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INFLUENCE OF OVARIAN HORMONES ON THE CENTRAL OXYTOCIN INDUCE STIMULATION OF LUTEINIZING HORMONE IN OVARIECTOMIZED RATS

by

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Influence of ovarian hormones on the central oxytocin induced stimulation of luteinizing hormone (LH) in ovariectomized rats

Central oxytocin (oxy) can stimulate LH secretion in cycling female proestrous rats, but not in metestrous or diestrous rats. Gonadal steroids play a critical role in regulating the influence of oxy on LH secretion. To examine the influence of estradiol (E2) and/or progesterone (P4) directly, the effects of dose and duration of E2 and P4 exposure on the oxy stimulation of LH release was examined in ovariectomized (OVX) rats. For chronic exposure, subcutaneous time-release pellets containing vehicle, E2, P4 or both hormones were implanted in OVX rats. For short term exposure, various doses of P4 were administered, s.c., at 900 hr in OVX, E2-primed or OVX animals. Central injections of oxy were given through stereotaxically-placed, intracerebroventricular (icv) cannulae. Plasma samples were obtained via jugular cannulae from the unanesthetized, freely moving rats at 10 min before (Pre) and at 5, 15, 30 and 60 min following icv injection. Basal LH levels were highest in OVX animals, and responded to icv oxy with a modest decrease at 30 and 60 min. E2 or P4 replacement lowered basal LH levels, and the combination of E2 and P4 lowered basal LH even more. The results of oxy injection in E2-treated animals were variable, but in most cases caused a small increase in plasma LH. In animals treated with P4, alone, oxy injection did not alter plasma LH. In E2-primed animals, the acute injection of P4 caused a bell-shaped dose-response curve with low doses allowing some stimulation of LH release in response to central oxy injection, 2.5 mg P4 causing a dramatic synergism with E2 on the LH response, and higher doses causing a diminished response. The results prove the stimulatory effect of centrally administered oxy can be seen in a pharmacological model of the proestrous female rat. In the absence of E2 or P4, oxy exerts an inhibitory influence on plasma LH. The level of stimulation by oxy on plasma LH was critically dependent on the optimal dose and time of P4 as well as E2 priming.
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I. Neurotransmitters/Neuropeptides and Luteinizing Hormone Secretion

The primary neuroendocrine factor responsible for triggering the preovulatory surge of luteinizing hormone (LH) in mammals is the hypothalamic factor, luteinizing hormone releasing hormone (LHRH), which directly stimulates LH release from gonadotrophs located in the adenohypophysis (1-3). It is also accepted that many other factors modulate this regulation. The important role which LH exerts in stimulating ovulation is universally recognized (3).

Several neuropeptide and neurotransmitter substances contained within the central nervous system have been reported to play a significant physiological role in the regulation of LHRH and LH release. Norepinephrine (NE) is a monoamine neurotransmitter which has been shown to consistently stimulate LH release in a dose-related fashion in estrogen and progesterone treated ovariectomized rats or in intact, cycling female proestrous rats (4-7). Other numerous neuropeptides may play a role in the neuroendocrine regulation of LH secretion which depends upon the presence of gonadal steroids. neuropeptide Y (NPY) and the related human
pancreatic polypeptide stimulate LH secretion, but the response depends upon the presence of gonadal steroids (8-10). Steele et al (11) demonstrated that angiotensin II can stimulate LH secretion by working at central sites. Glutamate has also been found to stimulate LH, but not follicle stimulating hormone (FSH), secretion in estrogen-treated, ovariectomized immature rats (12). Endogenous opioid peptide neurons are recognized as a hypothalamic network which exerts a powerful inhibitory influence on gonadotropin secretion. It is obvious that each of the opiate receptor subtypes in the hypothalamus can suppress LH release when activated by an appropriate opiate receptor agonist (13-14). Another inhibitory factor on LH secretion is GABA (15). This inhibitory system inhibits LH secretion via GABA receptors and it operates without mediation by the NE system (15). Corticotropin-releasing factor (CRF) can also suppress LH secretion, and this action is through its stimulating the release of hypothalamic opioid peptides (16).

II. Central role of oxytocin in LH secretion

Hormonal functions for oxytocin on the female reproductive system are well established. During parturition, oxytocin regulates the contraction of the uterine wall and, during lactation, oxytocin triggers milk-ejection from the mammary gland (17). Several studies on the maternal behavior (17-18) and female sexual behavior (19-21) of the rat indicate
that oxytocin also plays a role in these behaviors in the central nervous system. Other studies indicate a possible role for oxytocin in the neuroendocrine regulation of anterior pituitary hormone secretion. Synthetic oxytocin stimulates prolactin secretion from the anterior pituitary both in vivo and in vitro (21-22). Oxytocin can influence the secretion of ACTH from the pituitary gland (23-26). Oxytocin has also been reported to stimulate gonadotropin-releasing activity in vivo. For example, oxytocin induced an increase in number and size of gonadotrophs in rats and dogs, increased testicular weight, enhanced urinary excretion of 17-keto steroids in rabbits (27), and increased urinary gonadotropin excretion (28-29). Recent reports indicate a physiological role for oxytocin in the neuroendocrine regulation of LH secretion (30-35). The first evidence that oxytocin might stimulate gonadotropin secretion was proposed in 1988 (30). It was later demonstrated that oxytocin can stimulate gonadotropin secretion from rat anterior pituitary cells in static culture (36). These findings suggest that the observed accelerated follicular maturation and ovulation in mice given oxytocin might have been a result of oxytocin-induced release of pituitary gonadotropins, although the endocrine events were not elucidated (37). Oxytocin is released directly into the hypophyseal portal blood system and could, therefore, affect LH secretion directly from the anterior pituitary (35-36). Furthermore, oxytocin neurons send projections which terminate
in many areas of the brain containing cell bodies or terminals of LHRH neurons or other LHRH secretagogues (38-42). However, there is a controversy concerning how oxytocin influences LH secretion. Some reports have indicated that the actions of oxytocin on LH release are primarily inhibitory in nature and are mediated through actions on central LHRH neurons (36). Other reports suggest the action of oxytocin on LH release is minor at best (43). On the other hand, there is evidence supporting a physiologically relevant stimulatory influence of oxytocin on the secretion of LH from the anterior pituitary mediated primarily at a central site (30). These actions result in the preovulatory release of LH (32). The latter studies utilized a physiological animal model of the female cycling rat on proestrus (when plasma levels of estradiol are physiologically high). The earlier studies showing inhibitory or little action of oxytocin on LH release used male or ovariectomized female rats (both of which have low plasma estradiol levels). The results indicate that in order for oxytocin to stimulate LH secretion, physiologically high levels of estradiol must be present (32).

Much evidence suggests estradiol plays a predominant role in the excitation of preovulatory LH release. From a variety of experiments, it is apparent that estradiol is required for the stimulation of LH release in ovariectomized rats (6, 8, 9, 11). Estrogens and progesterone have also been shown to increase the number of oxytocin receptors in some
areas of the brain (44-45). Additionally, gonadal steroids, in particular estradiol, are known to have multiple effects upon central oxytocin neurons. Administration of estradiol enhances the electrical activity of magnocellular oxytocin neurons (44-45), stimulates oxytocin secretion into the pituitary portal circulation (46), and releases oxytocin associated neurophysin into the peripheral circulation (47-49). Finally, receptors for estradiol are found on central oxytocin neurons (3) but not on central LHRH neurons.

1) Distribution of oxytocinergic neurons in the central nervous system

Oxytocin, synthesized in the hypothalamus and released from the posterior pituitary, has been associated with immunoreactive neurons not only in the classical magnocellular groups (the paraventricular and supraoptic nuclei), but also in the anterior commissural nucleus, medial preoptic area, periventricular stratum, intersupraopticoparaventricular (internuclear) group, perifornical nucleus, nucleus circularis, zona incerta and the ventral nucleus of ansa lenticularis (50-51). Besides the well-known projections to the posterior pituitary, oxytocinergic neurons project to the external zone of the median eminence (52), to the brain stem and spinal cord (53), and to olfactory and limbic targets (54-56).
2) Estradiol interactions with central oxytocin neurons

Oxytocin's involvement with reproductive functions centering on parturition and maternal and receptive behavior may be, in part, influenced by estrogen. Concentrations of oxytocin in the pituitary have been found to fluctuate with the estrous cycle (57). Oxytocin levels in the portal blood increase on the afternoon of proestrus, correlating with the onset of sexual receptivity as well as the preovulatory LH surge (47). Estrogen treatment can increase oxytocin release from the pituitary (58). Correspondingly, oxytocin content of anterior commissural neurons decreases with estrogen treatment, perhaps associated with increased oxytocin release from these neurons (59). Some oxytocin immunoreactive neurons in portions of the paraventricular nucleus and a few cells in the anterior commissural nucleus accumulate estrogen in the cell nucleus (60-62). These immunocytochemical studies show both increased oxytocin immunoreactivity and increased distribution of oxytocin immunoreactive neurons following estrogen treatment in the anterior commissural nucleus, anterior hypothalamic area, septohypothalamic nucleus, medial preoptic area, perifornical nucleus, and zona incerta (50-51, 61).

Recent experiments have utilized in situ hybridization to study possible estrogen influences on oxytocin messenger RNA levels. These studies concentrated on preoptic and anterior hypothalamic levels because of the possible
involvement of oxytocin in LHRH function influencing LH release (30) and because of demonstrated preoptic sites of action of oxytocin in female sexual behaviors (63). Analysis of oxytocin-expressing neurons included the numbers of cells labeled with in situ hybridization procedures as well as the numbers of grains per labeled cell because these two parameters can change independently.

The possibility of direct involvement of estradiol with oxytocin or vasopressin-producing neurons was suggested by observations of tritiated estradiol binding in the cell nuclei of neurophysin-immunoreactive neurons (61-62). Using northern blot and dot blot analysis, Van Tol et al (64) demonstrated a significant increase in oxytocin mRNA levels during the estrous phase of the female rat's cycle to almost twice the levels seen on diestrus. Oxytocin mRNA in the brain of the female rat is increased concomitant with puberty, and ovariectomy of adult females decreased oxytocin mRNA values (65); both observations consistent with estrogen stimulation of oxytocin. Significant elevations of oxytocin mRNA during the late stages of pregnancy also suggest increased gene transcription under the influence of ovarian hormones (66). Radioimmunoassay of oxytocin in micropunches from the medial preoptic area revealed significantly increased levels in estrogen-progesterone-treated animals which were behaviorally receptive (59), while the number of immunocytochemically detectable oxytocin cell bodies actually decreased. This
configuration of results suggested a greater amount of expression per oxytocin-producing neuron. Jirikowski et al (51) reported a stimulatory effect of short-term estradiol treatment on the distribution of immunoreactive cell bodies. Greater numbers of oxytocin immunoreactive neurons were seen in the septohypothalamic nucleus, the lateral subcommissural area, the medial preoptic area, the perifornical region, the zona incerta, and the ansa lenticularis. Finally, a number of experimental approaches in different species have suggested strongly that estrogen treatment facilitates oxytocin release (48, 58). Under these circumstances, where release is increased by estrogen and immunoreactive content is either not changed or is increased, elevated transcription and/or mRNA stability is to be expected.

The mechanisms of estrogen effects on oxytocin mRNA levels are unclear. The possibility of a direct effect of the estrogen receptor on transcription is indicated by the presence of these partial estrogen-response-elements upstream to the oxytocin transcription start site (67-69). On the other hand, significant numbers of supraoptic neurons with nuclear estrogen receptors have not been identified (70). There may be an analogy to control of the LHRH gene, where a long estrogen treatment can lead to increased mRNA levels (71-72) and where the stimulatory effects of estrogen can be interpreted as consequent to the release of the neuroendocrine peptide (73). Co-existence of oxytocin and of estrogen receptor in the same
neurons raises questions regarding their possible functional significance. Changes in the oxytocin levels in circulation after estrogen treatment (58) and during the estrous cycle in the PVN (74) and in the pituitary (57) have been reported. Results of these studies suggest that physiological fluctuation in ovarian steroid levels affect the release of neurohypophyseal hormones. Although the intensity of oxytocin immunostaining of perikarya and processes in several brain sites vary after estrogen treatment, it is impossible to explain whether the increased immunostaining after estradiol treatment is due to an increased synthesis of peptide or to an inhibition of its secretion. Existence of estrogen receptor protein in an identical population of neurons does not represent complete proof that estrogen regulates the synthesis of that peptide. However, recent studies using a hybridization assay on the levels of rat hypothalamic oxytocin mRNA during gestation, lactation and the estrous cycle (64,75-76) have shown that the expression of oxytocin gene was stimulated concomitantly with an increase in serum estrogen levels during the estrous cycle and pregnancy (77). These observations suggest that estrogens may be involved in regulating oxytocin gene expression. Recent studies by Richard and Zingg (78), demonstrate that beta-estradiol administration stimulates the transcriptional activity of the human oxytocin gene promoter by a direct estrogen receptor dependent mechanism. Anatomical evidence that an estrogen-stimulated release of oxytocin from
the posterior pituitary is mediated by a direct effect of estrogen on magnocellular hypothalamic neurons has also recently been published (79).

3) Gonadal hormones and LH secretion

Although a lot of evidence gathered over the years has documented the feedback action of gonadal steroids in the hypothalamus and pituitary in the neuroendocrine regulation of LHRH and LH secretion, the mechanisms involved in these actions have not yet been precisely delineated.

Several lines of evidence indicate that estradiol is the critical stimulus for the preovulatory surge of gonadotropins. In intact cycling female rats, the serum levels of LH are low from early on the morning of estrus, shortly after ovulation, through metestrus, diestrus, and midday on proestrus (80). Though most studies have reported basal unchanging levels of LH over this time (80-84), one study reported a significant diurnal variation throughout the cycle (85). This circadian pattern from estrus through diestrus consisted of a small elevation of LH each day at the midpoint of the light period, and the lowest levels by midnight of each day. On the late afternoon of proestrus (actual time of initiation depending on the light cycle) the circulating levels of LH begin to increase rapidly and ultimately reach peak levels by 1700-1900 hr on that same evening. This rapid surge of LH induces follicular rupture and ovulation. Thereafter, the blood levels
of LH begin to decline and reach basal levels by early on the morning of estrus.

There is a voluminous literature indicating that ovarian estradiol, secreted on diestrus, is a critical positive-feedback stimulus for the ovulation-inducing surge of LH. Several experimental approaches have attested to this fact. Specifically, under either a 14:10 or 12:12 light-dark cycle, with noon as the midpoint of the light interval (86), ovariectomized rats will respond to a dose of exogenous estradiol by presenting a proestrous-like LH surge the following day with the same timing and duration as the intact female rat on proestrus (86-88). However, the magnitude of this induced surge differs from that of the spontaneous surge on proestrus. That is, base-line values are not as low and peak values are not as high as those obtained during the endogenous surge on proestrus. In addition, rather than stimulating a surge of LH on a single day, estradiol administration to ovariectomized animals results in an LH surge of equivalent timing on each of at least the following 3 days. Thus, a daily neural event must be coupled with the presence of estrogen to eventuate in a daily LH surge in the ovariectomized rat. This confirms Everett and Sawyer's original proposal (89) of a 24 hr. periodicity in the LH release apparatus. Other approaches have demonstrated that ovarian estradiol secreted on diestrus is required to activate the LH surge response on proestrus. Specifically,
administration of estrogen inhibitors (90) or estradiol antiserum (90-92) or ovariectomy (93) on diestrus blocks the proestrous surge of LH. On the other hand, administration of these agents or removal of the ovaries early on proestrus is ineffective in preventing this response later that same day. These data, taken together, suggest that the rising titer of estradiol secreted on diestrus represents a major stimulus for the increased secretion of LH on proestrus. The tonic levels of LH secreted on diestrus contribute to the stimulation of estradiol secretion by the ovarian follicle which, in turn, stimulates LH secretion (94).

The long-term ovariectomized rat responds to a brief exposure (only 4 to 5 days) to estradiol with daily LH surges, whereas the acutely ovariectomized rat requires chronic exposure to estradiol to secrete daily LH surges (88). Also, it has been shown that expression of the daily LH surge mechanism is limited to proestrus by the heightened secretion of progesterone on proestrus (87). Thus, there is every indication from the present data as well as previous reports that progesterone contributes in a positive way to the secretion of the surge of LH on proestrus. In fact, progesterone administered early on the morning of proestrus will advance LH release by several hours (95). Similarly, progesterone administrated to ovariectomized, estradiol-benzoate-pretreated rats will induce an LH surge during the afternoon of that same day (106). In addition, progesterone
will enhance the magnitude of the estrogen-induced LH surge (96). The effect of estrogen to increase LHRH release may not be exerted directly on LHRH-containing neurons since only 1 in 435 LHRH neurons reportedly have estrogen receptors (97). The effect of progesterone appears not to be mediated through the ovary since studies in the rat have shown progesterone stimulates gonadotropin secretion in bilaterally ovariectomized rats primed with estradiol (98-100). The stimulatory effect of progesterone does, however, require the presence of estradiol, as evidenced by the lack of effect of progesterone on gonadotropin secretion in ovariectomized rats not given estrogen replacement (101-102). The critical dependence of progesterone on estradiol for the induction of progesterone receptors is important (103-104); however, estradiol may also exert other important priming effects, such as setting the neural trigger for progesterone activation. On the other hand, the data also suggest that the proestrous surge of LH may be terminated, in part, by the secretion of progesterone.

Progesterone's effect upon gonadotropin secretion is dependent not only upon time of administration but also upon the dose of administration. Freeman et al (105) found that the injection of progesterone to estradiol benzoate-treated, long-term ovariectomized rats resulted in an enhancement of the LH surge on the first day, and elimination of the surge on following days (105). The role of endogenous progesterone to
limit the expression of the gonadotropin surge to a single day was further confirmed by the study of Smith et al (106), who demonstrated that blockade of progesterone's effects on the afternoon of proestrus with either progesterone antibody administration or the antiprogestin, RU486, caused a return of pulsatile LH secretion on estrus.

In addition to direct effects at the anterior pituitary, progesterone may regulate hypothalamic release and degradation of LHRH as a mechanism in its positive feedback effect on gonadotropin secretion; however, it appears unlikely that this action of progesterone is exerted directly on LHRH-containing neurons since progesterone receptors are not reported to be present on hypothalamic LHRH neurons (107). It appears more likely that progesterone's effect on LHRH is exerted through neurotransmitter/neuropeptide-containing neurons in the hypothalamus which interact with LHRH neurons.

Numerous studies have demonstrated that progesterone enhances the pituitary response to LHRH (108-113). Acute progesterone treatment enhances, while chronic treatment suppresses, the pituitary response to LHRH. The cellular mechanism involved in the acute progesterone enhancement of LHRH response is unclear. One possible mechanism could be the modulation of LHRH receptors. However, a recent study by Attardi and Happe (110) failed to show any effect of progesterone on LHRH receptor number in the pituitary, suggesting the site of progesterone action could be
independent of effects on the LHRH receptor. Krey and Kamel (111) recently reported that progesterone enhances the pituitary LH response to secretagogues which act separate from the LHRH receptor.

Progesterone can modify the action of estradiol by decreasing nuclear estradiol receptor retention (114-115). The reduction of occupied nuclear estradiol receptors is equivalent to causing a fall in serum estradiol levels, because estradiol's action is exerted through its occupied nuclear receptors.

These reports demonstrate the crucial role played by progesterone in the regulation of gonadotropin secretion. The regulatory role of progesterone on gonadotropin secretion depends on the time and dose of its administration in relationship to the adequacy of estrogen priming.

All this information suggests to us that the effects of estradiol and progesterone are partly exerted through other neurotransmitter(s)/neuropeptide(s), and the central role of those factors. Verification of this hypothesis requires more research. The review summarized here suggests that oxytocin neurosecretion, itself, as well as its central influence on LH secretion, is subject to modulation by gonadal steroids and may represent a significant portion of this neuroendocrine control.
CHAPTER II

OBJECTIVES:

The major objectives of this research are to 1) demonstrate the important role of central oxytocin in the regulation of LH secretion in the pharmacological model of the proestrous LH surge, 2) to determine and characterize if that stimulatory role is influenced by gonadal hormone levels in vivo and 3) to study the interaction of different gonadal hormones on the oxytocin-induced stimulation of LH secretion.

Previous experiments in our laboratory suggest that central oxytocin neurons may exert a physiologically important stimulatory influence on the release of LHRH and LH occurring just prior to ovulation on the afternoon of proestrus in the cycling female rat (when estradiol levels are physiologically high)(32). Another recent report showed that the in vitro LH response to oxytocin at the anterior pituitary level is steroid-dependent(106). This research tested the hypothesis that the central stimulatory role of oxytocin on LH secretion is subject to modulation by the levels, concentration ratios and time of exposure of gonadal hormones.

To investigate these possibilities, we utilized ovariectomized, estrogen, or both estrogen and progesterone-primed female rats as an in vivo pharmacological model for the proestrous female rat. The results were planned to further
confirm the central effect of oxytocin on LH release and the
dependence of its action on plasma estradiol and progesterone
levels in a pharmacological model for the preovulatory surge.

The knowledge gained from this research might advance our
basic understanding of the nature, site, mechanism and
physiological significance of oxytocin in regulating the
preovulatory surge of LH and the influence of gonadal hormones
on that neuroendocrine regulation. It might also offer an
ideal experimental model for the study of the interaction
between oxytocin and other physiologically relevant LHRH
secretagogues.
CHAPTER III

MATERIALS AND METHODS

1) Animals:

Young adult female, ovariectomized, Sprague Dawley rats (200-300 g, BW) were housed in a temperature (22±1°C) and light (light on 700-1900 h) controlled room. Ovariectomies were done at least 4 weeks prior to further experiments. The rats were supplied food and water ad libitum.

2) Surgery:

a. Third ventricular cannulae placement (icv):

Stainless-steel 23 gauge cannulae were stereotaxically placed into the third cerebral ventricle of the ovariectomized rats using the atlas of König and Klippel (107) as a guide under tribromoethanol (1 ml/100 g B.W. of a 2.5% solution in saline) anesthesia. Coordinates utilized for placement of the icv cannulae were lateral 0.0 mm from midline, 2.5 mm posterior to bregma, and approximately -8.5 mm ventral to the surface of the brain. Each cannula was secured with dental cement and fine microscrews, and was provided with a screw-in mandril to prevent its obstruction. Location of the cannulae were confirmed by the flow of cerebrospinal fluid through the cannulae after removal of the mandril and by a positive

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drinking response to the icv injection of 10 ng angiotensin II (a positive drinking response started not more than one minute after icv injection of angiotensin II with a total consumption of water of greater than 3 ml within 30 minutes).

At the time of the experiment, the mandril of the cannulae were removed and injections were made using a 10 microliter Hamilton (Hamilton Industries, Two Rivers, WI) microsyringe connected by polyethylene tubing (PE 10) to a 30 gauge stainless steel infusion needle which fits within and extends to the lower lip of the chronic third ventricular cannula. The tubing and needle were filled with freshly made solution to be injected just prior to the injection.

b. Jugular cannulae:

Silastic cannulae were placed in the right atrium via the external jugular vein under tribromoethanol (TBE) anesthesia (1 ml of 2.5% solution/100 gm BW), secured to the pectoral muscle, threaded subcutaneously and externalized at the base of the neck on the rat's back (108). The cannulae were connected to extensions running outside the cages 2-3 h before further experimentation.

3) Estradiol, progesterone and LH radioimmunoassays:

Plasma samples were assayed for estradiol and progesterone levels using a double antibody radioimmunoassay (RIA, Diagnostic Assay Services, Gaithersburg, ML). The
sensitivities of the RIAs for estradiol and progesterone were 1.0 ng/liter and 1.0 ng/ml, respectively, with intraassay coefficient of variation of 8 and 11%, respectively.

Serum LH levels were measured by double antibody radioimmunoassay. The sensitivity of this assay was 0.5 ng/tube, with an intraassay coefficient of variation of 7-8%.

All samples from the same experiment were assayed in the same RIA.

4) Statistical analysis:

Data were analyzed by one-way analysis of variance with the Student-Newman Keuls post-hoc test or the Student's t-test. The 0.05 level of probability was used as the minimum level of significance for differences between the means for all experiments.

5) Drugs and chemicals:

All the drugs to be employed in the proposed experiments were available from commercial sources. Purified preparations of angiotensin II and oxytocin were available from Peninsula Laboratories (San Carlos, CA). HPLC-purified 125-I rat LH was obtained from Hazleton Laboratories (Vienna, Virginia). Other chemicals and reagents including progesterone were purchased from Sigma Chemical Company (ST. Louis, MO). The pellets for 17-beta estradiol, progesterone and their placebo carrier-binder were purchased from Innovative Research of America (Toledo, OH). Chemicals for intravenous injections were
diluted in saline.

6) Experimental procedure

**Effect of gonadal hormones on the stimulatory effect of oxytocin on LH secretion.**

1) Long term exposure to high doses of estradiol and/or progesterone:

Ovariectomized female rats were separated into 4 groups (7-8 rats/group). Pellets containing placebo carrier-binder (control group), 17-beta estradiol (1 mg, E2 group), progesterone (35 mg, P4 group) or both estradiol and progesterone (1 mg 17-beta estradiol and 35 mg progesterone, E2/P4 group) were placed subcutaneously 4 days prior to further experimentation.

2) Short term exposure of varying progesterone doses in ovariectomized rats with or without estradiol-priming:

Ovariectomized female rats were separated into 6 groups (7-8 rats/group). Rats received subcutaneous 17-beta estradiol pellets (1 mg) 2 days prior to further experimentation. On the day of the experiment, various doses of progesterone (15 mg, 5 mg, 2.5 mg, 1.5 mg, 0.5 mg or 0.0 mg/rat in vegetable oil vehicle) were administered, subcutaneously [P4(2.5), P4(1.5), P4(0.5), control, E-P4(15), E-P4(5) groups] in the
ovariectomized rats primed with estradiol. Four other groups of ovariectomized rats (7-8 rats/group) received various doses of progesterone [25 mg, 15 mg, 5 mg/rat or 0 mg/rat in vegetable oil vehicle; P4(25), P4(15), P4(5) and control groups, respectively].

Rats were housed and maintained according to guidelines published in "NIH Principles for Use of Animals, Guide for Care and Use of Laboratory Animals". All surgical procedures were classified as USDA type D procedures. The surgery for jugular cannulae placement takes approximately 5-10 min and the stereotaxic ICV cannulae placement surgery takes 20-30 min. Sterilized materials and prophylactic antibiotics (intramuscular injection and local ointment) were administered to prevent infections for all recoverable surgeries. Any rats displaying signs of illness were euthanized so they did not suffer, consistent with the recommendations of the panel on Euthanasia of the American Veterinary Medical Association.

Approximately 145 female rats were used for these experiments. All precautions were taken to avoid any stress in handling the rats, which could affect the release of LH.

Plasma samples were obtained via jugular cannulae (placed 2 days earlier) from the unanesthetized, freely moving rats at 10 min before (PRE) and at 5, 15, 30, and 60 min following oxytocin (10 ng) icv injection. Blood obtained from samples were transferred to heparinized tubes and separated immediately at 4°C. Plasma samples were stored at -80°C until
analyzed by RIA. Icv experiments started at 1300 hr (4 hours after progesterone injection).
CHAPTER IV

RESULTS:

1. Long term exposure of high doses of estradiol and/or progesterone on oxytocin induced LH release

The results indicate that basal LH was higher in control, ovariectomized rats treated with subcutaneous pellets containing placebo carrier-binder than in groups treated with subcutaneous pellets containing estradiol, progesterone or both hormones (Fig 1). Furthermore, ovariectomized animals (control group) responded to icv oxytocin with a modest decrease in plasma LH at 30 and 60 min after icv injection (Fig 2). Estradiol or progesterone treatments, alone, decreased basal LH levels compared to control ovariectomized animals (Fig 2; Pre time point). ICV oxytocin caused a modest increase in plasma LH at 5, 15, and 30 min in estradiol-treated animals compared with the proestrous LH surge in the intact, cycling female rats (Fig 2). ICV oxytocin administration to progesterone-treated animals caused little change in plasma LH. In animals treated with both estradiol and progesterone, basal LH levels were significantly lower than in any other treatment groups (Fig 1 and Fig 2; Pre time point). The icv injection of oxytocin to chronic estradiol and progesterone treated animals resulted in no change in plasma LH (Fig 2; E2/P4 group).
Figure 1. Effect of chronic replacement therapy with subcutaneous pellets containing placebo carrier-binder (CNTRL group), 17β-Estradiol (1 mg, E2 group), progesterone (35 mg, P4 group), or both E2 and P4 (E2/P4 group) 2-4 days prior to further experimentation on basal plasma concentrations of luteinizing hormone. Column height represents mean and vertical bars represent SEM as determined from N = 7-8 rats. A, Significantly different from all other mean values (P<0.01). *, significantly different from CNTRL mean value (P<0.01).
EFFECT OF OVX & REPLACEMENT THERAPY ON BASAL LH

Figure 1

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Figure 2. Effect of a central injection of oxytocin (10 ug/5ul) on plasma LH concentrations in ovariectomized rats which have received subcutaneous pellets containing placebo carrier-binder (CNTRL group), 17β-Estradiol (1 mg, E2 group), progesterone (35 mg, P4 group), or both E2 and P4 (E2/P4 group) 2-4 days prior to further experimentation. At 1300 hr of the experimental day animals received an icv injection of oxytocin and plasma samples were collected 10 min prior to icv injection (Pre), or 5, 15, 30 or 60 min after the icv injection for plasma LH determination. Values represent mean plasma LH concentration as determined from N = 7-8 rats.
ICV OXYTOCIN ON LH IN OVX & HORMONE REPLACED RATS

Figure 2

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2. Short term exposure to various progesterone doses in ovariectomized rats with or without estradiol priming on oxytocin-induced LH release

The effect of short term exposure to various progesterone doses in estradiol-primed, ovariectomized rats on the oxytocin-induced stimulation of LH release were investigated. Once again, the results indicate that basal LH is highest in ovariectomized rats, next highest in animals treated with estradiol alone (Fig 3; Pre time values), and lowest (in a dose-related manner) in those treated with high doses of progesterone, alone [25 mg, P4(25) group; 15 mg, P4(15) group or 5 mg, P4(5) group; Pre time values in Fig 4]. Oxytocin injection did not alter plasma LH in animals treated with any of the progesterone doses, alone (Fig 4). Following estradiol treatment, alone, a very small decrease was seen at 5 min and a small increase in plasma LH was seen at 15 and 30 min after oxytocin injection (Fig 4, E group). In estradiol-primed, ovariectomized rats treated acutely with 15 mg [E-P4(15) group] or 5 mg [E-P4(5) group] progesterone at 900 hr, icv administration of oxytocin 4 hr later caused an increase in plasma LH (Fig 5). This increase was significantly higher than that seen in animals treated with estradiol, alone (E group, Fig 5), in animals receiving the lower dose of progesterone [E-P4(5) group, Fig 5] at 5 min-30 min. In animals receiving
Figure 3. Effect of a central injection of oxytocin (10 μg/5 μl) on plasma LH concentrations in ovariectomized rats which have received subcutaneous pellets containing placebo carrier-binder (CNTL group) or 17β-Estradiol (1 mg, E group) 2 days prior to further experimentation. At 1300 hr of the experimental day, animals received an icv injection of oxytocin and plasma samples were collected 10 min prior to icv injection (Pre), or 5, 15, 30 or 60 min after the icv injection for plasma LH determination. Values represent mean plasma LH concentration as determined from N = 7-8 rats.
E2 ON OXY LH RELEASE IN OVX RATS

Figure 3

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Figure 4. Effect of a central injection of oxytocin (10 ug/5 ul) on plasma LH concentrations in ovariectomized rats which have received subcutaneous pellets containing 17 β-Estradiol (1 mg, E group) 2 days prior to further experimentation, or an acute subcutaneous injection of progesterone at 25 mg/0.6 ml oil [P4(25) group], 15 mg/0.3 ml oil [P4(15) group], or 5 mg/0.3 ml oil [P4(5) group] at 900 hr of the experimental day. At 1300 hr an icv injection of oxytocin was administered and plasma samples were collected 10 min prior to icv injection (Pre), or 5, 15, 30 or 60 min after the icv injection for plasma LH determination. Values represent mean plasma LH concentration as determined from N = 7-8 rats.
E2 OR P4 ON OXY LH RELEASE IN OVX RATS

Figure 4

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Figure 5. Comparison of the effect of a central injection of oxytocin (10 ug/5ul) on plasma LH concentrations in ovariectomized rats which have received subcutaneous pellets containing 17 β-Estradiol (1 mg) 2 days prior to further experimentation either without (E group) or with an acute subcutaneous injection of progesterone at 15 mg/0.3 ml oil [E-P4(15) group] or 5 mg/0.3 ml oil [E-P4(5) group] at 900 hr of the experimental day. At 1300 hr an icv injection of oxytocin was administered and plasma samples were collected 10 min prior to icv injection (Pre), or 5, 15, 30 or 60 min after the icv injection for plasma LH determination. Values represent mean plasma LH concentration as determined from N = 7-8 rats.
E2/P4 ON OXY LH RELEASE IN OVX RATS

![Graph showing plasma LH levels (ng/ml) over time (min) post ICV injection.](image)

- □ E
- + E-P4 (15)
- ■ E-P4 (5)

**Figure 5**

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the higher dose of progesterone [E-P4(15) group, Fig 5] the LH stimulation resulting from icv oxytocin administration was smaller and of shorter duration than even in the estradiol, alone, treatment group; suggesting that higher doses of progesterone may inhibit the oxytocin-induced release of LH. To test this directly, ovariectomized, estradiol primed rats were given acute injections of 2.5 mg [P4(2.5) group], 1.5 mg [P4(1.5) group] or 0.5 mg [P4(0.5) group] progesterone or 0.3 ml oil vehicle at 9:00 hr and then given an icv injection of oxytocin at 1300 hr in order to evaluate the effect of the dose of progesterone on the oxytocin-induced increase in plasma LH in estradiol-primed rats. Oxytocin elicited a maximum response in plasma LH in animals given an acute injection of 2.5 mg progesterone, with a surge which increased 5 min after icv injection, peaked at greater than 60 ng/ml at 30 min and was still significantly elevated over control animals at 60 min [P4(2.5) group, Fig 6]. Oxytocin induced a much smaller, but significant, increase in plasma LH in ovariectomized, estradiol-primed animals treated acutely with 1.5 mg progesterone [P4(1.5) group, Fig 6] with increased plasma LH levels at 5-30 min after icv injection, peaking with a value of about 25 ng/ml at 5 min after icv injection. The effect of icv oxytocin in animals receiving the lowest dose of progesterone [0.5 mg, P4(0.5) group, Fig 6] showed a much smaller stimulation with small increases in plasma LH being observed at 5 min and at 60 min after icv injection of
oxytocin. Interestingly, in the animals receiving estradiol priming, alone, no significant effect of icv oxytocin on plasma LH was observed.
Figure 6: Comparison of the effect of a central injection of oxytocin (10 ug/5 ul) on plasma concentrations in ovariectomized rats which have received subcutaneous pellets containing 17β-estradiol (1 mg) 2 days prior to further experimentation and an acute subcutaneous injection of progesterone at 2.5 mg/0.3 ml oil [P4(2.5) group]; 1.5 mg/0.3 ml oil [P4(1.5) group] or 0.5 mg/0.3 ml oil [P4(0.5) group]; or received 0.3 ml oil vehicle (CNTRL group) at 900 hr of the experimental day. At 1300 hr an icv injection of oxytocin was administered and plasma samples were collected 10 min prior to icv injection (Pre), or 5, 15, 30 or 60 min after the icv injection for plasma LH determination. Values represent mean plasma LH concentration ± sem as determined from N=7-8 rats.
P4 ON OXY STIMULATION OF LH IN E2-PRIMED RATS

Figure 6

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CHAPTER V

DISCUSSION:

The data demonstrate that for the expression of the stimulatory role of oxytocin on plasma LH to be seen, serum levels, concentration ratios and exposure time of gonadal hormones are critical. This stimulatory action of oxytocin disappears when estradiol is removed. Furthermore, although a small dose of progesterone synergizes with estradiol to cause a greater release of LH by oxytocin, the action is reduced as the dose of acutely administered progesterone increases in the ovariectomized, estrogen-primed rats (Fig 6).

The data from the present study agree with previous reports (30, 32) that oxytocin exerts a physiologically relevant, stimulatory influence on the preovulatory release of LH on the afternoon of proestrus in cycling female rats (when the concentration of plasma estradiol level is high and progesterone is low, but increasing). Pharmacological manipulation of endogenous oxytocin with selective oxytocin antagonists and passive immunoneutralization with specific oxytocin antisera result in significant deficiencies in stimulated LH release in animal models with similar estradiol and progesterone concentrations (30, 32). Recently, Samson et
al (119) developed a novel method to selectively lesion central oxytocin neurons with a ricin-cytotoxin conjugate to reveal a physiologically relevant role for oxytocin within the hypothalamus to control LHRH release on the afternoon of proestrus in the steroid-primed LH surge model (119). Our results provide a possible explanation for data from several laboratories describing both inhibitory and stimulatory effects of oxytocin on LH. Changes in the concentration of plasma gonadal hormones are critical to the action and effect of central oxytocin on LH secretion. Two studies demonstrating inhibitory effects of oxytocin on LH release were carried out in animal models associated with low levels of ovarian hormones (34, 43). On the other hand, another group has provided evidence for a direct, stimulatory action of oxytocin on LH release from rat anterior pituitary cells in static culture (31, 35), which is steroid-dependent (106). We have shown pretreatment with either an oxytocin antagonist or an LHRH antagonist blocked the increase in plasma LH resulting from a central injection of oxytocin on proestrus (when plasma concentrations of gonadal steroids are high) (120). These results demonstrate that the oxytocin-induced increase in plasma LH is an oxytocin receptor-mediated event and is apparently mediated by influencing the release of LHRH. Additionally, using oxytocin-ricin A chain conjugates to selectively target and destroy cells in vivo and in vitro that possess oxytocin receptors, Samson and co-workers failed to
demonstrate that oxytocin acts directly on the anterior pituitary, and provided evidence that oxytocin acts through LHRH neurons centrally to affect LH release.

Evidence supporting the influence of plasma estradiol and progesterone on distribution, content, synthesis, and release of oxytocin in the CNS has been provided. Treatment with estradiol has been shown not only to enhance the oxytocin-like immunoreactivity contained in discrete regions of the rat brain (20), but also allows additional immunoreactive oxytocin perikarya and processes to be observed in areas which demonstrate no oxytocin-like immunoreactivity prior to estradiol treatment (50, 51, 58). The concentrations of oxytocin found in CNS nuclei fluctuate within the estrous cycle (57). Oxytocin levels in the portal blood increase on the afternoon of proestrus, correlating with the onset of the preovulatory LH surge (47). Studies examining the possible influence of estrogen on oxytocin messenger RNA levels using Northern blot and dot blot analysis provide evidence for a role at the molecular level (64-66). The possibility of a direct effect of estrogen on transcription is indicated by the presence of partial estrogen-responsive-elements located upstream to the oxytocin transcription start site (67-69). However, autoradiographic studies of estrogen binding have not revealed significant numbers of supraoptic neurons with nuclear estrogen receptors (70). This is unlikely to represent the only route of gonadal hormone action on the oxytocin
system. Other possibilities include the modification of hormone responsiveness by increasing the sensitivity of putative second messenger systems or membrane potential to oxytocin. For LHRH gene expression, a long estrogen treatment can induce increased mRNA levels and the stimulatory effects of estrogen can be consequent to the release of the neuroendocrine peptide (71-73). All these results suggest that the increasing activity of oxytocin neurons during proestrus (when plasma estradiol levels are high) is probably physiologically responsible for inducing the LH surge. The results from the present study support the fact that the stimulatory effect of centrally administered oxytocin can be seen in a pharmacological model of the proestrous female rat, and that the presence of physiologically high levels of estradiol are critical for the stimulatory effect of oxytocin to be seen.

Our results demonstrate that progesterone also plays an important role in this mechanism. In our study, both estradiol and progesterone chronically exerted additive negative feedback effects on basal LH levels. ICV oxytocin inhibited LH levels in ovariectomized rats, and modestly stimulate LH levels (in most cases) in estradiol treated rats compared with the proestrous LH surge in cycling female rats, but not in rats treated with the highest dose of progesterone. This suppressive action of progesterone is reduced and even begins to synergize with estradiol when the acute dose of
administered progesterone is reduced to 0.5-5 mg/rat (Fig 5, Fig 6). This result is consistent with the fact that progesterone administered early on the morning of proestrus advanced LH release by several hours and enhanced the magnitude of the estrogen-induced LH surge (96). The effect of progesterone appears not to be mediated through the ovary since studies in the rat have shown progesterone to stimulate LH release in ovariectomized rats primed with estradiol (98-100). However, our studies show that the stimulatory effect of progesterone required the presence of estradiol. These facts are consistent with the negative feedback of the gonadal hormones on the basal release of LH. The results also relate to the general mechanisms by which gonadal hormones exert their effects on different locations in the CNS to control LH secretion; including effects on neurotransmitter synthesis and release dynamics, to receptor number and affinity. The requirement of estradiol priming for progesterone's stimulatory effect to be exhibited is obvious and may be due to a critical dependence of progesterone on estradiol for the induction of its receptors (103-104). On the other hand, progesterone's action might be due to neurotransmitter release mechanisms while that of estradiol might be on synthesis of neurotransmitters. This possibility is supported by the fact that progesterone receptors are not found directly on hypothalamic LHRH neurons (107). Therefore, central oxytocin may represent one of the mediating factors in the action of
gonadal steroids on LHRH neurons. The effects of estrogen on hypothalamic LHRH release might be mediated, in part, through effects on oxytocin neurons surrounding LHRH neurons in the hypothalamus and median eminence. Indeed, estradiol and progesterone receptors have been identified on central oxytocin neurons (121). Estrogen also exerts important effects which sensitize the anterior pituitary to LHRH stimulation. Drouva et al (122) provided evidence that estradiol increases anterior pituitary protein kinase C activity both in vivo as well as in vitro. Direct effects of estradiol on the pituitary have been demonstrated in ovariectomized female rats in which the vascular connections between the hypothalamus and the pituitary were interrupted surgically, and regrowth was prevented by insertion of a piece of aluminum foil; as well as in experiments using dispersed pituitary cells in culture (123-124). On the other hand, at the very least, gonadal hormones modulate the oxytocin receptors and interactions involved in the stimulation of LH release in terms of its qualitative nature, quantity, affinity and distribution. The central influence of gonadal steroids was also proven in experiments showing pretreatment with an oxytocin antagonist, central administration of an oxytocin antisera or an LHRH antagonist blocked the increase in plasma LH resulting from a central injection of oxytocin. Several other neuropeptides and monoaminergic neurotransmitters have been implicated in the neuroendocrine regulation of LH secretion. They primarily
appear to work by altering the net release of LHRH from the median eminence into the hypophysseal portal blood. Surprisingly, most of the LHRH secretagogues or inhibitors more or less appear to be dependent on the levels of plasma gonadal hormones, especially physiologically high levels of plasma estradiol. These include NE, epinephrine, angiotensin II, glutamate, GABA, CRF, NPY, and the opiates (5-16). However, direct evidence for these interactions and their significance in the neuroendocrine regulation of LHRH and LH secretion, as well as the modulatory influence of plasma estradiol/progesterone levels on those possible interactions is controversial and largely unknown. Further work is needed to clarify these issues.

Besides the modulation by estradiol of the oxytocin stimulation of LH release, the acute administration of progesterone also acted synergistically to enhance LH release in estradiol-primed, ovariectomized rats in dose-related manner (Fig 5; Fig 6). How progesterone acts centrally to exert this action on LH release remains unresolved. The regulatory role of progesterone on the neuroendocrine regulation of LH secretion depends on the time and dose of its administration in relationship to the plasma level of estradiol. Long term administration of high doses of progesterone failed to show the synergistic effect when serum estradiol levels were similar to those attained in the most recent experiments demonstrating synergism (Fig 2, Fig 5, Fig
Such synergism was evident when progesterone levels were probably closer to the expected physiological progesterone level seen during proestrus. When progesterone was administered acutely (s.c.) to estradiol-primed rats, the resulting stimulation of LH release in response to centrally administered oxytocin demonstrated a bell-shaped dose response (Fig 5, Fig 6). The results indicate that the lower doses of progesterone synergistically enhance the ability of oxytocin to stimulate LH release in animals having physiologically high levels of serum estradiol. The enhancement was maximum after administration of 2.5 mg progesterone, but was also seen to a lesser degree in animals treated with 1.5 and 0.5 mg progesterone. As the dose of progesterone increased further, the synergism decreased. At the optimum progesterone dose, the level of LH stimulation was similar to that observed in intact proestrous rats during the spontaneous LH surge, and is higher than that observed in intact rats early on proestrus 30 min after oxytocin injection. These differences are most likely due to differences in the progesterone levels and duration in the current experiments compared to intact cycling rats. In particular, lower doses of progesterone may be important to stimulate the start of the oxytocin-induced LH release in estradiol-primed ovariectomized rats, whereas larger doses may help bring the LH surge back down to normal. Interestingly, the lowest dose of progesterone in our study (0.5 mg) stimulated oxytocin-induced LH release in a modest
biphasic manner (Fig 6). These data suggest low doses of progesterone might start the oxytocin-induced LH release in estradiol-primed ovariectomized rats, and that this stimulation of LH secretion could be brought back to normal as progesterone levels continue to rise.

The cellular mechanisms involved in the acute progesterone enhancement of oxytocin's stimulatory effect on LH release in estradiol primed, ovariectomized rats is unclear. No effect of progesterone on LHRH receptor number in the pituitary has been reported, suggesting the site of progesterone action must be at a level higher than the LHRH receptor (110). Other reports show that progesterone enhances the pituitary LH response to other secretagogues which act prior to the LHRH receptor (111). The results from our present study suggest that the action of progesterone may be required for a functional interaction between oxytocin, other neuropeptides and their receptors. Alternatively, progesterone may activate additional mechanisms which estradiol does not affect, which are then required for the facilitation of LH secretion by oxytocin.
CHAPTER VI
SUMMARY

In summary, both estradiol and progesterone exert negative feedback effects on basal LH secretion, and their combined effect was additive. Centrally administered oxytocin inhibited the high plasma LH levels seen in ovariectomized rats. In contrast, in most cases, oxytocin caused LH levels to increase in estradiol-treated rats following its icv injection, although the magnitude of that increase is very small compared to the proestrous surge or the estradiol-primed rat given 2.5 mg progesterone. In animals treated with progesterone, alone, there was little effect of centrally administered oxytocin on plasma LH. Oxytocin did not alter plasma LH concentrations in animals treated with long term progesterone and/or estradiol. The results support the fact that the stimulatory effect of centrally administered oxytocin can be seen in a pharmacological model of the proestrous female rat, and that the presence of physiologically high levels of estradiol are critical for the stimulatory effect of oxytocin to be seen. In the absence of estradiol or progesterone, oxytocin exerts an inhibitory influence on plasma LH. The level of stimulation by oxytocin on plasma LH was critically dependent on the optimal dose of progesterone. Progesterone's influence on the oxytocin-induced change in LH release was both dose and time dependent. Low doses of
progesterone synergistically enhance, while high doses inhibit, the ability of oxytocin administered centrally to stimulate LH secretion. The fact that the optimal dose of progesterone can influence oxytocin-induced LH release in a manner similar to that seen in cycling female rats on the afternoon of proestrus, and even stimulate LH release at 30 min after icv oxytocin suggests that the duration and/or dose of estradiol and progesterone exposure are critical for the expression of the stimulatory oxytocin effect. On the other hand, the progesterone levels seen in intact female rats on proestrus are dynamic, and are probably lower than that seen in most of the acutely progesterone-treated, ovariectomized groups. The dynamic changes occurring in plasma estradiol and progesterone in relation to each other throughout proestrus are probably critically important to the expression of the oxytocin stimulation of LH secretion. Alternatively, it is possible that if other critically important key elements/inputs were also stimulated in the present experiments, or if the oxytocin challenge could be given at the time of the endogenous surge, the resulting surge of LH might resemble even more closely that seen on the afternoon of proestrus prior to ovulation in intact, cycling rats. When ovariectomized rats are estradiol-primed, and the progesterone level is carefully controlled, oxytocin given centrally induces an increase in plasma LH which resembles the endogenous LH surge seen on the afternoon of proestrus.
in the cycling, intact female rat. The pharmacological model of the proestrous surge can be used to evaluate the influence of oxytocin on LH secretion, as well as the important interaction, occurring between oxytocin and other central neurotransmitters to provide that regulation.
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