et al., 1956; Schrieber, 1956). McCann and Friedman (1960) solidified evidence for the existence of a hypothalamic luteinizing hormone-releasing factor (LRF). Other experiments further confirmed and extended these results (Campbell et al., 1961; McCann, 1962; Nikitovitch-Winer, 1962; Ramirez and McCann, 1963). Igarashi and McCann (1964) reported the release of follicle stimulating hormone (FSH) from crude extracts of the rat hypothalamus under conditions similar to those that cause the release of LH. Mittler and Meites (1964) were able to demonstrate increased release of FSH into a medium of pituitary incubates using hypothalamic extracts, further proving the existence of an FSH releasing factor.
Meites et al. (1962; 1963) and Danon et al. (1963) showed that prolactin synthesis and release, which they and others have found to proceed at a high rate in vitro, could be inhibited by adding hypothalamic extracts to the media. Thus, a prolactin-inhibiting factor (PIF) was postulated to be present in the hypothalamus. A comprehensive review by McCann and Dhariwal (1966) is recommended for further study.

According to the current hypothesis, the neural control of gonadotropin secretion might depend on two mechanisms, each controlled by different levels of the central nervous system (Flerko, 1966). The first level of control involves the "hypophysiotropic mechanism" of the hypothalamus (Halasz et al., 1962), which stimulates the production of FSH and LH and their output at a basal level ("the tonic discharge" of FSH and LH according to Gorski and Barraclough, 1962). This area also functions in the inhibition of the secretion of LTH, at least in the rat. This system in itself can not enhance or inhibit the release of FSH and LH. This is the function of the second level of control, the "release regulating mechanism" which includes other brain structures that modify the activity of cells of the "hypophysiotropic area." Structures in this group are currently thought to be concentrated in the preoptic, anterior and dorsal regions of the hypothalamus and the limbic system. The term "hypophysiotropic area" was introduced by Halasz et al. (1962) to refer to their in vivo preparation of a neurally isolated hypothalamic-pituitary area. The pituitary must be in contact with this area for the maintenance of normal anterior
pituitary function and histology. The "hypophysiotropic area" of the hypothalamus corresponds cytologically and anatomically (Halasz and Szentagothai, 1962) to the arcuate nucleus, a ventral portion of the anterior periventricular nucleus, and the medial parvicellular parts of the so-called "retrochiasmatic area". This area also corresponds to the distribution of the nerve cells which form the tuberoinfundibular tract which terminates in the median eminence. Halasz and Pupp (1965) showed that the "hypophysiotropic area" not only relays the influence of other hypothalamic structures to the pituitary, but it also exerts a regulatory influence on the anterior lobe. When this area was isolated from the rest of the brain, female rats developed constant estrous with cystic ovaries and no corpora lutea. It appeared that FSH and LH releasing factors are reaching the pituitary, but the releasing factor mechanism of the "hypophysiotropic area" alone was unable to instigate normal sexual cycles. The origin of the neural stimulus responsible for the production of the burst of LH secretion that triggers ovulation must come from outside this area. The center for the neural mechanism that triggers the burst of LH secretion responsible for ovulation is probably located in the preoptic-suprachiasmatic area of the hypothalamus. The existence of this mechanism was postulated by Markee et al. in 1952. Large lesions of the anterior hypothalamus interrupting the neural connections between the preoptic and the hypophysiotropic area (Critchlow, 1958a) produced, in polycyclic animals, a state characterized by inability to mobilize enough LH for ovulation and formation of corpora lutea. Electrical stimulation of the preoptic
region, in rats made anovulatory by pentobarbital administration, induced ovulation (Critchlow, 1958b; Everett, 1961). This region could also be activated by progesterone (Barraclough and Yrarrazaval, 1961). It was concluded by Gorski (1966) that the preoptic-supra-chiasmatic area in the normal cycling female responds under proper environmental and hormonal circumstances by activating the more terminal "hypophysiotropic area" to elicit an ovulatory discharge of FSH and LH from the anterior lobe, and that this activation could be blocked in the adult female rat by testosterone administered at birth. In the absence of this mechanism, the hypophysiotropic structures still function normally but secretion is at a basal rate which evokes a constant estrous syndrome.

Before 1932 it was generally believed that the peripheral hormonal milieu influenced the production and release of gonadotropic hormones exclusively by direct feedback action on the adenohypophysis. Hohlweg and Junkman (1932) suggested that gonadal hormones might influence gonadotropic functions through a hypothetical "sexual center" located somewhere in the brain. In the light of the experimental data accumulated in the last few decades, it appears that the feedback action of gonadal steroids is mediated in part through multiple neuronal elements located in different parts of the hypothalamus. There may well be three levels at which feedback effects of sexual steroids act to modify gonadotropin secretion. The least complex of these is the adenohypophysis, while the other two are located in the central nervous system. Of
these neural centers, the first is the "hypophysiotropic area," and the other includes all higher brain centers which are capable of modifying the activity of the "hypophysiotropic area". Rose and Nelson (1957) showed that estrogen perfused into the hypophyseal fossa could inhibit the hypertrophy of gonadotropic basophils which normally follows castration. Bogdanove (1963) showed that estrogen implants in the anterior pituitary also block this response to castration. These experiments indicate, but do not prove unequivocally the existence of direct feedback to the pituitary. There is good evidence that the negative feedback control of FSH and LH release might act through neural structures located in the anterior hypothalamus and in the "hypophysiotropic area". The inhibitory effect of estrogen on the castration-induced rise in FSH and LH output can be diminished by lesions placed in the anterior hypothalamus of juvenile rats (Flerko, 1957). Estrogen released from small fragments of ovarian tissue autotransplanted into the anterior hypothalamus inhibits FSH and LH secretion (Flerko and Szentagothai, 1957). Hohlweg and Daume (1959) found that estrogen injected into the anterior hypothalamus of rats had an antifolliculotrophic effect 125 times larger than if estrogen was administered subcutaneously. Data indicating that neural structures in the "hypophysiotropic area" are affected by sex steroids in a negative feedback fashion include the following. Needles containing estradiol implanted into the arcuate nucleus (Lisk, 1960) induce cessation of estrous cycles and cause uterine atrophy. The ovaries of these animals contained no large follicles, and the interstitial tissue had become atrophic. Implantation of minute amounts of estra-
diol or testosterone into the hypothalamus of the rabbit (Davidson and Sawyer, 1961) was followed by failure of copulation-induced ovulation, and eventually by ovarian or testicular atrophy. Similar implants into the adenohypophysis did not do this. Using the ovarian ascorbic acid depletion test, McCann (1962) found that a single intravenous dose of estrogen (1-50 μg estradiol) caused LH to disappear from the plasma and that pretreatment with doses of estrogen, that were ineffective by themselves, plus small doses of progesterone had a similar inhibitory effect on LH release. In such animals, an injection of extract of the stalk median eminence region of the hypothalamus resulted in the reappearance of LH in the plasma within 10 minutes. Occurrence of aniline blue - and PAS positive, aldehyde fuchsin-negative "castration cells" in the pituitaries of spayed rats and rabbits was prevented by ovarian tissue or crystalline estradiol implanted in the ventral part of the hypothalamus, even when the amount of estrogen secreted by the graft or probe was too small to produce any stimulating effect on the reproductive organs of the host. Ovarian or estrogen implants in other parts of the hypothalamus were not effective (Kanematsu and Sawyer, 1963; Lisk, 1963). Another approach to the problem of establishing feedback sites of gonadal steroids is to study the uptake of radioactive labeled estrogen administered by systemic injection. Estrogen appeared to be taken up selectively by the "hypophysiotropic area," preoptic, and septal regions (Michael, 1964). Estrogen was virtually absent from other brain structures, although it was taken up by nonnervous target organs such as the uterus and vagina. Maximal
uptake by the hypothalamus occurred at 2-5 hours after injection and thereafter activity fell off.

Recently, attention has focused upon the limbic system as an area influencing gonadal function. In the past the rhinencephalon was thought to be associated only with olfactory function, but as its functions became known, (the regulation of a number of autonomic functions, certain endocrine actions, sexual behavior, reproduction, feeding, memory processes, and extrapyramidal activities) the label of "nose brain" no longer described its function. MacLean (1952) proposed that this phylogenetically old region of the forebrain should be called by the anatomically more descriptive and functionally noncommittal name of "limbic system".

The mechanism by which limbic structures modulate the function of the hypothalamo-hypophysial system is undoubtedly neural in character. DeGroot (1966), discussed the limbic system connections with the hypothalamus and neocortex, based on anatomical and electrophysiological studies. The connections of the fornix system has been studied extensively and the resultant clarification of the extent and location of fornix terminations underlines the importance of hippocampal efferents in hypothalamic function. Ipsilateral connections have been described between the hippocampal formation and the septal complex, certain thalamic nuclei, the lateral preoptic area, the lateral and other hypothalamic areas, the mammillary complex, the periventricular system, and the ventral tegmentum. Of particular interest is an efferent fornix fiber bundle terminating in the tuber cinereum close to the infundibulum of the pituitary
glan. The medial forebrain bundle extends between the olfactory tubercle cortex, the septal complex, the piriform cortex areas and the ventral grey matter of the mesencephalic tegmentum. This bundle is essentially an efferent limbic system projection pathway coursing through the lateral preoptic and hypothalamic areas. In addition, ascending fibers (mammillary peduncle) which form a distinct connection between the ventral tegmentum area and the mammillary nuclear complex, appear to play a role in the regulation of pituitary hormone release (Critchlow, 1958a). The fibrous connections with the amygdaloid nuclear complex include the stria terminalis as well as more direct pathways to and from the diencephalon. The latter form a more or less continuous, rather diffuse system of amygdalo-preoptic and amygdalohypothalamic fibers; the former relate to the septum complex and anterior hypothalamus. There are also connections with the basal ganglia and lower brain stem. Nauta (1956) confirmed the presence of a sizeable bundle of fibers in the fornix that project to the tuberal nuclei which sits astride the portal circulation of the pituitary. It was also learned that the limbic cortex has strong reciprocating connections with the central grey matter and the paramedian reticulum of the midbrain (Nauta and Kuypers, 1958).

It is likely that the functional state of the hypothalamus, as manifested by predominantly visceral and endocrine phenomena, essentially reflects the activation or inhibition of neural mechanisms represented in the limbic system-midbrain circuit. The overall function of this system appears to determine the 'attitude' of the organism toward its outer and inner environments. (Nauta, 1963)
Several authors have shown that separate structures within the limbic system can modify the secretion of gonadotropins. Koikegami et al. (1954) reported that stimulation of the medial portion of the amygdaloid nucleus in the rabbit induced ovulation. Ovulation has also been elicited by stimulation of the amygdala or septum pellucidum in rats (Bunn and Everett, 1957) and from stimulating the basal and lateral amygdala in cats (Shealy and Peele, 1957) in which spontaneous ovulation had been blocked by continuous illumination or pharmacological agents. Electrical stimulation of the medial amygdala or the "hypophysiotropic area" of the hypothalamus with parameters which induced ovulation was found by Hayward et al. (1964) to produce an immediate increase in ovarian progesterone output which continued to rise for a 30 minute period after stimulation. Control stimulation of closely adjacent regions of the hypothalamus and temporal lobes produced neither a rise in progesterone output or ovulation. Velasco and Taleisnik (1969) using rats maintained in persistent estrous induced by continuous illumination, could induce ovulation by stimulation of the medial nucleus or the basolateral complex of the amygdala. The ovulatory response to stimulation could be blocked by transection of the stria terminalis but not by interruption of the ventral amygdalo-fugal fibers. They also detected an increase in plasma LH and FSH after stimulation of the amygdala in either estrous or diestrous rats. These results give support to the idea that stimulatory influences on the release of gonadotropins can be evoked from the amygdaloid complex. This point of view is opposite to that
expressed by Elwers and Critchlow (1961) based on studies in immature rats, that the amygdala is an inhibitory structure, and the suppression of its influence causes precocious puberty and release of LH. Kawakami et al. (1968) showed that stimulating the alveus of the hippocampus induced ovulation in 23 out of 27 rabbits studied. Stimulation delivered to the cornus ammonis and fascia dentata failed to induce ovulation. These authors also found that stimulating the intermediate and medial nuclei of the amygdala would induce ovulation. Facilitation of biosynthesis of various progestin fractions accompanied ovulation but estrogen biosynthesis remained unaffected. However, the pattern of progestin biosynthesis was different from patterns obtained after copulation, LH administration, or stimulation of the arcuate nucleus. The affects of hippocampal stimulation on ovarian functions could be cancelled if the fornix or the periventricular arcuate nucleus was destroyed, thus indicating that a specific pathway is involved in the induction of ovulation from hippocampal stimulation. Lesions in the limbic system also affect sexual behavior. Lesions of the amygdala and in the underlying piriform cortex have been found to lead to hypersexualism (Goddard, 1964) or enhanced sexual behavior in males of various species (Green et al., 1957). Precocious ovarian stimulation following amygdaloid lesions in juvenile rats was reported by Elwers and Critchlow (1960). Lesions in the amygdala and the septum pellucidum of the female rabbit did not inhibit reproductive behavior or block copulation-induced ovulation (Sawyer, 1959), although reduced gonadotropin production
after bilateral ablation of the amygdala in the male rat were reported by Yamada and Greer (1960). Kim (1960) reported that rats with large dorsal hippocampal lesions increased the frequency of mounting acts by 50% over preoperative scores. Bermant et al. (1968) found that rats with dorsal hippocampal lesions progressively shortened the intervals between sexual responses. Lesions in the septal area also appear to effect sexual behavior. Hagamen et al. (1959) reported hypersexuality in 5 out of 13 cats with septal lesions, and Rasmussen et al. (1960) found that some rats with septal lesions would cross an electrified grid 400% more frequently than did control rats in order to reach a receptive female. Michal (1965) showed that animals with large septal lesions increased courtship activities and attempted mounts more often than animals with smaller lesions. A qualitative interpretation indicates that septal lesions increase courtship activities with no concomitant increase in the frequency of copulation.

Since it has been suggested from the stimulation and lesioning experiments that the limbic system, especially the hippocampus and amygdala, have an effect on ovulation, various groups have tried to find if the gonadal steroids can in turn influence limbic structures. While studying the effects of various hormones on the electrical activity of the whole brain, Selye (1942) reported a hypnotic effect following intravenous administration of ovarian steroids. Kawakami and Sawyer (1959) reported that progesterone injected into an estrous or estrogen
primed female rabbit lowered electroencephalogram (EEG) arousal and after reaction thresholds in the limbic system and hypothalamus. Kobayashi et al. (1962) found that when progesterone was injected intravenously it would induce a sleeplike EEG pattern lasting about one hour in the hippocampus of curarized rats. It was concluded from these two studies (Kawakami and Sawyer, 1959; Kobayashi et al., 1962) that progesterone suppressed the activity of the posterior hypothalamus via the limbic-midbrain area, thereby blocking ovulation. Selective effects of progesterone on hypothalamic neurons was postulated by Barraclough and Cross (1963) after reporting that progesterone blocked responsiveness to probing the vaginal cervix in rats but not to painful or thermal stimuli. Similar results have been attained by Chhina and Anand (1969) in the monkey. They found that the activity of single neuronal units located in different regions of the hypothalamus, amygdala and septum was suppressed following administration of estrogen when the vagina of mature and immature monkeys was stimulated with a glass rod. Komisaruk et al. (1967) attempted to analyze further the question of whether progesterone has a selective or generalized inhibitory effect on neurons. Single unit activity was recorded using monopolar electrodes in the thalamus, hypothalamus and arcuate nucleus. They found that progesterone inhibited neurons whose activity was linked to the general arousal level, but found no evidence for an effect of the steroid on independent neurons. Beyer et al. (1967) using microelectrodes recording from various diencephalic areas found no consistent effects of progesterone on
the electrical activity of the brain in rabbits. They did, however, detect transient spindling and a decrease in diencephalic background activity. Makino (1968) recorded hippocampal and amygdaloid activity in the rabbit using bipolar electrodes following an injection of 10 mgm of progesterone subcutaneously or 0.08 mgm estradiol intravenously. Makino found that progesterone had different effects on different parts of the limbic system; the amygdala was depressed while the hippocampus remained unchanged. Estrogen produced a different situation; the amygdala remained unchanged while the hippocampus was depressed. From these results, Makino suggested that progesterone and estrogen possess a different feedback action on the amygdala and hippocampus. Kawakami et al. (1966) set out to confirm the existence of a positive feedback loop between hippocampal activity and ovarian progesterone formation and output, as well as the existence of a negative feedback control loop between the medial amygdala activity and the ovarian progesterone formation and output. Using bipolar electrodes permanently affixed to the skull of rabbits, they reported that excitation of the hippocampus facilitated the release of gonadotropic hormones from the adenohypophysis via the hypothalamus. These discharged hormones caused an increase in both production and output of ovarian progesterone. This increased concentration of progesterone in the systemic circulation elevated the hippocampal activity while depressing that of the amygdala. They suggested that there may be a functional circuit between the hippocampus and the ovary having positive feedback control of the progesterone production and output, and a
circuit between the amygdala and ovary having negative feedback control. In 1967, Kawakami et al., in an extension of the above mentioned paper, showed that lesions in the dorsal fornix and arcuate nucleus but not the stria terminalis prevented ovulation and increased progesterone output following stimulation of the amygdala. Hippocampal induced ovulation could be blocked by bilateral lesions of the dorsal fornix or by massive lesions of the septum. From this work it was inferred that the enhanced activity of the hippocampus exerts influences somewhat different from those of the amygdala on the biosynthesis of sex steroids in the ovary. Tarasawa and Timiras (1968) utilized chronically implanted bipolar electrodes to follow the localized seizure threshold of the medial and lateral amygdala and dorsal hippocampus throughout the estrous cycle. Their data showed that localized seizure thresholds in the hippocampus and the amygdala change during the estrous cycle. These changes in seizure threshold may be interpreted to reflect changes in irritability of these areas. The time course of the threshold changes in the medial part of the amygdala was different from that in the hippocampus and lateral part of the amygdala. The threshold in the medial part of the amygdala decreased on the morning of proestrus and increased again by midnight of proestrus, while the thresholds in the hippocampus and lateral amygdala changed on the morning of estrus. It is possible that these alterations in thresholds reflect changes in plasma concentrations of gonadotropins instead of, or in addition to, changes in ovarian steroids. Tindal et al.
(1967) in an attempt to find structures in the limbic system which influence prolactin secretion implanted crystalline estradiol into the amygdala complex of pseudopregnant rabbits. They found that estrogen caused prolactin release as measured by increased mammillary development. Tindal's work showed that gonadal steroids implanted in the limbic system have an effect on the pituitary production of hormones.

These aforementioned results suggested that the hippocampus and amygdala play a role in the control of gonadal function. Little is known of the role played by the septum. For this reason, it was considered of interest to investigate the electrical activity of the hippocampus, amygdala and septum under the influence of gonadal steroids in a spontaneous ovulator, such as the rat. The first experiment utilized acutely implanted bipolar electrodes to follow the electrical activity of these brain areas subsequent to the administration of the steroids. The second experiment was concerned with locating sites in these structures that would respond to a direct hormonal stimulus and to determine the influence of this response on other structures of the limbic system.
CHAPTER II
METHODS AND MATERIALS

A. Animals

Ninety-six albino, virgin, female rats obtained from the Charles River Laboratories were used throughout the experiment. On the day of experimentation all animals weighed 200 to 300 gm. Vaginal smears were taken daily (between 8 and 10 A.M.) by lavage to determine the stage of the vaginal estrus cycle. Animals were housed in wire cages in a controlled environment of approximately 25.5° C, 40% humidity. A daylight period of 12 hours (6 A.M. to 6 P.M.) was maintained by using fluorescent bulbs attached to the four corners of the cage rack. Food and water were given Ad Libitum.

B. Surgery

On the day of appropriate vaginal smear (vaginal estrus or diestrus) animals were brought to the laboratory for surgery. The animals were injected with atropine sulfate (0.016 gm/100 gm body weight) and allowed to remain in their cages for 15 minutes. They were then placed in a Plexiglas etherization chamber, and anesthetized (Ether Squibb, anesthesia grade). The animal was placed on a surgery board and anesthesia maintained with an ether cone at a level conducive to surgery. The ventral skin over the trachea was cut, and
the trachea exposed by blunt dissection. The trachea was cut below the larynx and a polyethylene cannula (PE 280 – 3.2 mm O.D.) was inserted and secured with a cotton suture thread. The cannula was constructed with a small escape hole for expired air, one inch from the cannula tip. The animal was then connected to a Phipps and Bird small animal respirator (Model 70-886 positive pressure reciprocating piston) and respired with approximately 1 ml of room air 72 times per minute. The animal could be maintained under ether anesthesia by placing ether saturated cotton near the air intake of the respirator. Upon completion of this phase the animal was injected with tubocurare chloride (0.1 mgm/100 gm body weight).

C. Stereotaxis

A Kopf Small Animal Stereotaxis (Model 900) was used for implantation of recording electrodes. The animal was positioned in the stereotaxic apparatus and the tissue over the skull, around the ears, and in back of the neck was injected with xylocaine hydrochloride 2%. An incision was made to expose the skull. Bregma and lambda were leveled by elevating or depressing the tooth bar. Coordinates were determined from bregma according to the Slotnick-Thomas Atlas of the ratbrain (unpublished). The coordinates used were: lateral septum (ant. +0.5, lat. -0.9, vert. -5.5 mm); medial amygdala (ant. -2.0, lat. -4.0, vert. -10.0 mm); dorsal hippocampus (ant. -3.8, lat. -3.5, vert. -3.5 mm); and parietal cortex (ant. -2.0, lat. +2.0, vert. -1.5 mm). Holes were
drilled through the skull using a Foredom Model 7 Dental Drill and No. 33/4 dental burrs (right angle). Care was taken not to rupture the dura under the skull, so that during the course of the experiment drying of the exposed brain tissue would be kept to a minimum.

The trachea air escape hole was positioned so that expired air could escape. A thermister was inserted into the rectum, or a thermometer was placed under the abdomen to monitor body temperature. A 100 watt light bulb was positioned over the animal to maintain a body temperature of 35° C externally or 36.5° C rectally. Total time for surgery did not exceed twenty minutes.

**D. Electrodes**

Electrodes used in recording the electrical activity of the brain were made from strands of 36 gauge enameled nichrome alloy wire (27.63 ohms per foot, Driver-Harris Co.). To add strength, two strands of wire were twisted together to form bipolar electrodes. The insulation was scraped from each electrode 0.5 mm from the tip (Figure 1-A).

A cannula-electrode was prepared from 27 gauge stainless steel hypodermic tubing cut to two inches in length. A needle reamer for 27 gauge hypodermic needles was placed inside the hypodermic tubing and used as a trochar. The tip of the trochar was positioned so that it was just visible at the end of the tubing. The other end of the trochar was bent so that further movement inward was impossible. The nichrome electrode wire described above was twisted about the cannula so that bipolar
Figure 1. Bipolar Recording Electrode and Cannula-Electrode

A. Bipolar electrode used in recording the electrical activity of the brain. Prepared from two strands of nichrome alloy wire. B. Bipolar cannula-electrode. Prepared from hypodermic tubing and nichrome wire.
recordings could be made at the tip of the cannula. To insure adherence of the electrodes to the cannula, the assembly was dipped in varnish and dried overnight (Figure 1-B).

The cannula was filled by tamping it into powdered hormone and was then sealed by pressing paraffin wax over the opening at the tip of the cannula. Hormone and wax were removed from the outside of the cannula with the aid of a dissecting microscope.

Four electrodes, made from the twisted strands of wire, were cemented together with Caulk Dental Cement to form a single, rigid, re-usable recording unit. When the cannula-electrode was used, three of the bipolar electrodes were also implanted in the animal's brain. Construction of re-usable electrodes allowed quick completion of surgery in subsequent animals.

E. Hormones

In the first series of experiments animals were injected intraperitoneally (IP) with 1 ml of estradiol-17β (0.1 mg/ml) or progesterone (1.0 mg/ml) dissolved in propylene glycol. Hormones were obtained from the Sigma Chemical Company. Control animals were injected with 1 ml of propylene glycol. Six groups of six or more animals were used in the first series of experiments. The groups consisted of vaginal estrus animals injected IP with: 1) estradiol-17β, 2) progesterone, or 3) propylene glycol, and vaginal diestrus animals injected IP with: 4) estradiol-17β, 5) progesterone, or 6) propylene glycol.

In the second series of experiments those areas of the brain
which responded to the systemic injection of hormone were exposed to the hormones again, this time directly. The estradiol-17β or progesterone was tamped into the 27 gauge cannula, sealed with paraffin wax, and implanted into the brain of the experimental animals. The cannula always contained less than 25 µgm of hormone (Sauter Monopan Balance). Vaginal estrus animals were used exclusively in this part of the study since vaginal estrus animals responded more appreciably and consistently than vaginal diestrous animals to the systemic injection of the hormones. Control animals were implanted the same way, with cannulas containing only the paraffin wax. There were at least 7 animals per group.

F. Frequency Analyzing Equipment

The equipment used to record and analyze the electrical activity from the discrete areas in the brain is presented in block form in Figure 2. The electrical activity from the brain of the experimental animal was amplified with a Grass Model 5 Polygraph, and a recording was made from each of the four target areas. A record of the electrical activity of an animal could also be stored on magnetic tape (Scotch Brand 202) for later analysis using a Roberts 770X four track stereotape recorder with a FM adapter unit (A.R. Vetter Co., Model 2 WD).

The amplified electrical activity from the brain of the experimental animal was passed from the polygraph into the frequency analyzing apparatus. The analyzing apparatus was designed to switch consecutively through the four outputs of the
Figure 2. Block Diagram of the Equipment used in Recording and Analyzing the Electrical Activity of the Rat Brain.
polygraph and analyze the electrical activity of any one channel for as long as desired.

The frequency analyzing equipment utilized six band pass filters (White Instrument Co.) which allowed the passage of frequencies between 0.6-1.25, 1.25-2.5, 2.5-5.0, 5.0-10.0, 10.0-20.0, and 20.0-40.0 Hz. The center frequencies of these octave filters were 0.9, 1.7, 3.5, 7.0, 14.0 and 28.0 Hz. For convenience throughout the paper the output of the filters will be referred to as Band 0.9, Band 1.7, Band 3.5, Band 7.0, Band 14.0 and Band 28.0 respectively.

The filter outputs were passed through an AC to DC converter, sampled sequentially, and recorded as a histogram on the polygraph record. The filter outputs were also passed through analog to digital and digital to decimal converters so that the output levels could subsequently be printed on an electric Friden adding machine. Each channel of the polygraph was sampled for approximately one minute in order to obtain five sets of outputs from the band pass filters. Variations in the frequency bands could then be averaged.

The apparatus was capable of recycling itself, to repeat this procedure, every 30 minutes. Normally for the course of an experiment, recordings were made and analyzed at 0 (control), 15, 30, 60 and 90 minutes post injection.

Statistical analysis of the data was accomplished using the Wilcoxon Matched-Pairs Signed-Ranks Test (Siegel, 1956) as follows: Each band of the frequency analyzed signal was averaged, and the
corresponding control period band subtracted from it for each animal and each time period. The difference was divided by the control and converted to a percentage according to the formula:

\[
\frac{\text{Experimental} - \text{Control}}{\text{Control}} \times 100 = \text{percent change from control}
\]

The percent difference for all animals of the same experimental procedure were ranked according to size of change without respect to sign. To each rank the sign of the difference (+ or -) was then affixed. The sum of the ranks with a plus sign should equal the sum of the ranks with a negative sign if the Null hypothesis is correct (i.e., no change from control values). However, if the sum of the plus ranks is much greater than the sum of the negative ranks then the Null hypothesis is rejected. Rejection of the Null hypothesis was made at the 0.05 level in a two-tailed test.

G. Verification of Electrode Placement

At the termination of an experiment, the animal was disconnected from the recording assembly and the electrodes connected to a lesion producing device (C.H. Stoelting Co.). Each electrode site was marked with a DC current of 1 mA passing for 10 seconds. The animal was then removed from the stereotaxic instrument, the brain removed and placed in a 10% formalin solution, stored, and later transferred to a 4% potassium ferrocyanide-10% formalin solution for 48 hours, or placed fresh in the 4% potassium ferrocyanide-10% formalin solution for 48 hours to stain the deposited iron ions (Perls, M., 1867 quoted from Lillie, 1954). Upon removal
from the potassium ferrocyanide-10% formalin solution, the brains were quickly frozen in acetone-dry ice, trimmed, frozen to a sectioning planchet, and placed in a Harris International Cryostat (Model CT). All brains were sectioned at 50 micra and those sections showing a Prussian Blue stain at the site of the lesion were retained. Sections were then counterstained with Cresyl Violet. Electrode sites were ascertained using the Slotnick-Thomas Atlas in conjunction with the DeGroot (1959) Atlas of the rat brain.
CHAPTER III

RESULTS

A. Location of Recording Electrodes and Cannula-Electrodes

The sites of unilateral electrode placement are shown as hatched areas on the brain sections presented in Figures 3, 4, and 5. The reconstruction of the rat brain showing the septum, hippocampus, and amygdala were taken from the Slotnick-Thomas Atlas. Since the location of the recording electrodes and the cannula-electrodes showed considerable overlap between the two studies, the hatched areas represent the location of all electrodes placed in that area during the course of both experiments.

Only electrodes localized within the lateral septum, medial amygdala and doral hippocampus were included in this study. The location of the cortical electrodes is not shown since they were superficial (located 0.5 mm below the surface of the cortex) and difficult to locate, because the Prussian Blue stain washed out of the cortical lesion site following removal of the frozen sections from the cryostat. The cortical electrodes were positioned in parietal cortex as determined from electrodes which could be located and from the stereotaxic coordinates. The electrodes were located in an area of somatosensory function near the junction of the borders of areas 2, 4 and 6 according to the Krieg (1946) nomenclature.
Figure 3. Location of Recording Electrodes and Cannula-Electrodes in the Lateral Septum

Hatched area in the lateral septum includes the location of all recording electrode and cannula-electrode tips used in this study. CA: anterior commissure; CC: corpus calosum; CPU: caudate nucleus/putamen; LS: lateral septum. AP +0.5, 0.5 mm anterior from bregma.
Figure 4. Location of Recording Electrodes and Cannula-Electrodes in the Dorsal Hippocampus

Hatched area in the dorsal hippocampus includes the location of all recording electrode and cannula-electrodes tips used in this study. CC: corpus callosum; CI: internal capsule; HPC: hippocampus. AP -3.8, 3.8 mm posterior from bregma.
Figure 5. Location of Recording Electrodes and Cannula-Electrodes in the Medial Amygdala

Hatched area in the medial amygdala includes the location of all recording electrode and cannula-electrode tips used in this study. AME: medial amygdala; AL: lateral amygdala; CI: internal capsule; HPC: hippocampus. AP -2.0, 2.0 mm posterior from bregma.
B. Study I. The Effect of a Systemic Injection of Hormone on the Electrical Activity of the Brain

The averaged percent differences from the control period for each frequency band at different time intervals are shown in Figures 6-13. Frequency bands are plotted on the abscissa and percent differences from control values on the ordinate. Significant changes from the control periods (Wilcoxon Matched-Pairs Signed-Ranks Test) are represented by hatched areas on the graphs. The probability level of 0.05 or less was designated as significant using a two-tailed test.

With the above method of statistical approach each animal acts as its own control. To control carrier effects, two groups of animals, one vaginal estrus and the other vaginal diestrus were injected with propylene glycol to verify that the carrier had no effect on the animal.

1. Effect of a Systemic Injection of Propylene Glycol into a Vaginal Diestrus Rat.

Propylene glycol (1 ml) given IP to a vaginal diestrus rat produced no significant changes in the lateral septum, medial amygdala, dorsal hippocampus or parietal cortex, as shown in Figures 6-A, 7-A, 8-A, and 9-A. This lack of change following injection of the pure carrier substance into a vaginal diestrus rat indicates that the animal was not adversely affected by the injection itself and that the carrier substance (propylene glycol) had no effect on the electrical activity of the brain. Propylene glycol was then employed throughout the rest of this series of
Figure 6. Response of the Septum to IP Injection of Steroid Hormones in the Diestrous Rat

Hatched bars indicate significant changes in electrical activity (P<0.05, Wilcoxon Matched-Pairs Signed-Ranks Test). A. Response to carrier (propylene glycol) at 15, 30 and 60 minutes. Center frequencies of the filters used in measuring the electrical activity of the brain are given in Hertz. B. Response to estradiol-17β. C. Response to progesterone.
Figure 7. Response of the Hippocampus to IP Injection of Steroid Hormones in the Diestrous Rat

Hatched bars indicate significant changes in electrical activity (P<0.05, Wilcoxon Matched-Pairs Signed-Ranks Test). A. Response to carrier (propylene glycol) at 15, 30 and 60 minutes. Center frequencies of the filters used in measuring the electrical activity of the brain are given in Hertz. B. Response to estradiol-17\(^\beta\). C. Response to progesterone.
Figure 8. Response of the Amygdala to IP Injection of Steroid Hormones in the Diestrous Rat

Hatched bars indicate significant changes in electrical activity (p<0.05, Wilcoxon Matched-Pairs Signed-Ranks Test). A. Response to carrier (propylene glycol) at 15, 30 and 60 minutes. Center frequencies of the filters used in measuring the electrical activity of the brain are given in Hertz. B. Response to estradiol-17β. C. Response to progesterone.
Figure 9. Response of the Cortex to IP Injection of Steroid Hormones in the Diestrous Rat

Hatched bars indicate significant changes in electrical activity (P<0.05, Wilcoxon Matched-Pairs Signed-Ranks Test). A. Response to carrier (propylene glycol) at 15, 30 and 60 minutes. Center frequencies of the filters used in measuring the electrical activity of the brain are given in Hertz. B. Response to estradiol-17β. C. Response to progesterone.
experiments to act as a carrier medium for the hormones.

2. Effect of a Systemic Injection of Estradiol-17β into a Vaginal Diestrus Rat.

Following an IP injection of Estradiol-17β (0.1 mgm/ml) dissolved in propylene glycol into a vaginal diestrus rat, there were no significant changes in the electrical activity stemming from the dorsal hippocampus or medial amygdala. This can be seen in Figures 7-B and 8-B. However, by 30 minutes post-injection of the estrogen (Figure 6-B), the lateral septum showed a significant response to the hormone by an increase in the activity of the lower frequencies (Bands 3.5, 1.7 and 0.9). This response continued into the 60 minute recording involving more of the higher frequencies (Bands 7.0, 3.5, 1.7 and 0.9). The parietal cortex (Figure 9-B) showed no significant changes in the frequency bands studied at 15 and 30 minutes post-injection. At 60 minutes the lowest frequency band (Band 0.9) showed a significant increase in amplitude.

3. Effect of a Systemic Injection of Progesterone into a Vaginal Diestrus Rat.

After an IP injection of progesterone (1.0 mgm/ml) dissolved in propylene glycol into a vaginal diestrus rat, no significant changes were observed in the lateral septum or parietal cortex. This is shown in Figures 6-C and 9-C. The dorsal hippocampus (Figure 7-C) showed a significant increase in Band 7.0 by 15 minutes after the IP injection of progesterone. This response became more pronounced at 30 minutes by incorporating a larger number of frequency bands (Bands 7.0, 3.5 and 1.7). The signifi-
cant increase in these bands was still present at 60 minutes post-injection. The medial amygdala (Figure 8-C) displayed a significant decrease at 60 minutes in Band 3.5.

4. Effect of a Systemic Injection of Propylene Glycol into a Vaginal Estrus Rat.

Following an IP injection of propylene glycol (1 ml) into a vaginal estrus rat no significant changes were seen in the lateral septum, dorsal hippocampus or parietal cortex (Figures 10-A, 11-A, and 12-A). The medial amygdala (Figure 12-A) showed a significant change at 60 minutes post-injection in Band 0.9.

The lack of a major change following injection of the pure carrier material indicates that vaginal estrus animals do not respond to propylene glycol which is consistent with the lack of response shown by vaginal diestrus animals in section 1.

5. Effect of a Systemic Injection of Estradiol-17β into a Vaginal Estrus Rat.

In this part of the study no significant changes in the electrical activity occurred in the parietal cortex following an IP injection of estradiol-17β (0.1 mg/ml) dissolved in propylene glycol into a vaginal estrus rat. This is shown in Figure 13-B. The lateral septum displayed a significant increase in the middle frequency bands (Bands 7.0 and 3.5) at 30 minutes and in Bands 14.0, 7.0 and 3.5 at 60 minutes following the injection of estrogen (Figure 10-B). The dorsal hippocampus (Figure 11-B) also responded to the injection of estrogen showing significant increases in the center frequencies (Bands 7.0 and 3.5) by 30 minutes and in
Figure 10. Response of the Septum to IP Injection of Steroid Hormones in the Estrous Rat

Hatched bars indicate significant changes in electrical activity (P<0.05, Wilcoxon Matched-Pairs Signed-Ranks Test). A. Response to carrier (propylene glycol) at 15, 30 and 60 minutes. Center frequencies of the filters used in measuring the electrical activity of the brain are given in Hertz. B. Response to estradiol-17β. C. Response to progesterone.
Figure 11. Response of the Hippocampus to IP Injection of Steroid Hormones in the Estrous Rat

Hatched bars indicate significant changes in electrical activity (p < 0.05, Wilcoxon Matched-Pairs Signed-Ranks Test). A. Response to carrier (propylene glycol) at 15, 30 and 60 minutes. Center frequencies of the filters used in measuring the electrical activity of the brain are given in Hertz. B. Response to estradiol-17β. C. Response to progesterone.
Figure 12. Response of the Amygdala to IP Injection of Steroid Hormones in the Estrous Rat

Hatched bars indicate significant changes in electrical activity (P<0.05, Wilcoxon Matched-Pairs Signed-Ranks Test). A. Response to carrier (propylene glycol) at 15, 30 and 60 minutes. Center frequencies of the filters used in measuring the electrical activity of the brain are given in Hertz. B. Response to estradiol-17β. C. Response to progesterone.
Figure 13. Response of the Cortex to IP Injection of Steroid Hormones in the Estrous Rat

Hatched bars indicate significant changes in electrical activity (P<0.05, Wilcoxon Matched-Pairs Signed-Ranks Test). A. Response to carrier (propylene glycol) at 15, 30 and 60 minutes. Center frequencies of the filters used in measuring the electrical activity of the brain are given in Hertz. B. Response to estradiol-17β. C. Response to progesterone.
Bands 7.0, 3.5 and 1.7 by 60 minutes post-injection. By 60 minutes post-injection, Band 0.9 was significantly increased in the medial amygdala (Figure 12-B). The amygdala response is similar to that seen following control injections of propylene glycol (Figure 12-A).

6. Effect of a Systemic Injection of Progesterone into a Vaginal Estrus Rat.

Following a systemic injection of progesterone (1.0 mgm/ml) dissolved in propylene glycol into a vaginal estrus rat, all parts of the brain studied showed significant increases in electrical activity. The lateral septum did not respond significantly to the injection of progesterone until 60 minutes had lapsed (Figure 10-C). Significant increases occurred in Bands 7.0, 3.5 and 1.7 at the 60 minute recordings. The dorsal hippocampus responded significantly by 30 minutes post-injection (Figure 11-C) with increases in Bands 7.0 and 3.5, and at 60 minutes with Bands 14.0, 7.0, 3.5, 1.7 and 0.9. The medial amygdala (Figure 12-C), like the septum, did not show significant elevations until 60 minutes post-injection. At 60 minutes Bands 3.5 and 1.7 were significantly elevated over the control values. The parietal cortex (Figure 13-C) displayed an isolated increase at 30 minutes post-injection in Band 7.0 which was absent at the 60 minute recording time.

C. Study II. The Effect of Direct Hormonal Stimulation on the Electrical Activity of the Brain

The data derived from this study were analyzed in the same manner as in Study I. Again the data are presented graphically
to illustrate the changes in electrical activity which occurred in each experimental group (Figures 14-21).

The cannula-electrode, described in the section of Methods and Materials, was employed in this study to place hormones directly in contact with brain tissue, and to record the electrical activity of that area of the brain. The cannula-electrode was placed in one area of the brain in each animal studied. Recording electrodes were placed in the remaining three structures to record changes in activity that might occur. Only those areas of the brain which responded appreciably and significantly to systemic injections of the hormones were investigated in this study. The purpose was to see if these areas respond to direct hormonal stimulation.

Since the main portion of consistent positive results occurred in the vaginal estrus animals in the first study, it was decided to use only vaginal estrus animals for Study II.

Control experiments were run to see if changes in the electrical activity were due to damage resulting from placement of the cannula-electrode. Controls were implanted with the cannula-electrode covered at the tip with paraffin wax but without hormone. 1. Effect of Paraffin Wax Implanted Directly into the Lateral Septum in the Vaginal Estrus Rat.

Paraffin wax implanted into the lateral septum in vaginal estrus rats produced no significant changes in the electrical activity of the lateral septum itself, nor in the dorsal hippocampus, medial amygdala or parietal cortex (Figures 14-A, 15-A, 16-A and 17-A). The lack of significant change following implanta-
Figure 14. Response of the Septum to Implanted Steroid Hormones into the Septum of the Estrous Rat

Hatched bars indicate significant changes in electrical activity (P<0.05, Wilcoxon Matched-Pairs Signed-Ranks Test). A. Response to paraffin wax at 15, 30, 60 and 90 minutes. Center frequencies of the filters used in measuring the electrical activity of the brain are given in Hertz. B. Response to estradiol-17β. C. Response to progesterone.
Figure 15. Response of the Hippocampus to Implanted Steroid Hormones into the Septum of the Estrous Rat

Hatched bars indicate significant changes in electrical activity (P<0.05, Wilcoxon Matched-Pairs Signed-Ranks Test). A. Response to paraffin wax at 15, 30, 60 and 90 minutes. Center frequencies of the filter used in measuring the electrical activity of the brain are given in Hertz. B. Response to estradiol-17β. C. Response to progesterone.
Figure 16. Response of the Amygdala to Implanted Steroid Hormones into the Septum of the Estrous Rat

Hatched bars indicate significant changes in electrical activity (P<0.05, Wilcoxon Matched-Pairs Signed-Ranks Test). A. Response to paraffin wax at 15, 30, 60 and 90 minutes. Center frequencies of the filters used in measuring the electrical activity of the brain are given in Hertz. B. Response to estradiol-17β. C. Response to progesterone.
Figure 17. Response of the Cortex to Implanted Steroid Hormones into the Septum of the Estrous Rat

Hatched bars indicate significant changes in electrical activity (P<0.05, Wilcoxon Matched-Pairs Signed-Ranks Test).
A. Response to paraffin wax at 15, 30, 60 and 90 minutes. Center frequencies of the filters used in measuring the electrical activity of the brain are given in Hertz. B. Response to estradiol-17β. C. Response to progesterone.
tion of the cannula-electrode and the injection of the paraffin wax into the brain tissue via the trochar indicates that the brain tissue surrounding the cannula was not damaged sufficiently to cause a change in the electrical activity of this structure which would persist to the 15 minute recording. It also shows that the paraffin wax was inert as far as this study was concerned, and could be used with confidence to seal the cannula tip to prevent premature exposure of the brain to the hormones.

Since the other areas of the brain also remained significantly unchanged it is theorized that the damage to the septum did not affect their level of activity.

2. Effect of Estradiol-17β Implanted Directly into the Lateral Septum in the Vaginal Estrus Rat.

Following the injection of estradiol-17β (less than 25 µgm) into the lateral septum of a vaginal estrus rat, there were no significant changes in the electrical activity of the medial amygdala or the parietal cortex. This is shown in Figures 15-B and 16-B. At 15 minutes post-injection of estradiol-17β into the septum (Figure 14-B), there was a significant elevation of electrical activity in the lateral septum (Bands 7.0, 3.5, 1.7 and 0.9). This significant increase persisted at 30, 60 and 90 minutes post-injection and encompassed all the frequency bands studied (Bands 28.0, 14.0, 7.0, 3.5, 1.7 and 0.9). The dorsal hippocampus showed no significant increases in frequencies at the 15 minute recording (Figure 15-B). By 30 minute post-injection of estradiol-17β into the lateral septum, there was a significant increase in the
hippocampus in Bands 3.5 and 1.7 which continued to be significant at the 60 minute recording. At 90 minutes, only Band 3.5 remained significantly elevated above control levels.

3. Effect of Progesterone Implanted Directly into the Lateral Septum in the Vaginal Estrus Rat.

When progesterone (less than 25 µgm) was implanted into the lateral septum of vaginal estrus rats no significant changes occurred in the medial amygdala or the parietal cortex (Figures 16-C and 17-C). By 15 minutes after the injection of progesterone into the lateral septum (Figure 14-C) there was a significant elevation in the electrical activity in the lateral septum (Bands 14.0, 7.0, 3.5, 1.7 and 0.9). At 30, 60 and 90 minutes all Bands were significantly elevated (Bands 28.0, 14.0, 7.0, 3.5, 1.7 and 0.9). The dorsal hippocampus (Figure 15-C) also responded to the progesterone implanted into the lateral septum but not until 30 minutes post-injection. At the 30 minute recording there were significant increases in Bands 14.0, 7.0, 3.5 and 0.9 and at 60 and 90 minutes post-injection significant elevations were present in Bands 14.0, 7.0, 3.5 and 1.7.

4. Effect of Estradiol-17β Implanted Directly into the Dorsal Hippocampus of the Vaginal Estrus Rat.

When the cannula-electrode containing estradiol-17β (less than 25 µgm) was placed into the hippocampus of vaginal estrus rats, no significant changes occurred in the lateral septum, dorsal hippocampus or the medial amygdala (Figures 18-A, 19-A and 20-A). The parietal cortex remained unchanged during the 15, 30 and 60 minute recordings, but there was a statistically significant elevation
Figure 18. Response of the Septum to Implanted Steroid Hormones into the Hippocampus of the Estrous Rat

Hatched bars indicate significant changes in electrical activity (P<0.05, Wilcoxon Matched-Pairs Signed-Ranks Test). A. Response to estradiol-17β at 15, 30, 60 and 90 minutes. Center frequencies of the filters used in measuring the electrical activity of the brain are given in Hertz. B. Response to progesterone.
Figure 19. Response of the Hippocampus to Implanted Steroid Hormones into the Hippocampus of the Estrous Rat

Hatched bars indicate significant changes in electrical activity (P<0.05, Wilcoxon Matched-Pairs Signed-Ranks Test). A. Response to estradiol-17β at 15, 30, 60 and 90 minutes. Center frequencies of the filters used in measuring the electrical activity of the brain are given in Hertz. B. Response to progesterone.
Hatched bars indicate significant changes in electrical activity (P<0.05, Wilcoxon Matched-Pairs Signed-Ranks Test). A. Response to estradiol-17β at 15, 30, 60 and 90 minutes. Center frequencies of the filters used in measuring the electrical activity of the brain are given in Hertz. B. Response to progesterone.
Figure 21. Response of the Cortex to Implanted Steroid Hormones into the Hippocampus of the Estrous Rat

Hatched bars indicate significant changes in electrical activity (P<0.05, Wilcoxon Matched-Pairs Signed-Ranks Test). A. Response to estradiol-17β at 15, 30, 60 and 90 minutes. Center frequencies of the filters used in measuring the electrical activity of the brain are given in Hertz. B. Response to progesterone.
in Bands 7.0 and 3.5 at the 90 minute recording period.

5. Effect of Progesterone Implanted Directly into the Dorsal Hippocampus of the Vaginal Estrus Rat.

The implantation of less than 25 µg of progesterone into the dorsal hippocampus of the vaginal estrus rat resulted in no significant changes in the dorsal hippocampus, lateral septum and medial amygdala (Figures 18-B, 19-B, and 20-B). The parietal cortex remained unchanged until 90 minutes into the experiment. Band 1.7 was significantly elevated at this point.

Because there were basically no significant responses from implantation of estradiol-17β and progesterone into the dorsal hippocampus, no control injections of paraffin wax were employed.

D. Summary of Results

1. The parietal cortex did not show a consistent response to hormonal stimulation. There was a larger amount of variability in the electrical activity of the parietal cortex than in the other structures studied.

2. The medial amygdala responded to an IP injection of progesterone in vaginal diestrus animals and to estradiol-17β and progesterone in vaginal estrus animals but only after prolonged periods of time (60 minutes post-injection).

3. The dorsal hippocampus responded to IP injections of estrogen in vaginal estrus animals (30 minutes) while IP injections of progesterone produced increases in the electrical activity of the dorsal hippocampus in both vaginal diestrus (15 minutes post-injec-
tion) and vaginal estrus (30 minutes post-injection) animals. Following direct stimulation of the lateral septum with either estrogen or progesterone, there was an increase in the electrical activity of the hippocampus within 30 minutes. The hippocampus did not respond to direct gonadal steroid stimulation.

4. The medial septum responded with an increase in electrical activity following an IP injection of estradiol-17β into either vaginal diestrus or vaginal estrus animals within 30 minutes. The septum also responded (by 60 minutes) to IP injections of progesterone into vaginal estrus animals only. Following direct implantation of either progesterone or estradiol-17β into the septum, there was an immediate increase in electrical activity.
CHAPTER IV
DISCUSSION

In order to consider a neural area as a possible receptor site for gonadal steroids, certain criteria, determined during the course of this study, are suggested: 1) the structure must respond to direct hormonal stimulation; 2) changes in the electrical activity of the structure in question should occur within a relatively short period of time, and direct hormonal stimulation of the structure should elicit changes more rapidly than systemic hormonal stimulation; 3) more than one of the frequency bands employed should show a change, mainly within the normal frequency spectrum of the rat brain as observed in this study (Bands 3.5, 7.0 and 14.0); 4) the change in the frequency spectrum should be stable, once there is a response, this response should continue at least into the following recording period.

The lateral septum was the only structure studied that can be considered as a receptor for gonadal steroids using the above mentioned criteria. The septum responded to IP injections of estrogen in vaginal diestrus rats. The septum also responded to direct stimulation in estrous rats by both estrogen and progesterone with a rapid change in the electrical activity of the
normal frequency spectrum. This change in electrical activity
was stable and continued through 90 minutes of recording. Follow­
ing the IP injection of progesterone into estrous rats (Figure 10-C)
a discrepancy from the time to response criterion was noted. The
septum did not respond until 60 minutes post-injection. The septum,
however, did show an increase at 30 minutes post-injection in 5 out
of 6 animals tested, suggesting that the septum is responsive to
progesterone at this earlier time. The septum thus appears to be
a central nervous system receptor for estrogen and progesterone.
This thesis is supported by the works of Michael (1965) and Green et.
al. (1969) who showed that the lateral and medial septal area, as
well as the preoptic region and hypothalamus, accumulated tritiated
estradiol following a systemic injection.

The dorsal hippocampus did not respond to direct gonadal
steroid stimulation and, therefore, cannot be considered as a
receptor site. The hippocampus did show an increase in electrical
activity following IP injections of estrogen into diestrous rats,
and estrogen and progesterone into vaginal estrus rats. There
was also an increase in the electrical activity of the hippocampus
15 minutes after the response of the septum to direct hormonal
stimulation. It appears that gonadal steroids can affect the
hippocampus, not directly, but through other neural structures,
including the septum.

The response in vaginal diestrus rats to gonadal steroids
appears to be similar, in some respects, to the responses seen
in vaginal estrus rats. Diestrous rats were injected with the
gonadal steroids randomly throughout the vaginal cycle. This factor alone, variable levels of endogenous hormones, may have an effect on the sensitivity of the structures studied. Following IP injections of estrogen into diestrous rats, the septum responded as it did in estrous rats, but there was no significant increase in the electrical activity of the hippocampus. Progesterone administration IP into diestrous rats was able to elicit a response in the hippocampus without there first being a response in the septum (Figure 7-C). It is possible that in diestrous rats the hippocampus is able to respond to gonadal steroids differently than in estrous rats, or that other structures in the brain, which are able to respond to the gonadal steroids but are masked during estrous, may influence the hippocampus. In diestrous rats these other structures do not necessarily include the septum.

The results obtained in this study might be interpreted as being due to factors other than the ones suggested. The possibility exists that some of these effects are due to migration of the steroids away from the implantation site, causing activation of other structures which in turn stimulate the septum to respond. Michael (1961), using C14 labeled estrogen, fused to the tip of a needle which was implanted into the brain of a cat, showed that radioactivity migrated less than 500 micra from the edge of the implant site, even when the brain was exposed to the hormone for up to fifty days. Booth (1967), applying solutions of neurochemicals via 27 gauge needles, reported distances between effective and ineffective sites in the brain of as little as 100 micra. Routten-
berg and Bondareff (1969), using fluorescent histochemical and autoradiographic studies of chemicals applied to subcortical structures, reported that the sphere of chemical spreading of an isotonic solution from the cannula, may be at least 1 mm in diameter. Diffusion of the crystalline hormones in the present study would occur rapidly and might account for the results if the hormones diffused into the ventricle. An argument against this is that implants directly in the ventricle caused no changes in the electrical activity of the hippocampus, amygdala and cortex (four cannula-electrodes containing estradiol-17B and one containing progesterone). If the implanted hormones in the septum are indeed migrating to some other structure in the brain, this other structure could cause the septum to appear to be responding as if it were being stimulated directly. However, when the septum responded to implanted steroids, verified as being in the septum, there was always an associated increase in the electrical activity of the hippocampus. Steroid implants, verified as being in the ventricle, evoked no changes in the electrical activity of the hippocampus. Thus, it seems unlikely that the hormones were migrating into the ventricle and stimulating the septum via some other structure in the brain. Montgomery and Singer (1969) have found that stimulation of the brain could be induced by osmotic effects of hypertonic solutions, powders and crystals. In the present study, hormone powders might have caused this type of osmotic stimulation, however, hormone powders placed in the hippocampus did not induce changes in the electrical activity in this structure,
providing an argument against the thesis of osmotic stimulation. It may also be argued that the results obtained in this experiment were due partially to the decline in the viability of the preparation. All animals used in this study were alive through 90 minutes of recording and in some cases up to three hours of recording. Animals with control injections of propylene glycol did not vary from pre-injection values during the course of the experiment, indicating that the animals were viable. To insure that data were reliable, vital signs were monitored. Those animals which showed abnormal neural activity during the experiment (i.e., sudden depression, excessive spiking) are not reported.

The latencies between injection and response in the present study are similar to data reported by other investigators and help to establish the authenticity of the receptor site in the septum. Following the IP injection of the gonadal steroids there was a delay of approximately 30 minutes before significant changes occurred in the structures studied. This is in accordance with data obtained by Kawakami and Sawyer (1959) who showed that there was a drop in the cortical arousal threshold of the rabbit by one hour following a subcutaneous (SC) injection of progesterone in oil. Makino (1968) injected both progesterone and estrogen subcutaneously and found that the threshold of the EEG after reaction did not start to fall until one hour post-injection into rabbits. Beyer et al. (1967), Kobayashi et al. (1962), and Komisaruk et al. (1967) all reported effects on brain activities within 3-10 minutes following injections of progesterone and
estrogen, dissolved in propylene glycol, intravenously (IV) into rats and rabbits. In the second series of experiments in the present study, the effects of progesterone and estrogen placed in contact with the brain were reported to occur by 15 minutes post-injection. However, this was the time of the first post-injection recording. Monitoring of the records showed that the effects of the hormones were mediated within 3-5 minutes following injection. These time courses seem appropriate (30-60 minutes IP or SC, 3-10 minutes IV, 3-5 minutes implanted) for the injected material to reach a sufficient concentration to evoke a response in neural tissue. The time course of the response further supports the thesis that the responding structures react to gonadal steroids. The delay in response of the hippocampus following direct hormonal stimulation of the septum, is indicative of communication between the septum and the hippocampus. Studies of major hippocampal afferent pathways have been made by Petsche et al. (1962). In this work he showed that the control of the hippocampal arousal activity was affected by septal neurons, and that septal ablation prevented hippocampal arousal. Anatomical connections have been described between the septum and hippocampus (Daitz and Powell, 1954; reviewed by DeGroot, 1966) and are supplemented by electrophysiological studies (Brugge, 1965). Brugge showed that electrical stimulation of the septum would cause increased activity in the hippocampus. From these studies and others mentioned earlier it appears that the septum is able to influence the activity of the hippocampus. The present study indicates that septal driving of
the hippocampus may be accomplished by chemical stimulation as well.

Previous workers have shown that the limbic system is involved in the mechanism of ovulation. Experiments concerning stimulation and lesioning of the amygdala showed that the amygdala undoubtedly plays some role in the ovulating mechanism of the rat. Ovulation occurred following stimulation of the lateral amygdala (Bunn and Everett, 1957; Shealy and Peele, 1957) or the medial amygdala (Hayward et al., 1964; Koikegami et al., 1954; and Velasco and Taleisnik, 1968). Lesions placed in the amygdala that block ovulation (Yamada and Greer, 1960) have been rather extensive and have included portions of both the lateral and medial nuclei. Makino (1968), Kawakami et al. (1968) reported that progesterone implants in the medial amygdala were without effect in changing the steroid biosynthetic pattern of the ovary, and Kawakami et al. (1967) reported that progesterone decreased the activity of the amygdala and that this perhaps represented a negative feedback loop. The medial amygdala, as shown in the present work, failed to meet the criteria of a hormonal receptor site in the limbic system, but did show isolated, inconsistent changes in electrical activity following progesterone and estrogen administration. In light of the evidence that the amygdala plays a definite role in ovulation, and that it does not respond as a receptor site to gonadal steroids, the role of the amygdala must be neural in character. Experimental evidence indicates that the hippocampus must also be involved in ovulation. Kawakami et al. (1968) showed that following stimulation of the dorsal hippocampus ovulation
occurred in rabbits when the fimbral-fornix system was intact. Makino (1968), Kawakami et al. (1966), Kobayashi et al. (1962) and Tarasawa and Timiras (1968) reported evidence for a feedback loop between hippocampal activity and ovarian function. From the data presented in this study, the hippocampus does not appear to be a receptor site for the feedback control of gonadal steroids, but it may be important in the mechanism of ovulation since gonadal steroids influence its activity via other structures in the brain. Unfortunately, there has been little work on the role of the septal nuclei in regards to the endocrine system. Bunn and Everett (1957) stimulated the septum pellucidum in one rat and found evidence of ovulation. Lesions in the septum pellucidum (Sawyer, 1959) of the rabbit did not inhibit reproductive behavior or block copulation-induced ovulation, but similar studies have not been carried out in a spontaneous ovulator such as the rat. The lateral septum, with its ability to respond to gonadal hormones directly and also its ability to drive the hippocampus, must be considered important in neuroendocrine mechanisms. Whether this septal receptor is important in the endocrine control of the hypothalamus or in the expression of reproductive behavior, must be determined by future studies.
Female, virgin, albino rats were studied to determine areas in the limbic system that respond to gonadal steroids. Bipolar electrodes were stereotaxically placed in the lateral septum, dorsal hippocampus, medial amygdala and parietal cortex. Animals were injected with turbocurare and artificially respirated. Intraperitoneal injections of estradiol-17β and progesterone in propylene glycol were given and the electrical activity of limbic structures was recorded and a frequency analysis made. Those areas which responded to systemic treatment were implanted with the same gonadal steroids using a recording cannula-electrode. The parietal cortex showed no significant change in activity that was consistent throughout the experiment. The medial amygdala showed decreased electrical activity in one analysis Band following IP injection of progesterone into diestrous animals and increased activity in one analysis Band following estradiol-17β and progesterone in estrous animals. The responses occurred 60 minutes following the hormone injection and were not explored further. The dorsal hippocampus responded to IP injections of both estradiol-17β and progesterone in diestrous and estrous rats. The hippocampus did not respond to direct stimulation with the gonadal steroids. When the septum was
stimulated directly with either progesterone or estradiol-17β there was an increase in the electrical activity of the hippocampus within 30 minutes. The lateral septum responded to injections of both estradiol-17β and progesterone in diestrous and estrous animals. Following direct implantation of either estradiol-17β or progesterone into the lateral septum, there was an immediate increase in the activity of the septum. The conclusions obtained from this are:

1. Of the limbic structures studied, the lateral septum appears to be the only receptor for gonadal steroids.

2. Activity of the septal receptor is able to influence the level of activity of the hippocampus, however, the hippocampus may also be influenced by other brain structures which respond to the gonadal steroids.


