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CONDON, TIMOTHY PETER

REGULATION OF GONADOTROPIN SECRETION IN THE RAT: ROLE OF OVARIAN INHIBIN

The Ohio State University

Ph.D. 1981

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REGULATION OF GONADOTROPIN SECRETION IN THE RAT:
ROLE OF OVARIAN INHIBIN

DISSERTATION

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

By

Timothy Peter Condon, B.S., M.S.

****

The Ohio State University

1981

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DEDICATION

I dedicate this dissertation to my mother and my father whose love and sacrifices over the years have helped make this a reality.
ACKNOWLEDGMENTS

I wish to thank Dr. John "the chief" Curry for his support and often needed encouragement during my stay at Ohio State. I also wish to thank the other members of my advisory committee; Dr. Margaret T. Nishikawara, Dr. Jack Boulant, and Dr. Jim Grossie. I wish to express my sincere thanks to my co-worker Bob Leipheimer for his help and encouragements during these experiments.

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INTRODUCTION

Reproductive function has for many years been a topic that has aroused much interest and scientific investigation. This is perhaps because most individuals of the species *Homo sapiens* find themselves concerned with some aspect of reproductive function during periods of their lives, some perhaps much more so than others. In remote antiquity, the first observations of castrate men were made implicating the gonads in reproductive function. These early observations, as well as those which followed over the centuries, suggested that the gonads were solely responsible for reproductive function. These early observations led the way to scientific investigation of this topic eventually elucidating the presently accepted concept of nervous-pituitary-gonadal interaction in this process.

In the study of various aspects of reproductive function, the laboratory rat has become the model of choice. The female of the species displays a four or five day estrous cycle as first described by Long and Evans (2). It becomes apparent then, that this animal is a good model to study reproductive function in a spontaneous ovulator because of the frequency of ovulation. Because of pioneering work of Everett and co-workers (3-5), it is now generally accepted that the central nervous system (CNS) in this animal plays a major role in regulating reproductive cyclicity. Although many structures of the CNS may be involved in this process, particular importance has been given to various hypothalamic structures. The medial basal
hypothalamus (MBH) of the male and female rat contains peptidergic neurons which secrete gonadotropin releasing hormone(s) into the hypophysial portal system (6). This neural system is believed to be responsible for the tonic release of gonadotropins in both sexes. In the female rat the current thinking is that the neural signal responsible for cyclic release of luteinizing hormone (LH) and perhaps follicle stimulating hormone (FSH) arise from, or is integrated in, the preoptic-anterior hypothalamic area (POA-AHA) (7). Once releasing hormone(s) enters the portal vasculature, it is carried to the gonadotropin secreting cells of the adenohypophysis. There the releasing hormone(s) stimulates the release and/or synthesis of the gonadotropins from the adenohypophysis which then enter the systemic circulation and are carried to their target glands, principally the gonads. In the male, LH and FSH promote spermatogenesis and steroidogenesis in the testis. In the female, the target organs for the gonadotropins are the ovaries where they affect folliculogenesis, ovulation and as in the testis, steroidogenesis. The secretions from the testes once stimulated by the gonadotropins feed back to the pituitary gland and the hypothalamus via the circulation to regulate further gonadotropin release and/or synthesis by the former and release and/or synthesis of the releasing hormone(s) by the latter. In the female, however, the feedback regulation is more complex. The rhythmic nature of the reproductive process in the female rat is clearly demonstrated by the temporal pattern of change in the plasma levels of several hormones. In the intact cycling rat plasma, LH and FSH remain low throughout most of the
estrous cycle except for the precipitous rise of LH and FSH on the afternoon of proestrus, FSH remaining elevated until the morning of estrus (8,9,10). It is thought that the gonadotropins are held in a state of feedback inhibition by ovarian hormones throughout a large portion of the cycle. However, the rapidly rising levels of ovarian steroids, in particular estradiol, on proestrus in some manner exert a positive feedback on the pituitary and the hypothalamus which results in the proestrus surge of LH and FSH. Plasma LH levels remain elevated for a relatively brief period returning to near baseline values later in the evening of proestrus. FSH, however, shows a different pattern of release. Earlier studies (8,9) reported that FSH levels rise concomitantly with the proestrus LH surge, but remained elevated until the morning of estrus. More recent work reports that this FSH elevation is actually two distinct peaks (10). The first surge of FSH coincides with the proestrus LH surge approximately between 1500-1900 hrs with plasma FSH levels declining slightly later on proestrus. This decline in FSH is followed by a secondary FSH surge which peaks some time during the early morning of estrus (0400 hrs) and which remains elevated above baseline until the early afternoon of estrus. It has been suggested that this prolonged release of FSH serves to stimulate the ovary for the next estrus cycle rather than having a major role in ovulation that occurs early in the morning of estrus (11). This secondary FSH surge is of interest since it
demonstrates one instance in the normal cycle of the rat when there is a selective increase in one gonadotropin independent of the other. It becomes apparent then that the control of LH and FSH secretion in the female rat cannot be explained in terms of simple negative feedback control by ovarian steroids. Since there are periods when these ovarian hormones exert a positive feedback on the hypothalamic-pituitary system, and there are instances of selective release of one gonadotropin independent of the other, some other mechanism(s) most probably are involved.

Related to the selective rise in FSH seen in the intact rat is the pattern of gonadotropin release following bilateral ovariectomy in the female rat. For some time it has been known that removal of the ovaries in the rat leads to eventual increases in serum levels of LH and FSH (12). These increases are thought to occur in response to the removal of estrogen suppression. However, more recently, attention has been given to the temporal pattern of gonadotropin release immediately following removal of the ovaries. Serum LH shows a delay of 2-4 days before it responds to ovariectomy (13-16). In contrast, however, is the response of FSH to ovariectomy. Serum FSH rises rapidly within 5 hrs of surgery on metestrus and continues to rise for several weeks (15,16). In contrast to LH, the FSH rise post-ovariectomy is not affected by the stage of the estrous cycle in which surgery occurs (15). Estradiol administration has been shown to suppress ovariectomy induced high levels of serum LH (12,14) but only partially suppresses serum FSH (14,17,18). Administration of dihydrotestosterone or testosterone will decrease serum LH in ovariectomized rats but fails to fully suppress
serum or plasma FSH levels (17,19,20). Physiological doses of pro-
gesterone are ineffective in lowering post-castration rises in LH or FSH (12,17).

In view of the above findings it is not unreasonable to conclude that FSH secretion in the female rat may be under slightly different control mechanisms that is LH. Since removal of the ovaries results in such a rapid rise in FSH compared with LH and since steroid replacement cannot fully suppress the FSH rise, it has been suggested that FSH may be controlled by a different neural mechanism than LH or that perhaps the ovary secretes some other substance that controls FSH secretion. This latter possibility will be discussed later.

Although at the present time there is no general concensus as to the mechanism by which FSH may be selectively secreted independent of LH as stated above, a number of possibilities exist. One of the more controversial questions is whether the decapeptide LHRH is the releasing hormone for FSH as well as LH or whether there exists an addition FSHRH. With the isolation, sequencing and subsequent synthesis of LHRH by Schally and co-workers the decapeptide has been shown to stimulate both LH and FSH secretion (21,22). In support of their declaration that "one hypothalamic hormone designated LHRH/FSHRH could be responsible for stimulating the release of both FSH and LH from the anterior pituitary gland", Schally and co-workers demonstrated that differential secretion of LH and FSH in response to LHRH could be obtained by altering the steroid milieu (21-23). The results of other investigators obtained with pituitary cell cultures have supported this.
These studies showed that estrogens increased pituitary basal secretion and LHRH responsiveness of pituitary cell cultures while androgens markedly inhibited LH release while stimulating FSH secretion.

In contrast to these studies, several others have shown the existence of additional hypothalamic factor(s) which can selectively stimulate FSH secretion (27-30). Bowers and co-workers (27,28) have isolated fractions of porcine hypothalami which can stimulate secretion of greater amounts of FSH than LH whereas the decapeptide LHRH under the same conditions will stimulate release of more LH than FSH. This biologically active FSHRH is both antigenically and biochemically distinct from the decapeptide (27-30). If in fact a separate FSHRH does exist, it would seem reasonable that this hypothalamic hormone may be one of the factors responsible for selective FSH secretion under various conditions.

Support of the hypothesis that LHRH is also FSHRH comes not only from the studies discussed previously concerning changes in hormonal milieu but also from a convincing study by Wise, et al., (31). This study in rats demonstrated that by specific infusion regimens of LHRH, selective FSH secretion could be elicited. This regimen consisted of a priming infusion of LHRH followed by a rest interval and subsequent infusion of a concentration of LHRH which by itself would not elicit FSH secretion. They suggested that the brief initial
infusion period (30 mins) of LHRH renders FSH gonadotrophs responsive to LHRH concentrations which are normally ineffective in evoking FSH release. The initial infusion may mobilize a greater releasable pool of pituitary FSH failing to prime the LH gonadotrophs to a comparable degree. Alternatively, some investigators have concluded that both hormones (LH and FSH) are produced by one gonadotroph (32,33). If this is the case, then a plausible explanation might be that changing the concentrations and the duration of time that LHRH is presented to this cell may change its secretory characteristics from a cell which releases predominantly LH to one which secretes FSH.

In view of the studies presented above, the question arises that if indeed selective FSH secretion can be regulated by differential release patterns of LHRH, by what mechanism does this specific temporal release pattern come about. One possibility is related to the growing evidence suggesting a neural control system for FSH independent of that for LH. Numerous studies using brain stimulation, lesions and deafferentation in rodents support this concept (18,29,34-36) however, only a few pertinent studies will be discussed. In the same study cited above, Wise, et al., demonstrated that low intensity stimulation of the preoptic area in Nembutal-blocked proestrous rats also resulted in selective increases in plasma FSH. By the stimulation parameters used, an attempt was made to induce the endogenous release of LHRH in patterns and concentrations similar to those produced by their infusion protocols of exogenous LHRH. Chappel, et al., (18,34) showed that
female rats bearing electrolytic lesions of the dorsal anterior hypotalamic area including the paraventricular hypothalamus (DAHA-PVH) appear to cycle normally with normal patterns of proestrous gonadotropin release. In these animals, however, the post-castration rise in plasma FSH is suppressible by estradiol only when such lesions are present. These findings led Chappel, et al., to conclude that there exists two levels for regulation of FSH secretion in the CNS, one estrogen sensitive (preoptic-tubereral system) and the other estrogen "insensitive" (DAHA-PVH). In addition to these findings, is the fact that unilateral electrochemical stimulation of this area (DAHA-PVH) in pentobarbital blocked rats can cause selective release of FSH without LH increases (18). Lumpkin and McCann (29) reported that bilateral radiofrequency lesions placed in the dorsal anterior hypothalamic area - paraventricular nucleus (DAHA-PVN) of chronically ovariectomized female rats produced serum FSH levels 33% lower than those of control or sham operated animals. In these same animals serum LH values did not differ from control levels.

Using a different model, a recent report by Nance, et al., (36) provides additional evidence for a separate FSH neural control system in prepubertal male rats. In these animals unilateral castration results in a rapid rise of FSH followed by subsequent compensatory testicular hypertrophy (CTH) with little or no change in serum LH. In male rats bearing hypothalamic hemi-islands unilateral castration on the contralateral side results in marked increase in serum FSH comparable to unilaterally castrated rats with sham brain surgery. However,
hypothalamic hemi-island rats which were unilaterally castrated on the ipsilateral side failed to show an increase in serum FSH. Serum LH values were comparable in all groups.

Considering the studies reviewed above, it seems plausible that a separate neural system for FSH may indeed exist. The exact nature and mode of regulation of this system awaits further elucidation but in view of our current knowledge of the LH regulating system, we might speculate on the nature of a FSH neural control mechanism. If FSHRH is, in fact, a component of this system then the selective increases in FSH become relatively easy to explain. If, however, LHRH is also FSHRH, then an explanation, although possible, will by necessity be more complex. Essential to such an explanation would be some mode of modulation of the LHRH peptidergic neurons of the hypothalamus. This modulation, evoking specific releasing hormone discharge patterns would result in selective changes in gonadotropin release. It is generally accepted that ovarian secretions, in particular gonadal steroids, in some manner modulate the neural system responsible for the proestrus surges of LH and FSH. In addition, as discussed above, there is evidence that gonadal steroids can modulate pituitary responsiveness to releasing hormone(s). It is reasonable, therefore, to speculate that modulation of a specific FSH control system would also result from changes in hormonal milieu. These changes in hormonal milieu may act on the preoptic-tuberal LHRH neurons themselves or on other neural areas such as DAHA-PVH having afferent inputs on the LHRH neurons.
It becomes apparent that because of the complexity of these systems, fine control mechanisms may be exerted at a number of different levels, i.e., CNS, pituitary. However, the question arises as to the exact locus of this control and the nature of these factors. The gonadal steroids have been well studied as possible modulators but until recently, another likely candidate, gonadal inhibin, has been given little consideration.

The term "inhibin", from the Latin verb "inhibere", was first coined by McCullagh in 1932 to describe a water soluble substance present in testicular extracts which was characterized by its ability to prevent hyperfunction of the pituitary in castrate animals (37). Later investigations of this hormone demonstrated that testicular inhibin is capable of selectively suppressing plasma FSH in intact rats (38-40), long-term castrate males rats (41), sheep (42) and acutely castrated male rats (43-45). In addition to these in vivo studies, testicular inhibin preparations have been shown to selectively suppress FSH release from pituitary cell cultures (46) and hemipituitary short-term incubations (47). Most studies using inhibin preparations of testicular origin find little or no effect on plasma LH or LH release in vitro. In the study of testicular inhibin, a number of sources of inhibin activity have been tested including rete testes fluid (38,39), seminal plasma (40) and spermatozoa (48,41). Several in vitro studies have shown that spent media from enriched Sertoli cell cultures has FSH-suppressing activity, suggesting a possible site of production for testicular inhibin in the male. Although this
hormone's existence in the male was postulated 47 years ago isolation, characterization and purification of "inhibin" has progressed very slowly. The estimated molecular weight for inhibin ranges from less than 5000 daltons to greater than 100,000 daltons (49) and appears to vary with the source of starting material and purification procedures (49). It is believed to be a peptide or protein because it is inactivated by proteolytic enzymes and heat and its activity is not diminished by extraction with steroid solvents (49). These data have led to the suggestion that inhibin could be a polymer or could be bound to other proteins as are other hormones. The observation that testicular inhibin preparations administered to female rats could reduce plasma FSH levels (50) prompted several investigators to look for "inhibin" activity in the female. Since there are several instances when selective FSH secretion occurs in the female as discussed above, it seemed plausible that an ovarian inhibin might provide the "missing link" in gonadotropin control. At present, ovarian inhibin has been identified in a number of species. Ovarian follicular fluid or ovaries of pigs (51-55), coes (56), rats (57), hamsters (58) and humans (59) have been shown to contain a nonsteroidal agent capable of inhibiting FSH secretion. This agent appears to have many physiological and biochemical properties similar to inhibin of testicular origin (49). Although some laboratories have termed this agent "folliculostatin" (51), most investigators refer to it as ovarian inhibin - these agents most likely being one and the same.
The site of production of ovarian inhibin has not been conclusively determined. However, studies on cultured rat granulosa cells have demonstrated FSH-suppressing activity in the media of these cultures (60). This would seem reasonable since the granulosa cells of the female are analogous to male Sertoli cells, a suggested site of testicular inhibin production.

As with testicular inhibin, the purification of ovarian inhibin has not been conclusive. The molecular weights range from 10,000 to greater than 100,000 daltons. However, there are a number of reports which consistently find the active component to have an approximate molecular weight of 20,000 daltons (49,59,61).

In the female rat, ovarian inhibin preparations have been shown capable of attenuating the post-castration rics of FSH in estrogenized and non-estrogenized rats and to reduce plasma FSH in longterm ovariec-tomized rats (51). In the same study, injection of porcine follicular fluid (PFF), the most widely used source of ovarian inhibin, into ovariectomized estrogenized rats returned plasma FSH levels to baseline values while estrogenization alone could not. In addition, PFF administration to intact cycling rats suppressed the secondary FSH surge seen on estrus as well as the proestrous surge of FSH (52,53). In vitro studies using dispersed pituitary cell cultures have shown PFF and other ovarian inhibin preparations to inhibit the basla secretion of FSH and the LHRH stimulated release of FSH and LH, the effect being greater for FSH (25). In view of the finding that inhibin
decreases basal FSH secretion as well as pituitary FSH content, it has been suggested that inhibin is acting, at least in part, on FSH synthesis (25).

In support of inhibin as a physiological regulator of FSH secretion, is the fact that nonsteroidal FSH-inhibiting activity (FSH-IA) presumably ovarian inhibin, has been demonstrated in rat ovarian venous plasma (57). Ovarian venous plasma collected from rats acutely hemi-ovariectomized appears to show increase FSH-IA 32 hours after surgery as the FSH levels are decreasing. In this same study, FSH-IA activity at various stages of the estrous cycle was negatively correlated with plasma FSH. This provides essential evidence for the existence of a long-loop-feedback system between FSH and ovarian inhibin.

It is generally accepted that the gonadal steroids act both at the level of the pituitary and the CNS to regulate gonadotropin secretion. It then seems possible that inhibin too may act on neural structures as well as the pituitary. In fact, Lugaro, et al., (48) reported a decrease in in vivo FSH-releasing activity of the hypothalami of rats following intraventricular administration of testicular peptide factor isolated from bull spermatozoa. Although this suggested a possible CNS site of action for testicular inhibin little additional investigation of this hypothesis was reported.

Considering all the studies discussed above, it can be concluded that the mechanism of action of the inhibins as well as the exact nature of the molecule(s) remains unresolved. Part of the problem with
characterization of this molecule is attributable to the difficulty in designing standardized assay systems for detection of inhibin activity. In addition, the perspective of ovarian inhibin as a long term regulator of FSH secretion, hence folliculogenesis has not been thoroughly investigated. It was, therefore, the purpose of the following series of experiments to:

1) develop an in vivo bioassay model to detect inhibin activity;
2) investigate inhibin activity in a number of semi-purified preparations using this mode;
3) further investigate the mechanism of action of ovarian inhibin at the pituitary level;
4) investigate the possibility of a CNS site of action for ovarian inhibin;
5) investigate the mechanism of inhibin action in the CNS;
6) test the effects of chronic administration of ovarian inhibin containing preparations and the feasibility for its use as a long term regulator of FSH secretion.
METHODS AND MATERIALS

A. Animals

In all experiments, Sprague-Dawley rats were used. In experiments using virgin female rats, their weight ranged from 200-300 grams and in those experiments using male rats, weight range was approximately 400-500 grams. All animals were maintained on a 14/10 light/dark cycle with lights on at 0500 hours and were given food and water ad libitum. For studies using intact cycling, female rats daily morning vaginal lavages were performed and only rats displaying two consecutive four day estrous cycles were used.

B. Animal Surgical Procedures

1. Female rats were ovariectomized by means of bilateral incisions in the flanks under ether or methoxyflurane (Metofane) anesthesia. Animals were allowed at least one week for recovery before experimentation except where otherwise indicated.

2. Transplantation of ovaries under spleen or kidney capsule was accomplished by exteriorizing appropriate organ and making a small slit in capsule. Fine forceps were inserted into this slit and a pocket was formed by slowly teasing away capsule. In one group, animals received autotransplants consisting of one ovary. In the second group, previously ovariectomized animals received one ovary from donor rats at random stages of the estrus cycle.

3. In brain cannulation experiments, rats were implanted with bilaterally with 20 guage stainless steel cannulas fitted with trochars to
maintain patency when not in use. Cannulas were stereotaxically implanted into the dorsal anterior hypothalamic area (DAHA) and the medial preoptic area (MPOA) using the atlas of Pellegrino and Cushman (62). Coordinates were: DAHA .8 mm anterior to bregma, .75 mm lateral from midline and .8 mm down from dorsal surface; and MPOA, 2.4 mm anterior to bregma, .25 mm lateral from midline and 7.5 mm down from dorsal surface. Animals were allowed at least two weeks to recover before experimentation.

C. Estrogenization

Silastic capsules containing 17\(\beta\)-estradiol (Sigma) were constructed according to the methods of Legan and Karsch (63). Briefly, this consists of plugging one end of Silastic tubing (Dow Corning I.D. .020 in., O.D. .037 in) with a flat ended wooden applicator and securing it in place with Medical Adhesive (Dow Corning, Silicone Type A). Estrogen is subsequently introduced into tubing and tamped down to desired length of estrogen column. A second wooden applicator is inserted into other end and glued in place with adhesive. Capsules are preincubated for 1-3 hours in normal saline before implantation to minimize the initial surge of steroid release seen upon implantation of capsules not preincubated. The release rate of steroids from these preincubated capsules is relatively constant and is a function of their length, i.e., length of estrogen column. Capsules were implanted subcutaneously on the back of animal and incision closed with a wound clip.

D. Collection and Preparation of Test Materials

1. Porcine follicular fluid (PFF) was aspirated from more than 2000 pig ovaries obtained at a local abbatoir. Ovaries were removed
from pigs minutes after death and immediately placed on ice. Ovaries were returned to the laboratory, fluid collected into chilled vials from large and medium size follicles and subsequently centrifuged 1000 x g for 20 mins to remove granulosa cells and particulate matter. PFF was then either charcoal or ether extracted to remove steroids. Ether extraction consisted of adding 3 times the volume of ether to PFF three separate times and separating the aqueous phase. Charcoal extraction consisted of adding 100 mg charcoal (Norit-A, Fisher) per ml of fluid. This mixture was stirred at 4°C for at least one hour and subsequently centrifuged several times to remove charcoal (1000 x g, 30 mins.).

2. Collection of Pig Serum. [Barrow Serum (BS) and Gilt Serum (GS)]. Pig blood was collected from male (barrow) or female (gilt) pigs at the abbatoir as animals were exsanguinated. Upon return to the laboratory, blood was chilled and centrifuged. Serum was removed and treated similar to PFF in all respects.

3. Preparation of Porcine Follicular Fluid Extract (PFFX) and Barrow Serum Extract (BSX). An extract of PFF was prepared by subjecting charcoal treated PFF to ultrafiltration using Amicon PM10 and PM30 molecular weight filters and Amicon ultrafiltration chamber (Amicon Corp.). Charcoal extracted PFF was diluted with equal volumes of .15 m NaCl and placed in ultrafiltration chamber containing a PM30 filter (molecular weight exclusion 30,000 daltons). Filtration took place under approximately 40 p.s.i. pressure with constant stirring. When retentate volume was reduced by 80%, it was diluted with .15 m NaCl and filtered
again. This procedure was repeated one additional time. By this procedure, approximately 80\% of substances having molecular weights less than 30,000d are filtered during first pass. Second filtration allows 80\% of species remaining in first retentate having molecular weights less than 30,000d to pass through filter and so on. Pooled filtrate from all 3 passes was then filtered through a PM10 filter (M.W. exclusion 10,000d) using the same protocol. The resulting retentate and rinse of the PM10 filter was placed in cellulose dialysis sacks (Sigma) and dialyzed overnight against distilled water. Contents of the tubing was removed, tubing rinsed with distilled water and this mixture lyophilized and stored frozen at -20°C until use.

BSX was prepared by the same protocol using charcoal extracted barrow serum as starting material.

4. Ammonium Sulfate Fractionation of Porcine Follicular Fluid.

Whole follicular fluid (20 ml) was diluted with an equal volume of .15m NaCl. Enough cold (-20°C) absolute ethanol was added to give a final alcohol concentration of 86\% (V/V). The resulting precipitate was allowed to settle for a 24 hr. period, recovered by centrifugation and washed three times with cold acetone by re-suspension and re-centrifugation. Final material was recovered by lyophilization. This extract was dissolved in distilled water and dialyzed overnight against distilled water. The retentate was recovered and subjected to ammonium sulfate fractionation at 4°C according to the following protocol: With constant stirring, crystalline ammonium sulfate (reagent grade, Baker) was added to the extract to obtain a 20\% saturated solution.
This mixture was centrifuged and the supernatant subjected to addition of more ammonium sulfate until 40% saturation was reached. This procedure continued until 100% ammonium sulfate saturation was accomplished in 20% increments. The precipitate from each fraction was dissolved in and dialyzed against distilled water overnight to remove the ammonium sulfate. The dialysis tubing was emptied, contents lyophilized and stored frozen until use. Fractions were reconstituted with distilled water to give .5 ml equivalent of starting PFF in 1 ml volume. This volume was injected into animals at time of experimentation.

E. Collection of Blood Samples

Collection of blood via cardiac puncture was accomplished under ether anesthetic into heparinized syringes. In acute experiments, trunk blood was collected into heparinized or nonheparinized chilled conical centrifuge tubes subsequent to stunning and decapitation of animals. All blood samples were centrifuged at 4°C and plasma or serum frozen (-20°C) until hormone determination.

F. In Vitro Incubation Experiments

1. Anterior Pituitary Glands.

Male or female rats were stunned by a quick blow to the head and decapitated. Pituitary glands were exposed by removal of the brain from the dorsal aspect of the skull. The dural membrane was dissected away along with the posterior lobe of the pituitary. The anterior pituitary was then transferred to a petri dish containing modified medium 199 (Gibco) at room temperature, cut into quarters and two
quarters placed in incubation flasks containing 1 ml medium 199. Flasks were gassed with 95% O₂ - 5% CO₂, capped and placed in a Dubnoff water bath with gentle agitation at 37°C. After 30-45 mins preincubation period the medium was discarded and replaced with fresh medium with or without test substances depending on experimental design. When protocol called for LHRH in medium, LHRH (Beckman) was either dissolved in medium itself or in .15 m NaCl at high concentration and added to flasks. Final volumes in all incubation flasks were equal. Flasks were again gassed and incubation continued for 3-4 hrs. At the end of incubation period media were removed from flasks and snap frozen in a dry ice-methanol bath. Anterior pituitary glands were either quickly weighed and snap frozen or immediately placed in .5 ml cold 5% trichloroacetic acid (TCA) and homogenized in a ground glass tissue grinder. The homogenate was removed and grinder rinsed with an additional .5 ml cold 5% TCA. Rinse and homogenate were pooled and centrifuged at 2000 x g for 20 mins at 4°C. Supernatant was decanted into test tubes and stored frozen at -20°C until cyclic nucleotide determination. The pellet was dissolved in 1 ml 1.0N NaOH for protein determination by the protein-dye binding method (64).

G. Hypothalamic Tissue Fragments

Donor animals were decapitated and brains removed as described above and placed on a glass plate with ventral surface up. For incubation experiments, hypothalami were dissected using the following boundaries: anterior-optic chiasm, posterior-mammillary bodies, and lateral-internal carotid arteries. Removal of hypothalami was
performed either by "pinching" hypothalami with a pair of forceps using the above structures as guides or by fine dissection using micro-dissection scissors and watchmaker's forceps under a magnifying glass. Fragments were placed in 1 ml of phosphate buffered saline (PBS) containing $1.5 \times 10^{-2}$ m glucose, gassed with 95% $O_2$ - 5% $CO_2$, capped and incubated as above. After 15 mins. preincubation media were removed and discarded and 1 ml of fresh PBS plus glucose containing test materials was placed in appropriate flasks. Hypothalamic fragments were incubated for 1 hr at which time media and fragments were snap frozen.

For hypothalamic releasing activity experiments, hypothalami were removed by a slightly different procedure. Brains were removed as above and placed ventral surface up. An apparatus constructed in our laboratory (fondly referred to as the "Curry handy-dandy hypothalamic-ectomizer") consisting of a #2 cork borer (6 mm diameter) fitted with a stainless steel stylet 2 mm shorter than the shaft of the borer. With the ventral surface of the brain up, the sharpened borer was lowered over the hypothalamic area and a cylinder of neural tissue was sliced through the brain to the dorsal surface. The metal stylet was inserted into the opposite end of the borer shaft and expelled the cylinder of tissue except for the most ventral 2mm. At this point the expelled cylinder was sliced off with a scalpel leaving a 2 mm thick disc in the barrel which contained most hypothalamic structures. This disc was weighed and immediately snap frozen until extraction.
Acid extracts of hypothalamic tissue were prepared by homogenizing discs or fragments in 1 ml cold .1N HCl or .1N acetic acid. The homogenate was centrifuged and supernatant decanted into test tubes. Supernatant pH was adjusted to 7.4 with addition of 1N NaOH while vortexing tube and the volume of supernatant diluted appropriately for RIA or pooled and lyophilized for releasing activity experiments. All samples were stored frozen at -20°C until use.

H. Hormone Determination

1. Plasma, serum and media LH and FSH values were measured by using radioimmunoassay (RIA) kits provided by the NIAMDD Rat Hormone Distribution Program. LH and FSH values are expressed in terms of their respective NIAMDD rat standards (FSH-RP-1, LH-RH-1). The protocols for double antibody RIA as well as for iodination of antigens were those provided with the kits. The only modification of these procedures was that in early experiments RIAs were incubated at 4°C. All subsequent assays were incubated at room temperature as per kit directions.

2. Luteinizing Hormone - Releasing Hormone Radioimmunoassay.

LH-RH levels were determined by a double antibody RIA according to the methods described by Nett, et al., (65). Briefly 2.5 μg of LH-RH (Beckman) was radioiodinated with 1 mCurie of 125I (Amersham) for 15 secs in the presence of 20 μg chloramine T (Matheson, Coleman and Bell). The reaction was stopped with 40 μg of sodium meta-bisulfate (Baker). Labeled LHRH (125I-LHRH) was separated from free 125I by placing the reaction mixture on a Sepadex G-25 column (.6 x 20cm) and
eluted with .01M PBS containing .1% bovine serum albumin (Sigma). .5ml fractions were collected from the column into 10 x 75 test tubes containing 25 µl of 2% bovine serum albumin (BSA). Aliquots of 10 µl from each fraction were counted to detect elution pattern of labelled hormone and free iodine. Labeled hormone fractions were tested for binding to LH-RH antiserum (#R42) provided by Dr. T. Nett. Standard curves were generated with 18-22 points ranging from 1 to 500 pgs. To each assay tube 100 µl of sample, 200 µl of antiserum (1: 40,000 dilution) and approximately 25,000 cpm $^{125}\text{I}$-LH-RH was added. All tubes were incubated for 24 hours at 4°C. At this time, 200 µl of appropriately diluted anti-rabbit gamma globulin (raised in goat, Miles) was added and incubation continued for an additional 24 hr. period. At the end of this incubation period, 3 ml of cold PBS was added, the tubes centrifuged at 1000 x g for 30 mins., supernatant decanted and the precipitate counted.

I. Cyclic Nucleotide Determinations

c-AMP and c-GMP tissue concentrations were determined by using a single antibody RIA that was developed and validated in the laboratory of Dr. Richard Fertel, Department of Pharmacology at Ohio State University (66). TCA was extracted from supernatant of tissue homogenates using 3 volumes of water-saturated petroleum ether. After the extraction, the ether layer was aspirated off. This procedure was repeated 3 times. After the third extraction 100 µl/ml solution of 1.0M acetate buffer pH = 6.5 was added to aqueous phase. The RIA procedures for both c-AMP and c-GMP were as follows:
1. pipette 100-200 µl of sample at desired dilution into 12 x 75 mm test tubes.

2. pipette standards into tubes at same volume as sample.

3. acetylate all tubes with 10 µl acetylation reagent (400 µl acetic anhydride: 1.0 ml triethylamine) while vortexing. This procedure increases the sensitivity of the assay approximately 10-30 fold.

4. incubate tubes for 20 mins at room temperature.

5. add 50 µl of $^{125}$I-c-AMP or $^{125}$I-c-GMP to assay tubes.

6. add 50 µl of appropriate antiserum diluted to 1:2000 with 0.05M acetate-BSA buffer solution. Vortex all tubes and incubate overnight.

7. Add 2.5 ml of 60% ammonium sulfate (NH$_4$)$_2$SO$_4$ to each tube, vortex, incubate for 20 mins and centrifuge at 1500 x g for 30 mins.

8. decant and count precipitate.

J. Iodination of PFFX

A small sample of PFFX was subjected to radiiodination via a modified chloramine T method. Briefly this consisted of the addition of 25 µg of chloramine T to PFFX in the presence of 1 mCurie $^{125}$I (New England Nuclear). After 45 seconds the reaction was stopped by the addition of 50 µg sodium metabisulfate. Reaction mixture was placed on a Sephadex G-75 column (.75 x 20 cm) eluted with PBS and .5 ml fractions collected.
K. **Protein Determinations**

All protein-determinations were performed using the Protein-Dye Binding Technique (64). Coomassie Brilliant Blue G-250 (Sigma) was used as the dye and reference preparation was bovine serum albumin (Sigma).

L. **SDS-Polyacrylamide Electrophoresis of Ammonium Sulfate Fractions**

SDS-polyacrylamide electrophoresis was carried out by a modification of the method of Fairbanks (65). Molecular weight standards (Bio-Rad) included phosphorylase a (MW = 98,000), bovine serum albumin (MW = 68,000), ovalbumin (MW = 43,000), carbonic anhydrase (MW = 30,000) and α-lactalbumin (MW = 23,000). Gels were stained for protein with Coomassie Blue (Shandon) and subjected to densitometric analysis.

M. **Histology**

In brain cannulation experiments, cannula placement was verified histologically in a sampling of animals. This was accomplished by perfusing animals with .9% NaCl followed by 10% neutral formalin. Brains were removed and fixed in formalin for at least 1 week. After this time, brains were washed in running tap water for several hours. They were next subjected to dehydration by soaking in ascending concentrations of alcohol according to the following protocol:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% ETOH</td>
<td>1</td>
</tr>
<tr>
<td>70% ETOH</td>
<td>1</td>
</tr>
<tr>
<td>80% ETOH</td>
<td>1</td>
</tr>
<tr>
<td>90% ETOH</td>
<td>1</td>
</tr>
</tbody>
</table>
Solution | Time (hrs)
---|---
95% ETOH | 1
100% ETOH | 1
100% ETOH | 1
100% ETOH/xylene | 2
xylene | 2
xylene | 2

After last xylene solution, brains were placed in paraplast for 2 hrs and paraplast changed and soaked for an additional 2 hrs. At this time, brains were removed from vials and placed in histology paraffin boats and imbedded in wax overnight. Subsequent to this, paraffin blocks were mounted on wooden blocks. The brains were sectioned in 10 micron sections and every fifth section mounted on slides previously coated with albumin fixative (Harleco). Slides were dried on a warming tray at 58°C and subsequently deparaffinized and rehydrated according to the following protocol.

<table>
<thead>
<tr>
<th>Solution</th>
<th>time (mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylene</td>
<td>5</td>
</tr>
<tr>
<td>Xylene/100% ETOH</td>
<td>5</td>
</tr>
<tr>
<td>100% ETOH</td>
<td>5</td>
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<tr>
<td>100% ETOH</td>
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<td>80% ETOH</td>
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<tr>
<td>70% ETOH</td>
<td>5</td>
</tr>
<tr>
<td>50% ETOH</td>
<td>5</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>5</td>
</tr>
</tbody>
</table>
Slides were next stained by submerging in a 20% solution of Paragon Multiple Stain for 1 min and then dehydrated again by submersion for a few seconds in ascending ethanol concentrations as before. Cover-slips were mounted on slides with Permount and stored until examination.
EXPERIMENTAL DESIGN AND RESULTS

I. GONADOTROPIN PROFILES IN THE OVARIECTOMIZED ESTROGEN CAPSULE IMPLANTED AND INTACT CYCLING RATS.

A. Ovariectomized Estrogen Implanted Rats

1. Experimental Design

Although daily LH surges in the ovariectomized estrogen capsule implanted rat (OVX-E) had been previously described (63), FSH profiles had not been determined. It was therefore necessary to determine FSH profiles in OVX-E rats over a 24 hr period. Estrogen capsules of three lengths, 1mm, 3mm and 5 mm were constructed as described in Methods and Materials. Virgin female rats were ovariectomized and allowed at least one week to recover before estrogen capsule implantations. All animals were implanted 5 days before experimentation. On the day of experiment, 5-15 animals bearing various size capsules were decapitated and trunk blood collected at selected times over a 24 hr period. Plasma FSH and, when possible, plasma LH were determined by RIA. Statistical analysis was performed using analysis of variance followed by Duncan's Multiple Range Test.

2. Results

Plasma FSH and LH values in OVX-E rats bearing 5 mm silastic capsule are shown in Figure 1. Plasma FSH values at 1700 hrs (476 ± 36 ng/ml) are significantly different (p<.05) from 0900 hrs value (289 ± 70 ng/ml). At 1900 hrs a distinct drop in FSH
values was seen (288 ± 31 ng/ml) followed by a secondary rise at 2100 hrs (477 ± 58 ng/ml). The value observed at 1900 hrs was significantly different from the 1700 hrs and 2100 hrs values (p < .05) but not from the 0900 hrs morning value.

This profile appears analogous to the primary and secondary FSH surge reported by others (10) in the intact cycling rat except for a temporal shift.

The plasma LH profile was similar to that reported by other laboratories (63). Plasma LH levels peaked at 1700 hrs (361 ± 87 ng/ml) concurrent with the primary FSH rise, but remained near baseline throughout most of the remainder of the 24 hr period.

Animals implanted with 3 mm capsules were killed at 0900 hrs and 1700 hrs only. Plasma FSH values in these animals were not significantly different (391 ± 46 ng/ml and 376 ± 88 ng/ml respectively).

As shown in Figure 2 animals bearing 1 mm capsules showed similar plasma levels at all times studied. No primary FSH rise was seen at 1700 hrs as in 5 mm OVX-E rats indicating that estrogen levels were not sufficient in 1 mm or 3 mm implanted animals to exert a positive feedback on the hypothalamic-pituitary axis.

B. **Intact Cycling Rats**

1. **Experimental Design**

In another group of intact cycling animals daily vaginal lavages were performed over several weeks. After animals displayed 2 consecutive estrous cycles, they were decapitated at various selected times on proestrus and estrus and trunk blood collected.
Serum FSH were again determined by RIA.

2. Results

As seen in Figure 3 serum FSH values were determined over approximately the same period as in OVX-E rats. 0900 hrs and 1200 hrs proestrus values (235 ± 11 ng/ml and 230 ± 29 ng/ml respectively) correspond to those seen in OVX-E rats (5 mm) at 0900 hrs (289 ± 70 ng/ml). FSH levels peaked at 1900 hrs, fell briefly at 2100 hrs and rose again by 2300 hrs through 0100 hrs estrus. FSH levels had returned to near proestrous morning values by 0900 hrs estrus.
Figure 1. Circadian plasma FSH and LH profiles in ovariectomized estrogen-implanted rat * = significant at p < .05 level from 0900 hrs value. Φ = significant at the p < .05 level from 1700 hrs and 2100 hrs surge. (N = 5 animals/time).
Figure 2. Comparison of FSH levels at four selected times in 5 mm and 1 mm estrogen capsule implanted rats. *= Significant at p < .05 level from 0900 hrs, 1900 hrs and 0300 hrs. (N = 5/group).
Figure 3. FSH profile in the intact cycling rat from 0900 hrs proestrus to 0900 hrs estrus (5-6 animals/group).
II. EFFECTS OF SYSTEMIC INJECTIONS OF OVARIAN INHIBIN PREPARATIONS

A. Systemic Injection of Porcine Follicular Fluid (PFF)

1. Experimental Design

It was decided to test the effects of systemic injection of PFF on the primary FSH rise seen at 1700 hrs in the 5 mm OVX-E rat. Animals were ovariectomized and estrogenized as before. Each animal received test substances every two days thus acting as its own control. Morning blood samples (1.0 ml) were taken between 1100 - 1200 hrs via cardiac puncture and test substances injected subcutaneously at 1200 hrs. P.M. samples (1.0 ml) were collected 5 hrs later at 1700 hrs. Substances tested consisted of 1 ml of saline (Sal), charcoal extracted barrow serum (BS) and .25 or 1 ml of charcoal extracted PFF.

2. Results

As seen in Figure 4, A.M. FSH values on all days studied were similar to A.M. FSH values in Experiment I. Saline and BS appeared to have no effect on the primary FSH rise seen at 1700 hrs, the increase being similar to that seen in Experiment I. However, PFF treatment in either dose significantly attenuated the 1700 hrs rise of FSH as compared to saline and BS controls (p<.05).

B. Systemic Injection of Porcine Follicular Fluid Extract (PFFX)

1. Experimental Design

In the next experiment, the effect of an extract of porcine follicular fluid (PFFX) in 5 mm capsule OVX-E rats was tested. These animals were treated as before receiving test
substances every two days. Injections of test materials (PFF, 1 ml; BS 1 ml and PFFX equivalent of 1 ml PFF in .25 ml volume) were given interperitoneal. Timing of blood sampling and administration of test materials was as before.

2. Results

The results are shown in Figure 5 expressed as percent change of plasma FSH from morning to afternoon (5 hrs post-injection). Plasma FSH levels with BS treatment (N = 10) rose from 292 ± 73 ng/ml to 492 ng/ml between 1200 hrs and 1700 hrs. This increase was nearly identical to that seen in Experiment I between 0900 hrs and 1700 hrs indicating VS had no effect on this primary FSH rise. BS treated controls showed a mean percent change in plasma FSH of 123%, while PFF treatment severely attenuated the 1700 hrs rise, animals displaying only a 2% change. Of the ten animals treated with PFF, six showed a decrease in plasma FSH between 1200 hrs and 1700 hrs while the remaining four showed attenuated primary FSH rises (see Table 2, Appendix). PFFX treated animals (N = 6) showed a moderate attenuation of the primary FSH rise increasing only 54% between 1200 hrs and 1700 hrs. Only BS- and PFF-treatments proved statistically different (p < .05) although there was a clear difference between BS- and PFFX-treated which did not prove statistically different because of the large standard error. PFFX appeared to retain some of its FSH suppressing activity.
Figure 4. Plasma FSH levels in ovariectomized estrogen implanted rats bearing 5 mm capsules injected with 1 ml saline, barrow serum (BS), porcine follicular fluid (PFF) or .25 ml PFF. A.M. samples were taken at 1100-1200 hrs, P.M. values at 1700 hrs. * = significant at p < .05 level from saline or BS control. (N = 9-11/day)
Figure 5. Mean percent change in plasma FSH levels from 1200 hrs to 1700 hrs in ovariectomized estrogen-implanted rats after treatment with 1 ml barrow serum (BS), 1 ml porcine follicular fluid (PFF), or .25 ml extract of PFF (PFFX). * = significant at the p < .05 level from BS control. (N = 10 for BS and PFF, N = 6 for PFFX).
III. EFFECTS OF PFFX ADMINISTRATION TO THE CNS

A. Effect of Administration of PFFX to CNS areas on FSH

1. Experimental Design

Since PFFX appeared to retain some of its FSH-suppressing activity without large quantities of high (>30,000d) or low (<10,000d) molecular weight components, it was used to investigate a possible CNS site of action for ovarian inhibin. Virgin female rats were stereotaxically implanted with 20 gauge stainless steel brain cannulas (see Methods and Materials). Animals were ovariectomized at time of brain implantation and allowed at least two weeks to recover. Five mm estrogen capsules were implanted as before 5 days prior to experimentation. Cannulas were placed in the dorsal anterior hypothalamic area (DAHA) or medial preoptic area (MPOA). On the day of experiment, blood samples were collected at 1200 hrs and 1630 hrs. Immediately following first sample, cannulas of half the animals from each group were filled with PFFX and cannulas of the other half filled with BSX. Cannulas were capped with small pieces of polyethylene tubing crimped at one end. Following the 1630 hrs blood sample, all cannulas were emptied and gently flushed with normal saline and emptied again before replacing trochars. Two days later the entire process was repeated with each animal receiving the opposite treatment, each animal serving as its own control. Cannula placement was verified by histological examination in a sampling
of animals after experimentation. Statistical analysis employed was t-test for paired data.

2. Results

The effects of direct neural application of BSX and PFFX to the DAHA (N = 7) and MPOA (N = 8) are shown in Fig. 6, Table 3. PFFX treatment caused no significant attenuation of the primary FSH rise in OVX-E rats as compared to BSX-controls when administered to the MPOA. However, administration of PFFX to the DAHA resulted in significant suppression of the FSH rise when compared to BSX-treated controls at 1630 hrs (p<.05 levels).

B. Preparation and Administration of Radiolabeled PFFX

1. Experimental Design

Realizing that the FSH-suppressing actions of PFFX administered to the DAHA could possibly result from diffusion of active material from site of implantation to the pituitary, a small amount of PFFX was subjected to iodination with $^{125}$I according to the chloramine T method. Cannulas implanted into the DAHA and MPOA in a separate group of animals were filled with aliquots of labeled material. Animals were killed at intervals up to 4.5 hrs after administration and pituitaries, hypothalami, cerebellum, cerebral cortex, olfactory bulbs, thyroids and a blood sample were collected and counted to detect the presence of tracer.
2. Results

Fractions (.5 ml) collected from the Sephadex G-75 column showed the presence of some labeled material (Figure 7, fractions 6 and 7) separate from free iodide. Fractions 6 and 7 were pooled and aliquots of this material placed in cannulas. At no time up to 4.5 hrs did counts rise above background levels in any of the tissues sampled except in the hypothalamus.
Figure 6. Percent change in plasma FSH levels from 1200 hrs to 1630 hrs in ovariectomized, estrogen-implanted rats bearing chronically implanted dorsal anterior hypothalamic (DAHA) or medial preoptic (MPOA) cannulas. Cannulas were filled with either barrow serum extract (BSX) or PFFX following the 1200 hrs blood sample. * = significant at the p < 0.05 level vs appropriate BSX control value. (DAHA, N = 7; MPOA, N = 8).
Figure 7. Activity (CPM x $10^5$) of .5 ml fractions eluted from Sephadex G-75 column (20 cm x 0.75 cm) following $^{125}$I-iodination of PFX. Material in fractions 6 and 7 was pooled and used for administration.
IV. EXAMINATION OF POSSIBLE MECHANISMS INVOLVED IN THE ACTION OF OVARIAN INHIBIN

A. Comparison of Effects of Acute vs Chronic Administration of Ovarian Inhibin Preparations on Pituitary Function.

1. Experimental Design

Longterm ovariectomized female rats (3 weeks) were used in this series of experiments. Animals were injected intraperitoneally with 1 ml of BS, PFF or saline. Animals received acute treatment consisting of injection of test materials 8 - 9 hrs before sacrifice or chronic treatment consisting of .5 ml test substances twice a day for 3 days. Injections were approximately 12 hrs apart with the final injection given 8 - 10 hrs before sacrifice.

a. Animals were decapitated and trunk blood collected. Serum LH and FSH levels were determined by RIA.

b. Anterior Pituitary glands were removed and incubated with or without LHRH (2 ng) as described in Methods and Materials. From each animal, one-half of gland was incubated with LHRH to determine LHRH sensitivity and the other half without LHRH to determine basal secretion rate. Media LH and FSH were determine by RIA and expressed as ng hormone/ml media/mg anterior pituitary.

c. Anterior Pituitaries were homogenized in TCA at the end of incubation for determination of cyclic nucleotide by RIA.
2. Results

a. The effects of chronic and acute administration of test materials on serum FSH are shown in Figure 8. Acute administration of PFF (N = 18) resulted in significantly lower serum FSH levels $920 \pm 100$ ng/ml compared to BS (N = 19) and saline (N = 17) controls $1420 \pm 75$ ng/ml and $1599 \pm 125$ ng/ml respectively ($p<.01$). In animals chronically treated with test substances serum FSH levels were not significantly different between groups (PFF, $1254 \pm 89$ ng/ml; BS, $1455 \pm 98$ ng/ml; saline $1431 \pm 94$ ng/ml).

Serum LH values were not significantly different between treatment groups in either acute or chronic administration experiments as shown in Figure 9. Serum LH values in acutely treated animals, PFF, $1045 \pm 254$ ng/ml; BS, $829 \pm 201$ ng/ml; and Sal, $1153 \pm 181$ ng/ml, appeared to be slightly lower than their chronically treated counterparts, PFF, $1340 \pm 232$ ng/ml; BS, $1312 \pm 181$ ng/ml; and Sal, $1688 \pm 225$ ng/ml.

b. As shown in Figure 10, basal secretion of FSH from hemipituitaries of animals acutely treated with PFF showed a marked and significant decrease compared to BS or saline control hemipituitaries ($p<.05$). Basal secretion of FSH from hemipituitaries of animals chronically treated showed no significant differences.
Figure 8. The effects of acute (8 hrs) vs chronic (3 days) injection of porcine follicular fluid (PFF) on serum FSH levels in 3 week ovariectomized rats. All animals received 1 ml of test substance (PFF = charcoal extracted porcine follicular fluid, BS = charcoal extracted barrow serum, Sal = saline).

** = significant at p < .01 level from BS or Sal controls.
Figure 9. The effects of acute (8 hrs) vs chronic (3 days) injection of PFF on serum LH levels in the 3 week ovariectomized rat.
Figure 10. Basal FSH release from hemipituitaries of animals acutely and chronically treated. Hemipituitaries were incubated in 1 ml Medium 199 under 95% O₂ - 5% CO₂ for 4 hrs at 37°C. * = significant at p < .05 level from BS and saline controls.
Figure 11. Basal LH release from hemipituitaries of animals acutely and chronically treated. Hemipituitaries were incubated in 1 ml Medium 199 under 95% O₂ - 5% CO₂ for 4 hrs at 37°C.
Basal secretion of LH in the three acutely treated groups where not different nor was basal secretion of LH from hemipituitaries of animals chronically treated with test materials as shown in Figure 11. Hemipituitary sensitivity to 2 ng LHRH is shown in Figure 12 and 13. LH-RH stimulated FSH release was significantly reduced in the PFF group as compared to BS and saline controls (p<.01). Surprisingly however, hemipituitary sensitivity to LHRH in the group chronically treated with PFF was also significantly impaired as compared to BS and saline control (p<.05).

Unlike basal FSH secretion, basal LH secretion was not affected by acute injection of PFF as shown in Figure 11. However, LH-RH stimulated LH release was significantly decreased from hemipituitary of animals chronically injected with PFF as compared to BS and saline controls (p<.05).

c. Cyclic nucleotide content of anterior pituitary tissue was examined in acute injection experiments only. The results are shown in Table 1. Media FSH is expressed in terms of ngFSH/µg anterior pituitary tissue protein. As in previous experiment, both basal and LH-RH stimulated release of FSH from hemipituitaries of animals treated with PFF were depressed compared to BS and saline controls.
Figure 12. LHRH-stimulated release in vitro from hemipituitaries of animals acutely and chronically treated. (2 ng LHRH/ml) ** = significant at the p < .01 level from BS and saline controls, * = significant at the p < .05 level from BS and saline controls.
Figure 13. LHRH-stimulated LH release in vitro from hemipituitaries of animals acutely and chronically treated. (2 ng LHRH/ml). * = significant at the p < .05 level from BS and saline controls.
Table 1. Cyclic nucleotide content of hemipituitaries after 4 hrs incubation in vitro. Hemipituitaries from acutely treated animal only were examined both LHRH-stimulated and non-stimulated groups.

1) Significant at the p \leq 0.05 level from Sal-Basal.
2) Significant at the p \leq 0.05 level from BS-LHRH.
3) Significant at the p \leq 0.05 level from Sal-LHRH.

<table>
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<th></th>
<th>ng FSH/μg AP Protein</th>
<th>fmoles cAMP/μg Protein</th>
<th>fmoles cGMP/μg Protein</th>
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<tr>
<td>Sal</td>
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<tr>
<td>Basal</td>
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<td>27.59 ± 6.4</td>
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<tr>
<td>Basal</td>
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<td>19.45 ± 6.48</td>
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<tr>
<td>LHRH</td>
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<tr>
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<tr>
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<tr>
<td>LHRH</td>
<td>58.6 ± 11.4 (^2)</td>
<td>15.95 ± 3.2 (^3)</td>
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cAMP content of non-stimulated A.P. tissue was not
different among treatment groups. However, cAMP levels
in A.P. tissue stimulated with LHRH did show some
differences with the PFF group being lower than in BS or
sal controls (statistically significant between PFF-LHRH
vs Sal-LHRH only p ≤ .05). cGMP content showed no differ-
ence between LHRH stimulated pituitaries. In basal secre-
tion groups cGMP content was not significantly different
between goups.

B. Effects of Acute vs Chronic Administration of Ovarian Inhibin
Preparations on Hypothalamic FSH-Releasing Activity.

1. Experimental Design

Longterm ovariectomized rats injected acutely or chroni-
cally as in section A were hypothalamus donors. Acid extracts of
these hypothalami were pooled, lyophilized and reconstituted with
distilled water. Another group of longterm ovariectomized rats
were estrogen-progesterone primed for 3 days and served as
pituitary donors. Hemipituitaries from donor animals were incu-
bated with \( \frac{1}{2} \) hypothalamic equivalent (\( \frac{1}{2} \) Hx) from test animals.
By this bioassay, the hypothalamic FSH releasing activity of hypo-
thalami of acutely and chronically treated animals was tested in vitro.

2. Results

The amount of FSH released into media from donor pitui-
taries is shown in Figures 14, 15. Hypothalami from all chronically
treated animals showed no significant differences. Hypothalami
Figure 14. LH and FSH releasing activity of hypothalami from ovariectomized rats treated acutely with PFF, BS or sal. ½ hypothalamic equivalent incubated with donor pituitaries for 4 hrs under 95% O₂ - 5% CO₂ at 37°C.

* = Significant at p ≤ .05 level from PFF or Saline groups.
Figure 14. LH and FSH releasing activity of hypothalami from ovariectomized rats treated acutely with PFF, BS or sal. 1/2 hypothalamic equivalent incubated with donor pituitaries for 4 hrs under 95% O₂ - 5% CO₂ at 37°C.

* = Significant at p ≤ .05 level from PFF or Saline groups.
Figure 15. LH and FSH releasing activity of hypothalami from ovariectomized rats treated chronically with PFF, BS and saline. ½ hypothalamic equivalent incubated with donor pituitaries for 4 hours under 95% O₂ - 5% CO₂ at 37°C.

* = Significant at p < .05 level from Sal group.
from BS-acutely treated animals appeared to release more FSH than PFF or saline groups (p<.05). The serum FSH values for these animals shown in Figure 8 did not reflect the FSH releasing activity of hypothalami from these animals.

C. Effects of Ovarian Inhibin Preparations on Hypothalami In Vitro

1. Experimental Design

As a preliminary experiment, hypothalami from estrogen-progesterone primed ovariectomized rats were incubated in vitro with PFFX, BSX and without test substances. For PFFX and BSX a 1 ml equivalent of starting material was reconstituted in PBS plus glucose and incubated with hypothalami. LHRH content of tissues and amount released into media were measured by RIA.

2. Results

The results are shown in Figure 16 showing hypothalamic and media LHRH content after 1 hr incubation (N = 5 per group). Hypothalamic LHRH was lower in PFFX group compared to BSX and non-treated control. This value however, did not prove statistically significant. Likewise, LHRH in media from PFFX group was lower than both control groups but was not significant.
Figure 16. The effects of PFFX on hypothalamic fragments and media content of LHRH in vitro. Hypothalamic fragments from donor animals were incubated for 1 hr in PBS + glucose in the presence of PFFX or BSX (1 ml equivalent). Tissue and media LHRH content was measured by RIA (N = 5/group).
V. AMMONIUM SULFATE FRACTIONATION OF PORCINE FOLLICULAR FLUID

A. FSH-Suppressing Activity

1. Experimental Design

PFF was subjected to fractionation with crystalline (NH₄)₂SO₄ in 20% increments as described in Methods and Materials. FSH-suppressing activity of each fraction was tested by injection of .5 ml equivalent of starting material into longterm ovariec-tomized rats (N = 7/group). Pre- and post-injection blood samples were taken with post-injection cardiac punctures performed 9 hours after treatment. Data were expressed as percent changes in FSH between pre- and post-treatment samples.

2. Results

The results are shown in Figure 17. Normal (control data not shown) % changes of FSH under these conditions ranged from approximately 5 to 20%. It appeared from these data that some FSH suppressing activity was present in the 80% fraction and perhaps some activity in the 40% although this fraction's activity was not as distinct. In addition, the 100% fraction group showed a slight increase in FSH levels suggesting the presence of some substance in this fraction that may be stimulatory to FSH secretion.

B. SDS-Polyacrylamide Electrophoresis of Ammonium Sulfate Fractions

1. Experimental Design

Aliquots of each fraction were subjected to SDS-polyacrylamide electrophoresis to determine approximate molecular weights of substances present.
Figure 17. The effects of ammonium sulfate fractions of PFF on plasma FSH levels in ovariectomized rats. 0.5 ml equivalent starting material was injected i.p. and blood samples taken 9 hours later (N = 7/group).
2. Results

SDS-polyacrylamide gels of the 80% fraction showed six to eight bands of protein. There were three bands in the 10,000 to 30,000 dalton range. Several additional bands were present ranging from 38,000 to 95,000 daltons.
DISCUSSION

I. GONADOTROPIN PROFILES IN OVARIECTOMIZED ESTROGEN CAPSULE IMPLANTED AND INTACT CYCLING RATS

The plasma LH levels seen in the 5 mm OVX-E rat are in agreement with those seen in other laboratories (63). The plasma LH levels are completely suppressed by the estrogen levels produced by implantation of the 5 mm estrogen capsule. LH undergoes a daily surge seen at 1700 hrs returning to near baseline values by 1900 hrs. This finding is in agreement with others (4,63) that suggest a daily signal or 24-hour cyclicity in the "LH-release apparatus" of female rats. Numerous studies have shown that various brain nuclei display characteristic patterns of integrated multiple unit activity (MUA) throughout the estrous cycle of the rat (68,69). There appears to be a slight circadian rhythm of MUA in the basal hypothalamus and forebrain limbic system (68) and it has been suggested that this rhythm is a function of the light/dark cycle. In the rat, the rising levels of estrogen on proestrus in some manner amplifies this neural rhythm and if estrogen levels are sufficiently high, a sequence of neural events takes place ultimately leading to the release of hypothalamic releasing hormone and subsequent proestrous surges of LH and FSH.

The pattern of plasma FSH shown in Figure 1 suggests that FSH also undergoes a daily surge in the OVX-E rat at 1700 hrs. In addition, there is a secondary surge in FSH independent of LH which is analogous to the secondary FSH surge seen at 0400 hrs estrus as in Figure 3 and reported
by others (9,10). The magnitude of these primary and secondary surges in the OVX-E rat appear to be considerably less than those seen in the intact animal. One possible explanation for this finding is in differences between RIA procedures and reference preparations used in assaying these samples since they were not assayed in the same assay. Alternatively the smaller magnitude in the OVX-E rats could result from a depletion of pituitary FSH since it appears they undergo a daily release of FSH. Also, we cannot rule out the possibility that other ovarian secretions may play a role in the normal FSH surge which are missing with only estrogen replacement. In addition, as reported for the intact cycling rat (10) estradiol levels reach a peak and begin to decline prior to the peaks of the LH and FSH proestrous surge. In the OVX-E rat, it is plausible that the constantly elevated levels of estradiol resulting from sustained release of estradiol from capsules may in some way affect the release mechanism of the gonadotropins, in particular FSH.

It is not clear at present whether the neural "trigger" for the proestrous surge of the gonadotropins is a function of the rate at which estrogen levels rise on proestrus as suggested by some (70) or the absolute level of circulating estrogen. Our experiments comparing different size estrogen capsules support the latter hypothesis. Since no primary FSH surges were seen at 1700 hrs in the OVX-E rats bearing 1mm (Figure 2) or 3 mm estrogen capsules, it would appear that the "critical" level of circulating estradiol for FSH lies somewhere in between those produced by the 3 mm and 5 mm capsules. Of interest, however, is the fact that
1 mm and 3 mm OVX-E rats displayed an attenuated surge of LH at 1700 hrs (data not shown). This suggests that the "critical" or "threshold" level of estradiol may be different for FSH and LH. This may manifest itself at the pituitary level altering responsiveness to LHRH and/or basal secretion of FSH or at the hypothalamic level by some undetermined mechanism, perhaps a separate FSH neural network.

The primary FSH rise seen at 1700 hrs in the OVX-E rats bearing 5 mm estrogen capsules occurs concomitant with the LH surge. The FSH levels subsequently fall as do the LH levels at 1900 hrs followed by an abrupt secondary surge of FSH only at 2100 hrs through 0300 hrs the next day. Although the pattern of FSH release appears similar to that seen in the intact cycling rat, there is a distinctive temporal shift observed. Where as the primary FSH surge in OVX-E rats occurs at 1700 hrs, the same surge in intact rats is not seen until 1900 hrs (Figure 3). Similarly the secondary surge of FSH in OVX-E rats peaks at 2100 hrs whereas the FSH levels in intact rats actually appear to be declining at this time. This apparent temporal shift may be a result of the constantly elevated levels of estradiol in OVX-E rats allowing "threshold" for the neural apparatus to be reached as early in the day of proestrus as the circadian rhythm is present. Support for this idea comes from several studies comparing four and five day cycling rats (70,71).

Smith, et al. (71) reported that the LH and FSH surges occur earlier on the afternoon of proestrous in the five day rat than in the four day rat. They suggested that in the five day rat the "critical" estrogen level is reached too late after the previous ovulation to result in a four day
cycle. Because of the "24 hr clock" in these animals, they must wait one entire day before the neural events can be "triggered" by estrogen. In the meantime, the estradiol levels in the five day cycle rat continue to increase exposing the hypothalamic-pituitary mechanism to higher more prolonged estradiol levels. This results in "threshold" being reached sooner resulting in a temporal shift in gonadotropin proestrus surges. It is plausible that the prolonged high estradiol levels in OVX-E rats may also cause the temporal shift seen in our experiments.
II. EFFECTS OF SYSTEMIC INJECTION OF OVARIAN INHIBIN PREPARATIONS

Since the OVX-E rat bearing 5 mm estrogen capsules underwent a daily FSH surge seen at 1700 hrs, we used this model to investigate the effects of ovarian inhibin containing preparations on this FSH surge. Barrow serum (BS) collected from male pigs castrated when young was used as one control test substance since in the absence of the testes the serum would presumably have no inhibin activity. It was our intention to use a material similar to porcine follicular fluid as a control for non-specific effects of substances other than inhibin present in PFF. As seen in Figure 4, A.M. levels of FSH were comparable on all days tested, being similar to those seen at 0900 hrs in Experiment I. Saline and BS had no effect on the plasma FSH levels seen at 1700 hrs since they were comparable to those seen at 1700 hrs in Experiment I. However, injection of PFF at both doses, .25 and 1 ml, significantly attenuated the 1700 hrs surge of FSH compared to BS and saline controls. Since both doses of PFF were equally effective in this experiment, it would appear that the maximal effect was achieved with the lower dose. However, in subsequent experiments 1 ml of PFF was routinely used since there may have been slight differences in inhibin activity of the different batches of PFF collected. We concluded from this experiment that PFF does contain an agent, presumably ovarian inhibin, capable of suppressing FSH secretion. In addition, it appears that the model OVX-E rat would be acceptable for the bioassay of inhibin activity. In these experiments it was not possible to determine plasma LH values since we felt that sampling more than 1 ml blood every two days would be too
stressful to the animals. Since we routinely used 200 µl of plasma in duplicate in the FSH RIA insufficient quantities of plasma remained for LH determination. Therefore, in these experiments no conclusion can be drawn concerning the effects of PFF on plasma LH levels. However, as discussed in the Introduction, most laboratories report little or no effect on LH levels suggesting a reasonably specific effect on FSH secretion (49). This conclusion is supported by our later experiments showing no effect of PFF injection on serum LH in ovariectomized rats.

Since we had in mind to investigate the effects of ovarian inhibin on the CNS, it was desirable to "clean up" the test materials rather than apply whole PFF to neural tissue. Because ovarian inhibin had been reported by several laboratories to have a molecular weight around 20,000 daltons, we used molecular weight ultrafiltration to prepare an extract of PFF and BS termed PFFX and BSX respectively. The effects of systemic injection of PFF in OVX-E rats was tested, the data is shown in Figure 5. PFF and PFFX both appeared to attenuate the FSH surge in OVX-E rats at 1700 hrs. The data here are expressed as percent change in FSH from morning to afternoon samplings. Plasma FSH levels with BS rose 123% from morning to afternoon. This increase was nearly identical to that seen in this model in Experiment I suggesting to us again that BS had no effect. PFF treatment caused a marked reduction in plasma FSH, levels actually decreasing in 6 our of ten treated animals. In the remaining 4 animals severely attenuated surges were seen. PFF treatment day, although not significantly different from BS or PFF treatment days because of the large standard errors, appeared to retain some
of its activity. From these data it was apparent that FSH surges in OVX-E rats could be completely blocked by administration of PFF. Why PFF was more effective in six of ten animals is not readily apparent. Although care was taken during injection of these animals, others have suggested that similar discrepancies in their experiments can be explained as periodic injection into the gut rather than into the peritoneal cavity (72).

In addition, PFF in this experiment was much more effective than in the previous experiment. This probably resulted from the difference in the site of administration. PFF in the previous systemic injection experiment was given subcutaneously (s.c.) whereas here it was administered intraperitoneally (i.p.). The active agent in PFF may be inactivated more quickly when administered s.c. Alternatively, it may take longer to enter the circulation when given s.c. . This latter possibility would certainly explain the results since subsequent to these experiments it had been reported that the latency of action for PFF is 2-5 hours with a duration of 5-14 hrs, the maximal response occurring 8-12 hrs after administration i.p. (72). Since blood sampling was routinely performed 4½-5 hours after administration of test substances in these experiments it is probable that maximal suppression of FSH had not taken place. Therefore, it is plausible that a greater suppression of FSH might have been detected if the test period had been extended.

Although the plasma FSH levels following PFFX administration were not statistically different from BS controls, the extract clearly retained some of its FSH-suppressing activity. The loss of activity
suggests either that activity was lost through handling the materials during the extraction process or that some FSH-suppressing activity may exist in molecular species residing outside the 10,000 to 30,000 dalton molecular weight range. This latter possibility must be seriously considered in light of several reports describing the existence of FSH-suppressing material of ovarian and testicular origins which have molecular weights outside the range (49,73).
III. EFFECTS OF PFFX ADMINISTRATION TO THE CNS

In these experiments we investigated a possible CNS site of action for ovarian inhibin. The two areas chosen for investigation were the medial preoptic area (MPOA) and the dorsal anterior hypothalamic area (DAHA). The MPOA was chosen because of its suggested role in the proestrus LH and FSH surges. It is believed that this area is the integrator or initiator of neural signals that eventually lead to LHRH release into the hypophyseal portal system. Peptidergic neurons containing LHRH have been localized here in a number of studies and it is thought these neurons project to the median eminence with terminals near the portal vessels (74,75). In addition, stimulation, deafferentation, and lesion studies show this area to be essential for the cyclic discharge of gonadotropins (7,12).

The DAHA was selected because of numerous studies discussed previously implicating it in some control mechanism specific for FSH secretion. Using the model of OVX-E rat bearing 5 mm estrogen capsule PFFX appeared to be capable of suppressing the FSH surge at 1700 hrs when applied to the DAHA but not to the MPOA (Figure 6). The percent change in FSH seen in the MPOA animals when BSX was administered was similar in magnitude to control animals in previous experiments suggesting BSX had no effect when applied to the CNS. The plasma FSH levels in the MPOA group when administered PFFX were not significantly different from BSX although a small decrease was observed. In contrast,
PFFX administration to DAHA significantly attenuated the FSH surge compared to BSX treatment of the same animals. The generally lower percent increase in plasma FSH levels between 1200 and 1630 hrs in DAHA-cannulated animals on both treatment days as compared to MPOA-treated animals remains to be explained, however, it is probable that some destruction of neural tissue results from implantation. Whereas histological examination revealed that the DAHA sustained only minimal apparent damage, such minimal damage may have resulted in slight attenuation of the FSH surge in DAHA-implanted rats in much the same way that large lesions of this area attenuate the post-castration increases in plasma FSH (34).

Realizing that the FSH-suppressing actions of PFFX administered to the DAHA could possibly result from diffusion of material to the pituitary from the site of implantation, we subjected a small amount of PFFX to iodination with $^{125}\text{I}$ as described in Methods and Materials. Fractions collected from the column show the presence of some labeled material separate from free iodide (Figure 7, fraction 6&7). Since this labeled material did not diffuse as far as the pituitary in 4½ hrs but did diffuse into the hypothalamus we concluded that the effect seen on FSH secretion in DAHA rats treated with PFFX was a result of action on neural tissue. Although we cannot say that material collected from the Sephadex column was indeed radiolabeled inhibin the diffusion of substances in labeled and unlabeled PFFX were most likely similar.

We concluded from this experiment that ovarian inhibin may have a CNS site of action perhaps in or near the DAHA. Support for our
conclusion has recently been reported by Lumpkin, et al., (76). Using inhibin of testicular origin, they were able to show attenuation of the post-castration rise of FSH when animals were given inhibin by intraventricular administration.
IV. EXAMINATION OF POSSIBLE MECHANISMS INVOLVED IN THE ACTION OF OVARIAN INHIBIN

Since the potential of ovarian inhibin as a longterm regulator of FSH secretion and hence folliculogenesis in the female had not been well documented, we decided to examine the effects of chronic administration of PFF. The injection regimen consisted of 1 ml 8-9 hrs before sacrifice in acute experiments and .5 ml twice daily for 3 days the last injection given 8-10 hrs before sacrifice in chronic experiments. By this regimen in the chronic experiments we hoped to saturate the animals system with inhibin since the duration of its action has been reported to be at least 14 hrs (72). As seen in Figure 8 PFF was effective in suppressing FSH levels only when administered acutely, the serum levels of FSH being similar in all chronically treated animals. This finding was of interest since it suggested that ovarian inhibin may not be effective as a longterm regulator of FSH secretion. In addition, it suggested to us that some compensatory mechanism may be occurring during 3 days of treatment.

These findings are in agreement with another study reporting similar FSH levels in control and PFF injected animals after 3 days of treatment (50). Serum LH values were not different among groups in acute or chronic experiments indicating that the effects of PFF were largely on FSH.

To further elucidate by what mechanism this inhibin preparation was acting we incubated the pituitaries of acutely and
chronically injected animals in culture for four hours. As shown in Figure 10, basal FSH secretion of hemipituitaries from animals acutely injected with PFF was markedly reduced compared to BS and saline controls. Animals chronically treated show no such differences suggesting that the lack of effect on serum FSH in chronically treated animals may be a result of non-suppression of basal release of FSH and possibly synthesis. Although pituitary content of FSH was not determined, others have reported a decrease pituitary FSH content after inhibin treatment in vitro suggesting a decrease in synthesis of FSH. Since the basal secretion was not inhibited in chronically treated animals the pituitary gonadotrophs may be able to "over ride" the inhibitory effects of inhibin on synthesis and/or release of FSH. Basal secretion of LH did not appear to be effected, being similar among the three groups in each experiment. However, basal secretion of LH as well as serum LH of all chronically treated animals appeared to be slightly higher than their acutely treated counterparts. The reason for this remains to be determined but it is possible that stress of animals due to handling may be involved since chronic animals were handled twice a day for 3 days and acute animals only twice. LHRH responsiveness of hemipituitaries from treated animals was next compared. As seen in Figure 12 LHRH-stimulated FSH release was markedly suppressed in acutely and chronically treated PFF groups. In addition, as seen in Figure 13, PFF effectively inhibited LHRH-stimulated LH release in chronically treated animals with no differences evident between acutely treated groups. This latter finding remains to be explained. That PFF can inhibit LHRH-stimulated FSH and LH release is in agreement with other in vitro (25) and in vivo experiments (49).
However, it was surprising that PFF was almost equally effective on FSH release in acutely and chronically treated animals. Since PFF is capable of inhibiting LHRH-stimulated release of FSH even in chronically treated animals, it appears that the compensatory mechanism which occurs in chronically treated animals to "over ride" FSH suppression is not a result of changes in responsiveness to LHRH. This however, is not in agreement with a report by Wilkinson et al (77, personal communication) who demonstrated an increase in LHRH responsiveness of pituitaries from hemicastrated immature male rats chronically treated for 7 days with PFF. The explanation of these differences remains to be resolved however, the model used here is quite different from that used by Wilkinson, et al. Since steroid levels in the hemicastrated male rat would be higher than in bilaterally ovariectomized females, a difference in the responsiveness of pituitaries to LHRH from these different animals would be expected. In fact, an increase in LHRH responsiveness of pituitaries from ovariectomized animals chronically treated with PFF would very easily explain the "compensatory mechanism" evidenced by non-suppressed serum FSH values in these animals. However, since we did not find this to be the case, we must conclude that the lack of suppression of serum FSH in these animals is a result of an action on basal secretion and/or synthesis of FSH by the pituitary, an effect on hypothalamic releasing hormone(s) or both mechanisms.

Since there is considerable evidence for the involvement of cyclic nucleotides in the action of LHRH on LH and FSH release, it was of interest to investigate the possible involvement of inhibin in this
process. Table 1 shows the tissue cyclic nucleotides levels in anterior pituitaries from animals that had been acutely treated with PFF, BS and saline. cAMP of hemipituitaries of animals injected with PFF showed slightly lower values than saline control pituitaries. Only the LHRH stimulated tissue proved significantly different from saline LHRH group (p < .05) but not BS control group. There were no significant differences between basal secretion groups even though FSH basal secretion of the PFF group was significantly different from BS and saline controls. cGMP levels showed marked variation although overall it appeared that cGMP content of PFF and saline LHRH-stimulated pituitaries were lower than their non-stimulated hemipituitaries. From these data it is difficult to draw definite conclusions. The observed variability of these levels may be a result of processing of the pituitary tissue immediately after incubation or of tissue anoxia both of which have been reported to change cyclic nucleotide levels (78). The fact that there exists no general consensus of the exact nature of the involvement cyclic nucleotides have in LHRH stimulated release of LH and FSH adds to the difficulty in interpreting these data. In addition, the use of hemipituitaries in the study of cyclic nucleotides may be questioned since it is thought that only a small percent of pituitary cells are in fact gonadotropin secreting cells. Nevertheless, these data suggest that the action of PFF may involve cAMP and cGMP at the pituitary level. Clarification of this will have to await use of gonadotropin-enriched pituitary cell preparations and purer inhibin preparations.
Since we concluded that the "compensatory mechanism" which occurs after 3 days of treatment with PFF might involve a mechanism at the hypothalamic level, we investigated hypothalamic FSH and LH "releasing activity" of rats acutely and chronically treated. The releasing activity was measured by incubating donor pituitaries with acid extracts (HX) of hypothalami from treated animals and measuring the LH and FSH media content.

The pattern of HX-releasing activity for animals acutely treated was similar for LH and FSH. Surprisingly, a large amount of LH- and FSH-releasing activity was seen in the BS treated group compared with PFF and saline group. The explanation for this finding in unclear but the possibility of some effect of BS on hypothalamic LHRH or other releasing hormone species cannot be ignored. Alternatively, differences in releasing activity of hypothalami may be an artifact of hypothalamic tissue extraction procedures. Additional experiments will have to be conducted to clarify these results. Releasing activities of hypothalami from animals chronically treated showed completely different patterns for LH and FSH release (Figure 14,15). FSH-releasing activities for all chronically treated animals were similar, the saline group being slightly lower than PFF and BS groups.

The amount of LH released by HX of chronically treated animals differed from the LH released by HX's of their acutely treated counterparts. In addition, the pattern of LH release differed from the pattern of FSH released.

Since these data were inconclusive we decided to measure the effect of an inhibin preparation on hypothalami in vitro. The results of this
experiment are shown in Figure 16. Hypothalamic fragments were incubated with PFFX and BSX as described in Methods and Materials. These data suggest that hypothalamic fragments incubated with PFFX have lower levels of LHRH than fragments incubated with BSX or those incubated without addition of test materials. In addition, the amount of LHRH measured in incubation media was slightly less in PFFX group. These findings could be a result of PFFX degradation of LHRH or an actual decrease in LHRH synthesis. Similar results have been reported by Demoulin, et al., (79, personal communication) obtained with male rat hypothalami and testicular inhibin. In their experiment, bacitracin, which inhibits LHRH degradation in vitro, was added to the incubation media. They concluded from their experiment that inhibin may act at the hypothalamic level by inhibiting LHRH synthesis. Although out results with ovarian inhibin support their conclusion, further experiments with purified ovarian inhibin are necessary.
V. AMMONIUM SULFATE FRACTIONATION OF PORCINE FOLLICULAR FLUID.

As other experiments were conducted, further purification procedures for ovarian inhibin were investigated. Here PFF was subjected to classic ammonium sulfate precipitation and subsequent SDS-polycrylamide electrophoresis. Figure 17 shows the presence of FSH suppressing activity in fraction 80% since it was the only fraction which decreased plasma FSH values in ovariectomized rats. This is in agreement with some (49) but not with others (73). Although the SDS gels showed the presence of three bands within the 10,000 to 30,000 dalton molecular weight range, there were in addition, several higher molecular weight bands ranging from 38,000 to 95,000 daltons. Although this procedure does not produce a pure preparation of ovarian inhibin, it will be useful as a preparative step for further purification procedures.
CONCLUSIONS

The results of these studies demonstrate that the ovariectomized estrogen capsule implanted rat (OVX-E) undergoes daily primary FSH surges coincident with the daily LH surge seen in these animals. In addition, there appears to be a secondary FSH surge in OVX-E rats analogous to that seen in the intact cycling rat. There is a marked temporal shift in the occurrence of these surges in the OVX-E rat compared to intact rats, most likely resulting from constantly high levels of estradiol. The absolute estradiol level seems to be the key in eliciting these FSH surges in OVX-E rats since smaller estrogen capsule (1 mm and 3 mm) fail to elicit FSH surges. Since attenuated LH surges are seen in OVX-E rats bearing smaller capsules, this also suggests that the estradiol "threshold" level is different for FSH than LH.

Administration of porcine follicular fluid (PFF) or an extract of PFF (PFFX) is capable of suppressing FSH levels in the rat by either systemic injection or application to the dorsal anterior hypothalamic area. We conclude from this that PFF contains a non-steroidal agent, ovarian inhibin, which suppresses FSH secretion and provide the first preliminary evidence for a possible CNS site of action of this hormone in the control of FSH secretion. In addition, we conclude that extracts of PFF can decrease LHRH content of hypothalami incubated in vitro suggesting a possible mechanism at the CNS level. This latter conclusion, however, awaits further investigation with purer ovarian inhibin
preparations.

The acute versus chronic administration of PFF studies suggest that ovarian inhibin may not be useful as a long term regulator of FSH and hence of folliculogenesis in the female rat. We conclude that basal secretion of FSH appears to be acutely effected by PFF but not chronically. However, it appears LHRH-stimulated FSH release is inhibited by PFF when administered both acutely and chronically suggesting that the observed plasma FSH levels in rats chronically treated with PFF are a result of compensation by some other mechanism.
Table 2. Plasma FSH levels in ovariectomized estrogen-implanted rats at 1200 hrs (A.M.) and 1700 hrs (P.M.) after treatment with 1 ml barrow serum (BS), 1 ml porcine follicular fluid (PFF), or .25 ml extract of PFF (PFFX).

<table>
<thead>
<tr>
<th>BS</th>
<th>A.M. (ng/ml)</th>
<th>P.M. (ng/ml)</th>
<th>% CHANGE</th>
<th>A.M. (ng/ml)</th>
<th>P.M. (ng/ml)</th>
<th>% CHANGE</th>
<th>A.M. (ng/ml)</th>
<th>P.M. (ng/ml)</th>
<th>% CHANGE</th>
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<td>475</td>
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</table>

**Mean ± SE**

| BS       | 312 ± 67     | 528 ± 79     | 342 ± 88  | 315 ± 95     | 182 ± 50 | 235 ± 48 |

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Table 3. Plasma FSH levels at 1200 hrs (A.M.) and 1630 hrs (P.M.) in ovariectomized, estrogen-implanted rats bearing chronically implanted dorsal anterior hypothalamic (DAHA) or medial preoptic (MPOA) cannulas. Cannulas were filled with either barrow serum extract (BSX) or porcine follicular fluid extract (PFFX) following the 1200 hrs blood sample.

<table>
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<td>PFFX</td>
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<tr>
<td>A.M.</td>
<td>P.M.</td>
<td>Z CHANGE</td>
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<tr>
<td>(ng/ml)</td>
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<tr>
<td>NEAN ± SE</td>
<td>251 ± 29</td>
<td>382 ± 43</td>
<td>212 ± 42</td>
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|          | BSX  | PFFX     | BSX  | PFFX     |
| A.M.     | P.M. | Z CHANGE | A.M. | P.M. | Z CHANGE |
| (ng/ml)  | (ng/ml) | (ng/ml) | (ng/ml) | (ng/ml) |
| 100      | 240  | 140      | 106  | 253  | 139      |
| 80       | 266  | 233      | 206  | 220  | 7        |
| 193      | 353  | 83       | 180  | 413  | 129      |
| 60       | 193  | 222      | 133  | 173  | 30       |
| 133      | 140  | 5        | 135  | 260  | 93       |
| 140      | 273  | 95       | 153  | 473  | 209      |
| 196      | 382  | 95       | 158  | 453  | 187      |
| 173      | 392  | 127      | 123  | 248  | 102      |
| NEAN ± SE | 134 ± 19 | 280 ± 34 | 149 ± 12 | 312 ± 44 |
Table 4. Mean plasma FSH levels and percent change of the mean from 1200 hrs (A.M.) to 1700 hrs (P.M.) in animals described in Experimental Design and Results, Section II, B (Data shown in Table 2).

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<td>(ngFSH/ml)</td>
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Table 5. Mean plasma FSH levels and percent change of the mean from 1200 hrs (A.M.) to 1630 hrs (P.M.) in animals described in Experimental Design and Results, Section III, A (data shown in Table 3).

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<td>MEAN ± SE</td>
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<td>MEAN ± SE</td>
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<td>109</td>
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47. Setchell, B.P., R.V. Davies and S.J. Main. Inhibin, In: The Testes. Eds Gomes,


