Central neurochemical mechanisms involved in the generation of the preovulatory GnRH surge in normally cycling female rats

Daniel John Selvage
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Central Neurochemical Mechanisms Involved in the Generation of the Preovulatory GnRH Surge in Normally Cycling Female Rats

By

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BSc The University of Oregon, 1986
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A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

The University of Montana
Department of Pharmaceutical Sciences

Approved by

[Signatures]
Chairman of Supervisory Committee
Dean of Graduate School

3-30-01
Date
Central Neurochemical Mechanisms Involved in the Generation of the Preovulatory GnRH Surge in Normally Cycling Proestrous Female Rats

Committee Chairman: Craig A. Johnston

A number of central neurotransmitters have been implicated in the cyclic control of gonadotropin-releasing hormone (GnRH) secretion from the median eminence (ME) of the basal hypothalamus (BH) in normally cycling female rats. The large surge of this hormone released on the afternoon of proestrus stimulates secretion of luteinizing hormone (LH) from the pituitary, thereby driving ovulation. Among the central neurotransmitters thought to play a stimulatory role in the preovulatory, proestrous release of GnRH are norepinephrine (NE), oxytocin (OT), and nitric oxide (NO). The purpose of this research was to investigate the role of these neurotransmitters in stimulating GnRH release from BH explants in vitro, in order to better understand how they may be working in vivo.

It was found that OT stimulated GnRH release from BH explants, but not ME fragments harvested on the afternoon of proestrus from normally cycling female rats. This suggests that the connectivity of the intact BH is required for OT to stimulate proestrous GnRH release. The ability of OT to stimulate proestrous GnRH release from BH explants was blocked by OT receptor antagonism, and by NO synthase (NOS) inhibition. NE was also found to stimulate GnRH release from proestrous BH explants. Alpha-1, but not alpha-2 adrenergic receptor antagonism blocked the ability of NE to stimulate proestrous GnRH release from BH explants. OT receptor antagonism attenuated, but did not entirely block the effect of NE on GnRH release. When used in combination, NE/OT synergistically stimulated GnRH release from proestrous BH explants. This is the first report of this effect. NE/OT- induced stimulation of proestrous GnRH release from BH explants was blocked by OT receptor antagonism, and by inhibition of NOS. The results suggest that combined NE/OT stimulate GnRH release from proestrous BH explants in a synergistic manner, using OT-receptor and NO mediated mechanisms.
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<td>AVP</td>
<td>arginine vasopressin</td>
</tr>
<tr>
<td>CNOS</td>
<td>constitutive nitric oxide synthase</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CRF</td>
<td>corticotropin releasing factor</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
</tr>
<tr>
<td>GABA</td>
<td>y-aminobutyric acid</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>ICC</td>
<td>immunocytochemistry</td>
</tr>
<tr>
<td>ICV</td>
<td>intraventricular cannula</td>
</tr>
<tr>
<td>IEG</td>
<td>immediate early gene</td>
</tr>
<tr>
<td>IP3</td>
<td>1,4,5-tris-phosphate</td>
</tr>
<tr>
<td>KRBG</td>
<td>kreb's ringer buffer</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>LHRH</td>
<td>luteinizing hormone-releasing hormone</td>
</tr>
<tr>
<td>LNAME</td>
<td>N-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>MBH</td>
<td>medial basal hypothalamus</td>
</tr>
<tr>
<td>ME</td>
<td>median eminence</td>
</tr>
<tr>
<td>MPO</td>
<td>medial preoptic area</td>
</tr>
<tr>
<td>NBQX</td>
<td>kainate receptor antagonist (trade name)</td>
</tr>
<tr>
<td>NE</td>
<td>norepinephrine</td>
</tr>
<tr>
<td>nNOS</td>
<td>neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
</tr>
<tr>
<td>ODQ</td>
<td>1H-(1,2,4)oxadiazolo(4,3a)quinoxalin-1-one</td>
</tr>
<tr>
<td>OT</td>
<td>oxytocin</td>
</tr>
<tr>
<td>OTA</td>
<td>oxytocin receptor antagonist [d(CH2)5, Tyr (Me)2, ORN8] vasotocin</td>
</tr>
<tr>
<td>OVLT</td>
<td>organum vasculosom of the lamina terminalis</td>
</tr>
<tr>
<td>OVX</td>
<td>ovariectomized</td>
</tr>
<tr>
<td>POA</td>
<td>preoptic area</td>
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Abbreviations (continued)

PRZ............ prazosin
PVN............. paraventricular nucleus
RH.............. rostral hypothalamus
SON............. supraoptic nucleus
VMN............ ventromedial nucleus
YO............. yohimbine
Acknowledgements

A number of people made large contributions to the work presented in this dissertation. Foremost among them I would like to thank Craig Johnston, who introduced me to the subject of neuroendocrinology, patiently advised and mentored me as my research progressed, and always encouraged me to strive for excellence. I would also like to express my gratitude for the contribution of Vernon Grund, who opened the door for me to do my PhD at the University of Montana, and who played an important role during my time here as a committee member, and as Department Chairmen and Graduate Student Advisor. I would also like to thank my other committee members, Diana Lurie, Keith Parker, and Brent Ruby, whose advice and ideas contributed greatly to this work. Others who contributed to this work include the faculty of the department of Pharmaceutical Sciences, nearly all of who went out of their way to help me in some manner over the years, and my fellow students (past and present), most notably ‘Uncle’ Bret Bessac, Travis Denton, and Celine Wishcamper. Finally, I would like to thank my parents, Toby and Carlene Armstrong, and especially my wife, Mei Yang Selvage. Their love and support made the completion of this project possible.
Chapter 1: Introduction

Scientists have studied the control of reproductive cycles since the discovery and characterization of the gonadal endocrine steroids, estradiol and progesterone, in the 1930's. In the late 1940's and early 1950's, connections between the brain and the pituitary were elucidated, and the field of neuroendocrinology founded (Harris 1955; Bargmann and Scarrer 1951). In 1955, the possibility that the brain controls anterior pituitary gland function via the hypothalamic hypophysial portal blood system was first suggested (Harris 1955). Central nervous system (CNS) regulation of fertility-related pituitary function was first shown when Kalra (1971) demonstrated that electrochemical stimulation of the medial preoptic area (MPO) of the hypothalamus evoked the release of luteinizing hormone (LH), which drives ovulation, from the pituitary gland. In the early 1970's it was demonstrated that CNS-controlled pituitary function depended on circulating levels of estradiol and progesterone produced by the ovaries (Tepperman 1980), and the primary hypothalamic LH-releasing factor, gonadotropin-releasing hormone (GnRH), was characterized (Schally et al. 1972; Yen and Jaffe 1986). Since that time, the effect of many endogenous CNS neurotransmitter systems on GnRH release and fertility cycles have been described, but a clear picture of how the brain controls these cycles has not been determined. Also unresolved is how the peripheral endocrine system signals the brain that the peripheral reproductive organs are prepared for ovulation, and the timing for the preovulatory surge of LH is perfect. Figure 1-1 summarizes the feedback loops involved in the generation of the preovulatory GnRH surge and ovulation.
Regulation of Fertility: Gonadal Steroids and the Estrous Cycle

Because of its overall physiological similarity to the human and short estrous cycle, the rat is frequently used as a model for studying female fertility. Rats have a four to five day estrous cycle, which is characterized by cyclically changing hormone levels (Figure 1-2), and corresponding changes in vaginal cytology. The first day of the cycle is called metestrus (or diestrus one). Reproduction-specific gonadal and hypothalamic hormone levels remain low during metestrus. The second day of the cycle is diestrus (or diestrus two). Hormone levels remain quiescent until very late in diestrus, when estradiol levels slowly begin to rise. During proestrus, the third day of the cycle, estradiol levels continue to rise, reaching a peak in the late afternoon. On the afternoon of proestrus, progesterone levels also rapidly rise. The timing of the increases in these two gonadal hormones (especially estradiol), in turn, somehow signals the hypothalamus to secrete a large pulse of the hypothalamic hormone, GnRH, from the median eminence (ME). This hypothalamic hormone stimulates the preovulatory surge of LH release from the anterior pituitary. The final day of the cycle is estrus, the fertile period. During estrus, levels of LH and GnRH are low, whereas estradiol and progesterone levels remain physiologically high. It is the unknown signal(s) that mediate this important feedback of peripheral gonadal hormones to the brain, signaling that the time is right for ovulation, and stimulating GnRH release on proestrus, which is the particular focus of the studies outlined in this dissertation.
Luteinizing Hormone and Ovulation

LH is a 30,000 MW glycoprotein that is synthesized and secreted from gonadotropes in the anterior pituitary. In female rats and other mammals, LH and follicle stimulating hormone (FSH), another glycoprotein made in the anterior pituitary, are necessary for final follicular growth and maturation leading to ovulation. Both hormones consist of two polypeptide chains linked by hydrogen bonds and internal cross-linking disulfide bonds. LH and FSH are composed of identical 89- amino acid alpha chains, and a 115- amino acid beta chain unique to each hormone. Complex carbohydrate side chains are attached to specific locations on both the alpha and beta chains. The alpha and beta chains are synthesized separately and are combined before addition of the carbohydrate moieties.

FSH and LH bind to high affinity receptors in the ovaries that are glycoprotein encoded by homologous genes and are characterized by seven transmembrane-spanning domains. A large extracellular N-terminus region forms the specific binding site for each gonadotropin. The C-terminus is intracellular and mediates receptor down-regulation, and the generation of second-messenger signals. Both LH and FSH receptors demonstrate distinctive calcium signaling properties, and when bound by agonists activate adenylyl cyclase. The cAMP generated then triggers the activation of its protein kinase and subsequent phosphorylation of proteins necessary for steroidogenesis and ovulation.

During the follicular phase of the estrous cycle, FSH stimulates several follicles to accelerate maturation by binding to its receptors on the granulosa cells, leading to an
increased enzymatic activity to aromatize androgens to estradiol. As estradiol levels increase and follicles develop, FSH levels drop and the developing follicles begin to synthesize increased numbers of LH receptors. On proestrus, positive feedback from estradiol causes a large surge of GnRH release, which then stimulates LH release. This LH induces the production of prostaglandin F2-alpha, progesterone and proteolytic enzymes at the follicular level. These ultimately cause follicular rupture and ovulation. Recent data indicate that increases in progesterone itself, or a similar adrenal steroid, may actually precede the LH surge and contribute to its stimulation and timing. At any rate, following ovulation, the corpus luteum continues to make and secrete progesterone to prepare the uterus for implantation of a fertilized ovum. The high concentrations of estradiol and progesterone present just after ovulation then cause negative feedback upon GnRH, FSH and LH release. The resulting drop in LH secretion prevents further production of estradiol and progesterone. As the concentration of these gonadal steroids decrease, the degenerating uterine endometrial cells are attacked by leukocytes, preparing the reproductive system for its next cycle.

**GnRH**

GnRH, also known as luteinizing hormone-releasing hormone (LHRH), is a decapeptide hormone and neurotransmitter. GnRH neuron cell bodies originate in the MPO and the organum vasculosum of the lamina terminalis (OVLT) of the rostral hypothalamus (RH). They are widely distributed and few in number (Carillo 1980). GnRH projections are sent from the MPO to the ME, where GnRH is secreted into the
hypophysial portal blood, which carries the hormone to a second capillary bed in the sinusoids of the anterior pituitary, where GnRH binds to specific seven-transmembrane, G-protein coupled receptors.

In addition to being located in the anterior pituitary, GnRH receptors are also found in various other areas of the brain. These include the ventromedial nucleus of the hypothalamus (VMN), the lateral septal nucleus, the anterior cingulate cortex, and the subiculum (Jennes and Conn 1994). GnRH stimulation of these receptors is thought to mediate sex-related behaviors such as lordosis in the female rat. Thus, GnRH has central effects in addition to its role in stimulating LH and FSH release from the pituitary gland.

Activation of the GnRH receptor is coupled to the Gq/G11 signal transduction system, which activates phospholipase C. This enzyme leads to the generation of several second messenger molecules. Among these, diacylglycerol (DAG) and inositol 1,4,5-tris-phosphate (IP3) are critically important. Increased in DAG leads to activation of protein kinase C, which causes a series of intracellular changes, and IP3 causes increased release of calcium from intracellular pools (Diedrich 1999). GnRH receptor synthesis is subject to a positive feedback loop, whereby the levels of receptor-bound GnRH positively influence GnRH receptor synthesis (Stojiljkovic et al. 1994). Thus, one result of the signal transduction pathway for GnRH receptor stimulation is that transcription and/or translation of GnRH receptor genes is increased upon binding.

GnRH synthesis and release in the female rat is linked both to increased intracellular calcium levels in GnRH neurons, and transcription of a number of important immediate early genes (IEG), especially Fos and Jun (Finn, Steiner, and Clifton 1998).
This is important to note, as a number of studies using IEGs as markers of cellular activity have identified areas in the medialbasal hypothalamus (MBH), other than the ME, which are important for the cyclic synthesis and release of GnRH in the female rat. Further, increased Fos induction is seen in GnRH neurons on the afternoon of proestrus in female rats (Hoffman, Smith and Verbalis 1993; Hoffman 1993), and GnRH genes are plausible targets for activation by Fos and Jun (Berriman 1992).

**Control of GnRH Release**

Pulsatile GnRH release is regulated, in part, by circulating gonadal steroid levels. At most times during the reproductive cycle estradiol and/or progesterone provide negative feedback to GnRH and LH release. However, GnRH release can be stimulated by progesterone administered to estradiol primed, ovariectomized (OVX) animals (Levine 1997). This effect can be blocked when progesterone receptors are antagonized (Chappel and Levine 2000), which indicates an important role for both gonadal steroid hormones in the generation of the proestrous GnRH surge. Interestingly, GnRH neurons do not express appreciable estradiol or progesterone receptor protein (Simonian and Herbison 1997), and thus are thought to indirectly sense changing levels of gonadal steroids. However, estradiol and progesterone receptors are present on a number of other neuronal phenotypes, many of which make connections with GnRH neurons. It is hypothesized that it is through these neurons that the brain detects peripheral estradiol and/or progesterone levels in order to regulate GnRH secretion.
A number of central neurotransmitters have been shown to influence GnRH synthesis and release, and thus LH synthesis and release. These are summarized in Table 1-1. Norepinephrine (NE), neuropeptide Y (NPY), glutamate, oxytocin (OT), and galanin all have been shown to stimulate GnRH release. NE seems to play a role in both the preovulatory, proestrous GnRH surge, and in regulating basal secretion throughout the estrous cycle (Legan and Callahan 1999). Both NPY and glutamate are involved in stimulating the proestrous surge at the level of the ME, where GnRH is released (Prevot et al. 1999). OT’s role is negligible on all days of the cycle other than proestrus. However, on the afternoon of proestrus, OT has been shown to stimulate the preovulatory LH surge when given directly into the CNS (Johnston et al. 1992). Interference with endogenous OT via antibodies, OT receptor antagonism, conjugation of OT with the neurotoxin ricin, or OT antisense administration on the afternoon of proestrus blocks or attenuates the preovulatory GnRH and/or LH surge (Johnston et al. 1992; Johnston et al. 1990; Cheesman et al. 1977; Samson et al. 1992). Galanin has also been implicated in the stimulation of GnRH release on proestrus. Galanin mRNA has been localized on GnRH neurons and is dramatically upregulated during proestrus and estrus (Finn et al. 1998). Further, upregulation of galanin gene expression has been linked to the GnRH and LH surges (Finn et al. 1998). The galanin gene may be a target for activation by Fos and Jun, the two IEGs associated with estradiol-induced GnRH release (Hrabosvky 1995). Thus, several neurotransmitters have been implicated in the stimulation of GnRH release.

Agents inhibitory to GnRH secretion include beta-endorphin, gamma-aminobutyric acid (GABA), and corticotropin releasing factor (CRF). Beta-endorphin is involved in the tonic regulation of GnRH secretion, and administration of a beta-endorphin agonist
attenuates the GnRH surge (Polkowska, J and Przekop, 1997). CRF, which is secreted in response to stress, is also a potent inhibitor of proestrus GnRH secretion (Polkowska and Przekop 1997). The fact that stress is one of the most common causes of female infertility relates directly to this phenomenon.

That so many neurotransmitter systems influence GnRH release makes sense from an evolutionary standpoint, as reproduction can continue despite the loss of one the controlling factors of GnRH release. For instance, both chronic blockade of alpha-adrenergic receptors, and lesions of noradrenergic neurons in the locus ceureleus abolish normal reproductive cycles in the rat. However, normal reproductive cycles return after a few weeks, and further manipulations of the noradrenergic system have little effect on re-established pulsatile GnRH release. Thus, it appears that the loss of one neuronal system involved in control of cyclic GnRH release can be compensated for by the actions of others. This represents an important principle of redundancy, which enhances the likelihood of survival of the species (Berriman, 1992).

**Norepinephrine**

NE has been implicated in the regulation of both tonic and pulsatile GnRH release (Legan and Callahan 1999; Gallo 1980). This catecholamine is made primarily in the locus ceuruleus of the caudal midbrain and upper pons of the brainstem. From there, A1 and A2 NE projections are sent to targets throughout the brain, including several involved in reproductive behavior. These include the preoptic area (POA), MPO (Legan and
NE neurons in the AI field have estradiol receptors (Simonian and Herbison 1997b), and NE turnover in the MPO as well as the ME parallels changes in circulating LH concentrations, such that NE secretion increases around the time of the preovulatory LH surge (Crowley et al. 1978; Honma and Wutke 1980). Moreover, immunocytochemistry (ICC) and in situ hybridization studies reveal there are synaptic contacts between noradrenergic terminals and GnRH cells in the POA (Barraclough and Wise 1982; Kalra and Kalra 1983).

NE binds to several different receptor subtypes. In terms of NE's effects on fertility and sexual behavior, alpha- adrenergic receptors have been most implicated. Antagonism of NE alpha- adrenergic receptors by administration of phenoxybenzamine blocks the LH surge (Weick 1978), as does blockade of NE synthesis (Legan and Callahan 1999) in female rats. In OVX female rats, intracerebroventricular (ICV) administration of 5-amino-2,4-dihydroxy-methylphenylethylamine, a selective depletor of brain norepinephrine, reduces GnRH mRNA levels (Kang et al. 1998). In vitro administration of the catecholaminergic depletor, 6-hydroxydopamine, to MBH explants reduced GnRH mRNA levels, and this reduction was reversed by treatment with NE (Kim et al. 1993), indicating a direct effect of NE on GnRH synthesis. N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP4), a highly selective NE neurotoxin, suppressed estradiol stimulated GnRH release in vitro, reduced levels of GnRH mRNA in the POA, and caused a reduction of GnRH-receptor mRNA in the pituitary (Kang et al. 1998). In the OVX ewe, another animal frequently used in fertility studies, levels of statically expressed dopamine β-hydroxylase, the enzyme which converts dopamine to NE within synaptic vesicles, decreased in the MPO in estradiol-primed, OVX animals as
visualized by ICC (Kotwica et al. 1996). This was thought to indicate an increase in conversion of dopamine to NE during the late follicular stage of the ewe, which parallels proestrus in rats. In the rabbit, LH surges are induced following coitus, and this induction has been linked to increased release of NE in the MBH (Kaynard et al. 1990). Thus, data from a variety of species indicate an important role for NE in the GnRH and LH surges.

**Oxytocin**

Another putative stimulator of GnRH is OT. OT is a nanopeptide hormone and neurotransmitter, and is very similar to arginine vasopressin (AVP) in structure but not function. OT cell bodies are concentrated in the anterior portion of the paraventricular (PVN) and in the supraoptic (SON) nuclei of the hypothalamus (Peterson and Guillemin 1974). The main projection of OT neurons is to the posterior pituitary, where it is secreted as a hormone in response to suckling in nursing mammals, at the onset of parturition, and in response to stress. Projections are also sent to the ME, where it has been postulated that OT stimulates GnRH secretion (Rettori et al. 1974), and where OT is released into the hypophysial portal blood to influence anterior pituitary activity. Other OT projections are sent to areas in the hypothalamus containing GnRH cell bodies including the MPO, the OVLT, and the VMN. Sub-populations of OT neurons display nuclear estradiol receptors, and these may be involved in OT’s role in stimulating GnRH (Dufau and Catt, 1978).

The OT receptor is very similar to that for vasopressin. It belongs to the G-protein coupled, seven transmembrane receptor family. In the periphery, OT receptors are
found in the uterus, mammary gland, and pituitary lactotropes and corticotropes. In the brain, the receptor is found in a variety of nuclei involved in both the GnRH surge and sexual behavior. These include the VMN, the MPO, the OVLT, and the SON (Ludwig 1995). Activation of the OT receptor is coupled to the inositol phosphate (IP3)/calcium second messenger system. OT binding thus causes an increase in IP3, DAG, and ultimately, free intracellular calcium. It has been postulated that there are subtypes of OT receptors specific for 4-9OT, a potent metabolized version of OT in the brain, but these have not been characterized (Lopes-da Silva, Adan and Burbach 1993).

OT and OT receptor mRNA both are influenced by circulating estradiol levels (Bale et al. 1995; Quinones-Jehab et al. 1997). In OVX animals, estradiol-priming increases OT mRNA levels in the rat hypothalamus, and this phenomenon is greatly enhanced when progesterone is initially administered with estradiol, then subsequently is slowly withdrawn (Bale et al. 1990). Message expression of the OT receptor varies throughout the estrous cycle, with a 15-fold increase in that expression being seen in the VMN from the morning to the afternoon of proestrus (Bale, Dorsa, Johnston 1995). This supports a role for changing estradiol and progesterone levels in the regulation of OT synthesis. Further, the rat OT gene contains a consensus sequence for an estradiol response element (Quinones-Jelab et al. 1997).

While central administration of OT has previously been shown to stimulate elevated plasma LH on proestrus (when estradiol levels are high), but not on metestrus or diestrus (Johnston et al. 1992), whether OT directly stimulates GnRH on proestrus in the female rat is not known. Presumably it does, since the effect of OT on LH stimulation is a centrally mediated phenomenon and can be blocked by central administration of both OT.
antisera and a GnRH antagonist (Johnston et al. 1990). However, this has yet to be proven. Another unknown is the site in the brain where OT is working to stimulate LH release. These represent two of the primary questions examined in the research for this dissertation.

**Nitric Oxide**

Nitric oxide (NO) has been shown to play a role in cyclic GnRH release in the female rat both at the level of the ME and in the MBH (Herbison et al. 1996). NO is synthesized by a number of related NO-synthase (NOS) molecules in neurons, endothelial cells, platelets, and neutrophils in response to homeostatic stimuli (Moncada 1992). NOS is a complex enzyme which acts on molecular oxygen and arginine to produce NO and citrulline. This process involves five essential cofactors and two divalent cations (calcium and heme iron). NO interacts with the heme prosthetic group of guanylate cyclase, activating the enzyme and leading to increased cyclic GMP levels. Evidence has been presented for a role of constitutively expressed neuronal NOS (nNOS) and endothelial NOS (cNOS), in the stimulation of LH and GnRH release on the afternoon of proestrus in female mammals (Lamar et al. 1999; Lozach et al. 1998).

Several lines of evidence also provide evidence that NO plays a role in the stimulation of GnRH during proestrus. NO feeding via the nitrogen donor, sodium nitroprusside, enhances proestrous GnRH secretion in vitro (Bonavera et al. 1993; Mani et al. 1994). NO synthesis in the MBH, as measured by the by-product of NOS synthesis of NO, citrulline, is highest on the afternoon of proestrus (Canteros et al. 1996).
ME, estradiol has been shown to stimulate NO release from endothelial cells in ME explants, and this stimulation of NO was accompanied by increased release of GnRH from GnRH nerve terminals. These effects were blocked by administration of the estradiol antagonist, tamoxifen (Prevot et al. 1999). Further, it has been shown that when NO-stimulated guanylyl cyclase activation was inhibited using 1H-(1,2,4)oxadiazolo(4,3a)quinoxalin-1-one (ODQ), a potent and selective inhibitor of NO sensitive guanylyl cyclase, estradiol stimulated GnRH release from the ME is eliminated (Prevot et al. 1999). nNOS mRNA has been demonstrated to be present in the immortalized GnRH synthesizing cell line, GT1, and these cells also produce the by-product of NO synthesis, citrulline.

No evidence for nNOS in GnRH cells has been demonstrated immunocytochemically in vivo. However, Herbison et al. (1996) reported a distinct band of nNOS containing neurons in the MPO abutting GnRH cell bodies. Neurons containing neural NO synthase (nNOS) are also found in various locations in the hypothalamus that have been linked to reproduction, especially the PVN and SON (Amico et al. 2000). These neurons have axons projecting to the ME and extending into the neural lobe, where the highest concentration of NOS immunoreactivity is found in the rat brain (McCann 1998). Further, NOS-like activity has been demonstrated in the MPO and VMN, and this, in conjunction with the previously discussed biochemical information, indicates that NO could be involved in both the generation of the GnRH pulse, and in GnRH mediated reproductive behaviors.
Interaction Between the OT, NE, and NO Systems on Reproductive Behaviors

Interaction between the OT, NE, and NO systems on GnRH release has been demonstrated in several different ways. Anatomically, two or more of these systems have been shown to overlap in the VMN, MPO, SON, PVN, and ME regions via histochemical techniques (Mcann et al. 1998; Rettori et al. 1997; Daftary, Boubadaba, and Tasker 2000). As mentioned earlier, Fos induction in the hypothalamus has been shown via ICC to be a precursor of GnRH synthesis and release in female rats on the afternoon of proestrus. Both NE and OT have been linked to induction of Fos in the SON and the PVN (Conde et al. 1996; Flanagan et al. 1993), and it has been shown that NE stimulates OT release as well as Fos induction in the PVN (Bealer and Crowely 1999). Further, NOS-containing neurons in the SON have been shown to synthesize OT as well as express Fos (Hatakeyama et al. 1996). Thus, there is strong anatomical proof of overlap between these three neurotransmitter systems in hypothalamic areas known to be involved in the generation of the GnRH surge.

In addition to anatomical evidence for interaction between NE, OT, and/or NO, links between the three have been made via biochemical investigation. As previously mentioned, the NE, OT and NO neurotransmitter systems have been shown to be sensitive to changing estradiol levels. Other evidence indicates that these responses to estradiol might be interlinked. Vincent and Etgen (1993) demonstrated that steroid priming via estradiol promotes OT- induced NE release in the VMN in female rats in vivo. OT has been shown to influence NO synthesis in the MBH, and NE influences NO

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at both the level of the ME and the MBH in males (Rettori et al. 1997). Taken together, this evidence indicates significant interaction and interplay between these three systems in the generation of GnRH release, and GnRH mediated reproductive behaviors. These interactions are the major focus of the research presented here.
Figure 1-1. Schematic of events involved in ovulation. Estrogen and progesterone levels are sensed by the hypothalamus, resulting in the secretion of gonadotropin-releasing hormone (GnRH) into the portal blood at the median eminence. GnRH travels from there to the pituitary, where it stimulates its receptors. This in turn causes the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary, which stimulate receptors in the ovaries, ultimately causing follicular rupture and ovulation.
Figure 1-2. (opposite page). Approximate secretion levels of gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), estradiol, and progesterone throughout the rat estrous cycle. The vertical dotted line indicates the time of ovulation.
Estrus  Metestrus  Diestrus  Proestrus  Estrus

Day Of Estrous Cycle
### Table 1-1 Neurotransmitters Involved in the Central Control of GnRH Release

<table>
<thead>
<tr>
<th>Neurotransmitter</th>
<th>Action on GnRH/LH</th>
<th>Site(s) of Action</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norepinephrine</td>
<td>Stimulatory/Inhibitory</td>
<td>ME, MBH</td>
<td>Brainstem</td>
</tr>
<tr>
<td>Glutamate</td>
<td>Stimulatory</td>
<td>ME, Pituitary (?)</td>
<td>MBH, many other brain areas</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>Stimulatory on proestrus</td>
<td>BH(?), ME(?), Pituitary (?)</td>
<td>PVN, SON</td>
</tr>
<tr>
<td>Nitric Oxide</td>
<td>Stimulatory on proestrus</td>
<td>ME, MBH, Pituitary</td>
<td>nNOS, iNOS, cNOS</td>
</tr>
<tr>
<td>Galanin</td>
<td>Stimulatory</td>
<td>ME, MBH</td>
<td>VMN</td>
</tr>
<tr>
<td>B-endorphin</td>
<td>Inhibitory (Tonic)</td>
<td>ME, MBH</td>
<td>VMN (terminals)</td>
</tr>
<tr>
<td>GABA</td>
<td>Inhibitory</td>
<td>ME, MBH</td>
<td>Hypothalamus</td>
</tr>
<tr>
<td>CRF</td>
<td>Inhibitory</td>
<td>ME, MPO, OVLT</td>
<td></td>
</tr>
</tbody>
</table>
Specific Aims

The specific aims of this research are to:

1. Investigate the role of OT in the generation of the preovulatory GnRH surge by determining whether OT is able to stimulate GnRH release from ME or BH explants harvested on the afternoon of proestrus from normally cycling female rats.

2. Develop a dose-response curve for OT- induced stimulation of GnRH from proestrous BH explants.

3. Examine the mechanisms of OT- induced stimulation of GnRH release by testing whether OT and alpha- adrenergic receptor antagonists, or NOS inhibition, can influence OT- induced stimulation of GnRH release from proestrous BH explants.

4. Investigate the role of NE in the generation of the preovulatory GnRH surge by determining if NE can stimulate GnRH release from BH explants harvested on the afternoon of proestrus from normally cycling female rats, and develop a dose-response curve for NE- induced stimulation of GnRH release from proestrous BH explants.
5. Examine the mechanisms of NE- induced stimulation of GnRH release by testing whether OT and alpha- adrenergic receptor antagonists can influence NE- induced stimulation of GnRH release from proestrous BH explants.

6. Investigate whether there is interaction between NE and OT on stimulating GnRH release by examining the ability of sub-maximal doses of OT or NE alone, or in combination, to stimulate GnRH release from proestrous BH explants.

7. Examine the mechanisms of combined NE/OT- induced stimulation of GnRH release by testing whether OT and alpha- adrenergic receptor antagonists, or NOS inhibition, can influence NE/OT- induced stimulation of GnRH release from proestrous BH explants.

8. Investigate the role of NO in NE/OT- induced stimulation of proestrous GnRH by determining whether NE and/or OT can stimulate NO synthesis in proestrous BH explants, and test the ability of OT receptor antagonism on NE/OT- induced NO stimulation.

9. Examine the ability of NE and/or OT to stimulate GnRH and NO from BH explants harvested on the afternoons of estrus and diestrus versus proestrus.
Chapter 2: Materials and Methods

Animals

Young adult female Sprague-Dawley rats (Simonson Labs, Gilroy, CA) weighing 200-250 grams were housed four to a cage, with food and water ad libitum, in an AAALAC accredited vivarium. Room temperature was kept at 23 ± 1°C, with lights on from 0700hr to 1900hr. Only animals displaying two consecutive, normal four to five day estrous cycles were used in these experiments.

After at least one week of acclimatization to new housing and lighting conditions, rats were handled daily in order to accustom them to being manipulated by the researcher. Following several days of handling, tails were marked for individual identification. Vaginal cytology was then examined daily using a vaginal lavage method: A) the animal was picked up and placed feet first on the tester’s forearm, B) the tail was raised and a glass eyedropper with a small amount of water (approximately 0.5ml) was inserted into the vagina, C) after lavage, the eyedropper fluid was smeared onto numbered, clear glass slides and left to air dry, D) slides were then immersed for five seconds in 100% methanol for fixation, and after drying, in 0.05% methylene blue in ddH20 for approximately two minutes, then, E) slides were washed three times in fresh cold water, and set to air dry. Slides were examined using a light microscope at 10X magnification in order to determine the changes in vaginal cytology, and stage of the cycle.
Figure 2-1 demonstrates how the estrous cycle is readily discernable via light microscopy examination of vaginal cytology. The first day of the cycle is called diestrus one, or more commonly, metestrus. The influence of estradiol or progesterone is minimal on metestrus, and vaginal cytology reveals a sea of leukocytes descending upon, surrounding and destroying the cornified cells of estrus to begin another cycle. The second day of the cycle is called diestrus two, or simply diestrus. On diestrus, when estradiol exerts a limited influence, vaginal cytology consists of a small number of any or all cell types, including leukocytes, epithelial and cornified cells. On the third day of the estrous cycle, proestrus, high levels of estradiol and increasing levels of progesterone stimulate induction of the preovulatory GnRH surge. Vaginal cytology on proestrus reveals an abundance of small, round, nucleated epithelial cells, which often clump together or align in strands. On the fertile day of the cycle, estrus, vaginal cytology, under the strong influence of progesterone and estradiol, reveals large, enucleated cornified cells, which tend to clump together. These cells are rich in nutrients, and should an egg be fertilized, provide an excellent environment for implantation and nutrition.

**Tissue Preparation**

At 1500hr, animals determined to be at the proper stage of the estrous cycle were decapitated. Brains were quickly removed and the basal hypothalamus (BH) or ME was rapidly dissected using a dissecting microscope and fine iris scissors. The tissues were immediately placed in 0.5 ml preincubation Krebs-Ringer Bicarbonate buffer medium (pH 7.4) supplemented with 1.8 mg glucose, 10 ug/ml ascorbic acid, 1.3 mM Ca\(^{2+}\), and 1
mg/ml aprotinin (KRBG) for 30 minutes. All incubations were carried out in a metabolic shaker (30 cycles/min, supplied with 95% O₂/5% CO₂ at 37°C). The 30 minute preincubation period was determined empirically as the minimum time necessary for stable, basal GnRH secretion to be restored following harvesting of tissue. The preincubation media was discarded and replaced following 30 minute increments with 0.5 ml KRBG, alone, to determine basal (control) GnRH secretion levels, or KRBG containing OT (10⁻⁹M to 10⁻¹⁵M), NE (10⁻⁶M to 10⁻¹⁰M), OT (10⁻¹¹M) and NE (10⁻⁸M) combined, the NO synthesis inhibitor, N-nitro-L-arginine methyl ester (LNAME, 10⁻⁶M), the kainite receptor antagonist, NBQX (10⁻⁶M), the alpha-1 adrenergic receptor antagonist, prazosin (PRZ, 10⁻⁶M), the alpha-2 receptor adrenergic antagonist, yohimbine (YO, 10⁻⁶M), the OT receptor antagonist, [d(CH₂)₅, Tyr (Me)², ORN₈] vasotocin, (OTA, 10⁻⁶M), or combinations thereof. Following each 30 minute test incubation, the medium was removed and stored at -80°C until assayed for GnRH and/or nitrate/nitrite content.

**Lowry Protein Assay**

The amount of protein for each tissue sample was determined using a modified Lowry protein assay. Following overnight incubation in 0.5 ml 1N NAOH, tissue samples were vortexed until the solutions were homogenous. Tubes were labeled Blank, A-D in duplicate (to construct a standard protein curve), and 1-N in duplicate for the number of samples. For determination of a standard protein curve, standard stock solutions were prepared from a stock solution of one gram bovine serum albumin (fraction V) (BSA)/ml H₂O. The standard curve was constructed by adding 50 ul of
distilled H₂O (blanks) and 50 ul of distilled H₂O containing 0 ug - 50 ug protein to the appropriate tubes, followed by 50 ul 1N NAOH. The proper dilution aliquot of sample was then added to the appropriately labeled tubes, followed by distilled H₂O to bring total volume up to 100 uL. Reagent ‘A’ was then prepared by adding Sodium Carbonate: Cupric Sulfate: KNa Tartrate in that order in the following proportion - 100: 1:1. One milliliter of reagent A was then added to each assay tube, with each tube vortexed following addition. Ten minutes following the addition of reagent ‘A’ to each tube, 100 ul folin reagent (phenol: distilled H₂O at 1:1) was added, and each tube was vortexed following each addition. Thirty minutes following the first addition of folin reagent, standards and samples were read at a 660 nm wavelength using a Stafford III spectrophotometer (Gilford Instruments, Oberlin, OH). A standard curve was manually constructed by taking the average number of fluorescent units of each pair of standard, subtracting out the average blank, then dividing the result with the known amount of protein (in micrograms) for each pair. The average of these numbers was divided into the counts for each dilution of unknown sample (minus blank), and the total micrograms of protein determined from the dilution factor.

Radioimmunoassay

Radioimmunoassays (RIA) were performed using standard double-antibody GnRH RIA kits (Peninsula Laboratories, Inc., San Carlos, CA). The assay is based on the competition between ¹²⁵I-GnRH and unlabeled peptide (either standard or unknown) binding to a specific primary antibody added at a dilution of 1:20,000. An increase in the
concentration of standard or unknown GnRH peptide reduces the amount of $^{125}$I-GnRH able to bind to the antibody. By measuring the amount of labeled GnRH bound as a percentage of total binding possible for each standard reaction mixture, a standard curve was constructed. The amount of GnRH in unknown samples was then determined using the standard curve.

Following reconstitution of RIA buffer, standard GnRH peptide, and rabbit-anti GnRH, a standard dilution series was created (Table 2-1).

Table 2-1: GnRH Peptide Dilution Series

<table>
<thead>
<tr>
<th>Tube</th>
<th>Sample</th>
<th>RIA Buffer</th>
<th>GnRH</th>
<th>GnRH/100ul</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock powder</td>
<td>1 ml</td>
<td>12.8ug/ml</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>O</td>
<td>40ul stock</td>
<td>960ul</td>
<td>5.12ng/ml</td>
<td>512pg/100ul</td>
</tr>
<tr>
<td>A</td>
<td>10ul O</td>
<td>990ul</td>
<td>2.56ng/ml</td>
<td>256pg/100ul</td>
</tr>
<tr>
<td>B</td>
<td>500ul A</td>
<td>500ul</td>
<td>1.28ng/ml</td>
<td>128pg/100ul</td>
</tr>
<tr>
<td>C</td>
<td>500ul B</td>
<td>500ul</td>
<td>0.64ng/ml</td>
<td>64pg/100ul</td>
</tr>
<tr>
<td>D</td>
<td>500ul C</td>
<td>500ul</td>
<td>0.32ng/ml</td>
<td>32pg/100ul</td>
</tr>
<tr>
<td>E</td>
<td>500ul D</td>
<td>500ul</td>
<td>0.16ng/ml</td>
<td>16pg/100ul</td>
</tr>
<tr>
<td>F</td>
<td>500ul E</td>
<td>500ul</td>
<td>0.8ng/ml</td>
<td>8pg/100ul</td>
</tr>
<tr>
<td>G</td>
<td>500ul F</td>
<td>500ul</td>
<td>0.4ng/ml</td>
<td>4pg/100ul</td>
</tr>
<tr>
<td>H</td>
<td>500ul G</td>
<td>500ul</td>
<td>0.2ng/ml</td>
<td>2pg/100ul</td>
</tr>
<tr>
<td>I</td>
<td>500ul I</td>
<td>500ul</td>
<td>0.1ng/ml</td>
<td>1pg/100ul</td>
</tr>
</tbody>
</table>

Tubes were then labeled in triplicate TA (total activity), NSB (non-specific binding), and in quintuplicate TB (total binding), and appropriately for the standard curve. Two hundred microliters of RIA buffer was added to the TA and NSB tubes, and
100 ul to TB tubes. One hundred microliters of standard H through A was pipetted in triplicate into tubes #12-44, starting with the lowest concentration (tube H) and continuing with higher concentrations through tube A. Ten microliters, 5 ul, or 1 ul of unknown sample was then added to tubes #45 and up, and the total amount in each tube was brought up to 100 ul by adding RIA buffer. Primary antibody was then added to all but the TA and NSB tubes, and each tube was vortexed. All tubes were then covered with aluminum foil and allowed to incubate overnight (16-24 hrs) at 4°C.

On the second day of the assay, ^125^I-GnRH was reconstituted with 1 ml of RIA buffer, and mixed thoroughly. The tracer was diluted to a concentration of approximately 25,000 cpm/100 ul. One hundred microliters of tracer was added to each test tube in the assay, they were vortexed, covered and allowed to incubate overnight (16-24 hrs) at 4°C.

On the third day of the assay, a secondary Goat Anti-Rabbit IgG serum was reconstituted to a concentration of 1:22, and 100 ul was added to every test tube. Normal Rabbit Serum was then reconstituted to a concentration of 3% and 100 ul added to each tube. All tubes were vortexed and allowed to incubate at room temperature for 90 minutes. Five hundred microliters of RIA buffer was then added to each tube, and all but the TA tubes were vortexed, then centrifuged in a Beckman Model J-6B centrifuge for 35 minutes at 2,500 RPM. Supernatant from the centrifuged tubes was carefully aspirated, leaving a pellet. Pellets containing bound ligand-antibody from all tubes were then counted using a gamma counter (Apex Automatic Gamma Counter, ICN Micromedia Systems, Model 28026). Software by Isodata was used in order to quantitate a standard curve, and calculate the number of picograms GnRH per tube for unknown samples. Final data were reported as nanograms GnRH/mg protein/ml/hour.
Measurement of Nitric Oxide: The Griess Reaction

NO was measured using nitrate/nitrite assay kits (Cayman Chemical, Cat # 780001). NO is produced in trace amounts in neurons and other cells, and is rapidly scavenged to nitrates and nitrites (T1/2 = 4 seconds). For this to occur, NO undergoes a series of reactions with several molecules present in biological fluids, including:

\[
\text{NO} + \text{O}_2 \rightarrow \text{ONO}_2^- + \text{H}^+ \rightarrow \text{NO}_3^- + \text{H}^+
\]

\[
2\text{NO} + \text{O}_2 \rightarrow \text{N}_2\text{O}_4 + \text{H}_2\text{O} \rightarrow \text{NO}_2^- + \text{NO}_3
\]

\[
\text{NO} + \text{NO}_2 \rightarrow \text{N}_2\text{O}_3 + \text{H}_2\text{O} \rightarrow 2\text{NO}_2.
\]

The final in vivo products of NO are nitrite (NO$_2$) and nitrate (NO$_3$). The relative proportion of nitrite/nitrate is variable and cannot be predicted with certainty. Thus, the best index for total NO production is the sum of both, and that is what was measured in these assays. Once conversion of nitrate to nitrite was completed, Griess Reagents are added. These convert nitrite into a deep purple azo compound. Photometric measurement of the absorbance due to this azo chromophore accurately determines nitrite concentration when compared to standard curves.

The assay was run as follows: the assay buffer was diluted to 100 ml with HPLC-grade water. This buffer was used to reconstitute nitrate reductase, enzyme cofactors, and
nitrate standard. One-tenth of the reconstituted nitrate standard was then added to 0.9 ml assay buffer and vortexed. Using this diluted standard, a standard curve was made in a 96 well plate as depicted in Table 2-2 (with the addition of two blank wells filled with 200 ul of buffer only):

Table 2-2. Preparation of Standard Nitrate/Nitrite Curve

<table>
<thead>
<tr>
<th>Well</th>
<th>Nitrate Standard Assay Buffer</th>
<th>Nitrate Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>0ul</td>
<td>80ul</td>
</tr>
<tr>
<td>B1</td>
<td>3ul</td>
<td>77ul</td>
</tr>
<tr>
<td>C1</td>
<td>6ul</td>
<td>74ul</td>
</tr>
<tr>
<td>D1</td>
<td>9ul</td>
<td>71ul</td>
</tr>
<tr>
<td>E1</td>
<td>12ul</td>
<td>68ul</td>
</tr>
<tr>
<td>F1</td>
<td>15ul</td>
<td>65ul</td>
</tr>
<tr>
<td>G1</td>
<td>18ul</td>
<td>62ul</td>
</tr>
<tr>
<td>H1</td>
<td>21ul</td>
<td>59ul</td>
</tr>
<tr>
<td>I1</td>
<td>24ul</td>
<td>56ul</td>
</tr>
</tbody>
</table>

Eighty microliters of unknown sample was then added to the necessary number of wells. Ten microliters of enzyme cofactor mixture was then added to each well, followed by 10 microliters of nitrate reductase (except blank wells). The plate was then covered with a plate cover, and incubated at room temperature for 2 hours. After this incubation time, 50 ul of Griess reagent 1 was added to the standards and unknowns, followed by 50 ul of
Griess reagent 2. Photometric measurement of absorbance was then made at 540 nM using a microplate reader (Molecular Devices, Thermomax Microplate Reader) running Softmax Pro v1.2 software. A standard curve was generated using the software, and the micromolar content of nitrite in each sample well was determined.

**Statistics**

Statistics for these experiments were performed using either Microsoft Excel (raw data entry, mean), or Prism Graph Pad software. Comparisons between pairs of individual groups were made using the student’s t-test, and comparisons between 3 or more groups by one-way analysis of variance plus the Newman-Keuls post-test. The level of probability used as the minimum criterion of significance was $p \leq 0.05$ for all experiments. Because basal levels of nitrate/nitrite release varied between animals, but relative patterns of reactivity to the treatments did not, in the case of the nitrate/nitrite assays, each animal was it’s own control. Basal secretion was considered as 100%, and nitrate/nitrite levels were graphically expressed as percent of basal control. Levels of significance for the NO studies were determined using raw data.
Figure 2-1. Vaginal cytology on metestrus, the first day of the estrous cycle

Vaginal cytology on metestrus reveals a sea of leukocytes, which devour the cornified cells of estrus as the animal begins another estrous cycle.
Figure 2-2. Vaginal cytology on diestrus, the second day of the estrous cycle

Vaginal cytology on diestrus reveals a small number of cells, of mixed type. Cells from the previous estrous cycle are nearly gone, and a new uterine lining is forming.
Vaginal cytology on proestrus, when the GnRH surge occurs, reveals small, round, nucleated epithelial cells which are often clumped together in strands.
**Figure 2-4.** Vaginal cytology on the final day of the cycle, estrus

Vaginal cytology on estrus reveals large, cornified cells lacking nuclei. These provide nutrition for any ovum that might have been fertilized and implanted.
Chapter 3: Results

**Oxytocin Stimulates GnRH Release From the Proestrous BH But Not ME**

Whether or not OT stimulates GnRH release from hypothalamic explants in order to cause its stimulation of proestrous LH in vivo is unknown. To address this question, the ability of OT to stimulate GnRH release from proestrous ME explants, where GnRH nerve terminals are located and GnRH is released into portal capillaries was tested. Also tested was the ability of OT to stimulate GnRH release from proestrous BH explants, which included the ME as well as areas of the rostral hypothalamus containing GnRH cell bodies.

As illustrated in Figure 3-1, OT (10^-13M and 10^-11M) stimulated GnRH release from BH explants harvested on the afternoon of proestrus from normally cycling female rats. However, OT in the same concentrations failed to stimulate GnRH release from ME explants on proestrus. Since OT did not stimulate GnRH release from isolated ME fragments, a dose-response curve for OT- (10^-15M to 10^-9M) induced stimulation of GnRH from intact proestrous BH explants was constructed (Figure 3-2). The dose-response curve showed that OT- induced GnRH release from the BH occured at concentrations as low as 10^-13 M, and and increased in a dose-dependent manner from 10^-11M to 10^-9M.

In summary, the data demonstrate that the connectivity found in the intact BH is necessary for OT to stimulate GnRH release in a dose-dependant manner.
Figure 3-1. OT- induced stimulation of GnRH from the ME versus the BH

*In vitro* release of GnRH from either median eminence (ME) or basal hypothalamus (BH) explants obtained at 1500hrs from normally cycling, proestrous female Sprague-Dawley rats. GnRH release is in response to buffer alone (control), or buffer containing oxytocin (OT, $10^{-11}$M or $10^{-13}$M). Column heights represent mean GnRH (ng/mg protein/ml/hr) released, and vertical lines above columns denote the S.E.M. N=8 animals for all treatments. *, significantly different from basal control values (P<0.01).
Figure 3-2. Dose-response curve for OT- induced stimulation of GnRH release from proestrous BH

*In vitro* release of GnRH from basal hypothalamus (BH) explants obtained at 1500hrs from normally cycling proestrous female Sprague-Dawley rats. GnRH secretion is in response to buffer alone (control), or buffer with oxytocin (OT, $10^{-9}$M to $10^{-15}$M). Column heights represent mean GnRH (ng/mg protein/ml/hr) released, and vertical lines above columns denote the S.E.M. N=8 animals for all treatments. *, significantly different from basal control values (P<0.01).
OT - Stimulation of Proestrous GnRH release: Mechanism of action

In order to examine the mechanisms by which OT induces stimulation of GnRH release from proestrous BH, the ability of antagonists of neurotransmitters/receptors thought to be involved in the generation of the preovulatory GnRH surge were tested. The antagonists used included: 1) the OT receptor antagonist, [d(CH\textsubscript{2})\textsubscript{5}, Tyr (Me)\textsuperscript{2}, ORN\textsuperscript{8}] vasotocin (OTA); 2) the NO synthesis inhibitor, LNAME; 3) the alpha-1 adrenergic receptor antagonist, prazosin; 4) the alpha-2 adrenergic receptor antagonist, yohimbine; and 5) the glutamatergic kainate receptor antagonist, NBQX.

As depicted in Figures 3-3 thru 3-7, OT's ability to stimulate GnRH release from proestrous BH explants was blocked by both OT receptor antagonism (P<0.01) and by inhibition of NO synthesis (P<0.01). These results indicate that OT stimulates GnRH secretion from proestrous BH explants via an OT receptor mediated mechanism which works, at least in part, by the stimulation of NO. Receptor antagonists for alpha-1 and alpha-2 adrenergic receptors did not attenuate OT's ability to stimulate GnRH release \textit{in vitro} from proestrous BH explants, nor did the glutamatergic kainate receptor antagonist NBQX. These results indicate that alpha-1 and alpha-2 adrenergic receptors do not mediate OT's stimulation of GnRH from proestrous BH explants. This would appear to rule out direct OT-induced stimulation of NE as a cause of OT's effects on GnRH release. Finally, glutamatergic kainate receptor antagonism failed to attenuate OT's stimulation of GnRH release from BH tissues on proestrus. The data support the theory that OT does not require activation of alpha-adrenergic, nor kainate receptors to stimulate GnRH release. It does not, however, diminish the possibility that these systems
may influence OT neurons, or work together or independently in order to cause the normal preovulatory surge of GnRH and LH.
**Figure 3-3.** Effect of kainate receptor antagonism on OT- induced GnRH release from proestrous BH

*In vitro* release of GnRH from basal hypothalamus (BH) explants obtained at 1500hrs from normally cycling proestrous female Sprague-Dawley rats. GnRH release is in response to buffer alone (control), or buffer containing oxytocin (OT, $10^{-11}$M) and/or the kainate receptor antagonist NBQX ($10^{-6}$M). Column heights represent mean GnRH (ng/mg protein/ml/hr) released, and vertical lines above columns denote the S.E.M. N=8 animals for all treatments. *, significantly different from basal control values (P<0.01).
Figure 3-4. Effect of alpha-1 adrenergic antagonism on OT-induced GnRH release from proestrous BH

*In vitro* release of GnRH from basal hypothalamus (BH) explants obtained at 1500hrs from normally cycling proestrous female Sprague-Dawley rats. GnRH release is in response to buffer alone (control), or buffer containing oxytocin (OT, $10^{-11}$M), and/or the alpha-1 adrenergic receptor antagonist prazosin, (PRZ, $10^{-6}$M). Column heights represent mean GnRH (ng/mg protein/ml/hr) released, and vertical lines above columns denote the S.E.M. N=8 animals for all treatments. *, significantly different from basal control values (P<0.001).
**Figure 3-5.** Effect of alpha-2 adrenergic antagonism on OT- induced GnRH from proestrous BH

*In vitro* release of GnRH from basal hypothalamus (BH) explants obtained at 1500hrs from normally cycling proestrous female Sprague-Dawley rats. GnRH release is in response to buffer alone (control), or buffer containing oxytocin (OT, $10^{-11}$M), an/or the alpha-2 adrenergic receptor antagonist yohimbine (YO, $10^{-6}$M). Column heights represent mean GnRH (ng/mg protein/ml/hr) released, and vertical lines above columns denote the S.E.M. N=8-9 animals for all treatments. *, significantly different from basal control values (P<0.001).

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Figure 3-6. Effect of NOS inhibition on OT-induced GnRH release from proestrous BH.

*In vitro* release of GnRH from basal hypothalamus (BH) explants obtained at 1500hrs from normally cycling proestrous female Sprague-Dawley rats. GnRH release is in response to buffer alone (control), or buffer containing oxytocin (OT, $10^{-11}$M), and/or the nitric oxide synthesis inhibitor LNAME, ($10^{-11}$M). Column heights represent mean GnRH (ng/mg protein/ml/hr) released, and vertical lines above columns denote the S.E.M. N=8 animals for all treatments, *, significantly different from basal control values (P<0.001).

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Figure 3-7. Effect of OT receptor antagonism on OT- induced GnRH release from proestrous BH

In vitro release of GnRH from basal hypothalamus (BH) explants obtained at 1500hrs from normally cycling proestrous female Sprague-Dawley rats. GnRH release is in response to buffer alone (control), or buffer containing oxytocin (OT, 10^{-11}M), and/or an oxytocin receptor antagonist (OTA, 10^{-6}M). Column heights represent mean GnRH (ng/mg protein/ml/hr) released, and vertical lines above columns denote the S.E.M. N=8 animals for all treatments. *, significantly different from basal control values (P<0.01).
**Norepinephrine Stimulates GnRH Release On The Afternoon Of Proestrus**

NE has previously been implicated in the control of proestrous GnRH release. In order to further examine its role in stimulating GnRH release, the following studies were performed to 1) determine whether NE stimulates GnRH release from proestrous BH explants *in vitro*, 2) examine the dose-response relationship for NE stimulation of GnRH from proestrous BH explants, 3) determine whether NE stimulates GnRH from BH explants via alpha-1 or alpha-2 adrenergic receptors, and 4) determine the effect of OT receptor antagonism on NE- induced stimulation of GnRH from proestrous BH explants.

As depicted in Figure 3-8, NE stimulated GnRH release from proestrous BH explants *in vitro*. The effect of NE on GnRH did not dramatically increase as the dose rose from $10^{-8}$M to $10^{-6}$M, indicating the system was near saturation. The ability of NE to stimulate GnRH release was blocked by the alpha-1 adrenergic receptor antagonist, prazosin (Figure 3-9, $P<0.001$), but not the alpha-2 adrenergic receptor antagonist, yohimbine, (Figure 3-10). Administration of an OT- receptor antagonist attenuated the NE- induced release of GnRH from proestrous BH explants such that NE- induced stimulation of GnRH release was no longer significant in the presence of an OTA (Figure 3-11).

These results confirm that NE stimulates the release of GnRH from proestrous BH. That this effect was blocked by alpha-1, but not alpha-2 adrenergic receptor antagonism indicates NE is working through alpha-1 adrenergic receptors to stimulate GnRH secretion from proestrous BH explants. That OT- receptor antagonism also the inhibited NE- induced GnRH stimulation indicates that NE may, in part, be working...
through activation of the OT neurotransmitter system to stimulate GnRH release from proestrous BH explants.
Figure 3-8. Dose-response curve for NE-stimulation of GnRH release from proestrous BH

In vitro release of GnRH from basal hypothalamus (BH) explants obtained at 1500hrs from normally cycling proestrous female Sprague-Dawley rats. GnRH secretion is in response to buffer alone (control), or buffer with norepinephrine (NE, $10^{-6}$M to $10^{-10}$M). Column heights represent mean GnRH (ng/mg protein/ml/hr) released, and vertical lines above columns denote the S.E.M. N=8-10 animals for each treatment. *, significantly different from basal control values ($P<0.01$).
**Figure 3-9.** Effect of alpha-1 adrenergic antagonism on NE-induced GnRH release from proestrous BH

*In vitro* release of GnRH from basal hypothalamus (BH) explants obtained at 1500hrs from normally cycling proestrous female Sprague-Dawley rats. GnRH release is in response to buffer alone (control), or buffer containing norepinephrine (NE, $10^{-8}$M), and/or the alpha-1 adrenergic receptor antagonist prazosin, (PRZ, $10^{-6}$M). Column heights represent mean GnRH (ng/mg protein/ml/hr) released, and vertical lines above columns denote the S.E.M. N=8 animals for all treatments. *, significantly different from basal control values (P<0.001).
**Figure 3-10.** Effect of alpha-2 adrenergic antagonism on NE- induced GnRH release from proestrous BH

*In vitro* release of GnRH from basal hypothalamus (BH) explants obtained at 1500hrs from normally cycling proestrous female Sprague-Dawley rats. GnRH release is in response to buffer alone (control), or buffer containing norepinephrine (NE, $10^{-8}$M), and/or the alpha-2 adrenergic receptor antagonist yohimbine (YO, $10^{-6}$M). Column heights represent mean GnRH (ng/mg protein/ml/hr) released, and vertical lines above columns denote the S.E.M. N=9 animals for all treatments, *, significantly different from basal control values (P<0.01).
**Figure 3-11.** Effect of OT-receptor antagonism on NE-induced GnRH release from proestrous BH

*In vitro* release of GnRH from basal hypothalamus (BH) explants obtained at 1500hrs from normally cycling proestrous female Sprague-Dawley rats. GnRH release is in response to buffer alone (control), or buffer containing norepinephrine (NE, $10^{-8}$M), and/or an OT receptor antagonist (OTA $10^{-8}$M). Column heights represent mean GnRH (ng/mg protein/ml/hr) released, and vertical lines above columns denote the S.E.M. N=5 animals for all treatments, *, significantly different from basal control values (P<0.05).
**Interactions Between NE and OT in Stimulating GnRH Release In Vitro**

Recent literature suggests that there is an interaction between NE and OT on the stimulation of GnRH release from male BH explants (Rettori et al. 1997). In order to test whether such an interaction occurs in a female model, the ability of combined OT/NE to stimulate GnRH release from proestrous BH explants was tested. Also examined was the ability of several pharmacological agents to block the combined NE/OT stimulation of GnRH from proestrous BH explants. These agents were the alpha-1 adrenergic antagonist, prazosin; the alpha-2 adrenergic antagonist, yohimbine; the NO synthesis inhibitor, LNAME; and the OT receptor antagonist [d(CH₂)₅, Tyr (Me)², ORN⁸] vasotocin (OTA).

As depicted in Figure 3-12, both NE and OT, on their own, were able to significantly stimulate GnRH release from proestrous BH explants above basal control levels (P<0.01 for NE, P<0.001 for OT). When used in combination, NE/OT stimulated GnRH to a greater extent than either alone, and the increase in GnRH released when the two neurotransmitters were used together was synergistic rather than additive. Overall, combined NE/OT stimulated GnRH to 12.94 ± 3.2 ng/mg protein/ml/hour above basal control levels, whereas the sum of the stimulation of GnRH above basal control levels by each neurotransmitter alone was 6.36 ± 1.39 ng/mg protein/ml/hour. NOS inhibition blocked this synergistic effect (Figure 3-13, P<0.0001), as did OT receptor antagonism (Figure 3-14, P<0.01). These results indicate that the combination of NE/OT works to stimulate GnRH from proestrous BH explants by utilizing NO, and that this is driven, at least in part, by an OT receptor mediated mechanism.
Administration of the alpha-1 adrenergic antagonist, prazosin, reduced the level of stimulation of GnRH by NE/OT 3.74 ng/mg protein/ml/hour, which is slightly greater than the stimulation of GnRH by NE alone above basal control values (3.11 ng/mg protein/ml/hour). The level of stimulation of GnRH by NE/OT in the presence of PRZ was not significantly different than that by OT alone, and GnRH stimulation by NE/OT above OT alone was reduced 72% by PRZ (Figure 3-15). This result corroborates previous findings indicating NE stimulation of GnRH is at least in part due to alpha-1 adrenergic receptor activation. Alpha-2 adrenergic antagonism by administration of yohimbine did not affect the ability of NE/OT to stimulate GnRH release from proestrous BH explants (Figure 3-16).

These results indicate that NE and OT act in a synergistic manner to stimulate the release of GnRH by an OT receptor mediated mechanism, which appears to involve NO. That alpha-1 adrenergic receptor antagonism by prazosin attenuated the NE/OT stimulation of GnRH to the level of OT alone and alpha-2 adrenergic antagonism by yohimbine had no affect on the NE/OT induced GnRH stimulation indicates NE is working at least in part through an alpha-1 adrenergic receptor mediated mechanism to stimulate proestrous GnRH in vitro, and that this mechanism is not directly involved in the OT-mediated stimulation of GnRH from proestrous BH explants.
Figure 3-12. Effect of OT and/or NE on GnRH stimulation from proestrous BH

In vitro release of GnRH from basal hypothalamus (BH) explants obtained at 1500hrs from normally cycling proestrous female Sprague-Dawley rats. GnRH release is in response to buffer alone (control), or buffer containing norepinephrine (NE, $10^{-8}$M), and/or oxytocin (OT, $10^{-11}$M). Column heights represent mean GnRH (ng/mg protein/ml/hr) released, and vertical lines above the columns denote the S.E.M. N=8 animals for all treatments. *, significantly different from all other treatment values ($P<0.001$).
**Figure 3-13.** Effect of NOS antagonism on NE/OT- induced GnRH release from proestrous BH

*In vitro* release of GnRH from basal hypothalamus (BH) explants obtained at 1500hrs from normally cycling proestrous female Sprague-Dawley rats. GnRH release is in response to buffer alone (control), or buffer containing norepinephrine (NE, $10^{-8}$M), oxytocin (OT, $10^{-11}$M), and/or the nitric oxide synthesis inhibitor LNAME ($10^{-11}$M). Column heights represent mean GnRH (ng/mg protein/ml/hr) released, and vertical lines above columns denote the S.E.M. N=8 for all treatments, *, significantly different from all other treatment values (P<0.001).
Figure 3-14. Effect of administration of an OT receptor antagonist on NE/OT stimulation of GnRH from proestrous BH

In vitro release of GnRH from basal hypothalamus (BH) explants obtained at 1500hrs from normally cycling proestrous female Sprague-Dawley rats. GnRH release is in response to buffer alone (control), or buffer containing norepinephrine (NE, $10^{-8}$M), oxytocin (OT, $10^{-11}$M), and/or an oxytocin receptor antagonist (OTA, $10^{-6}$M). Column heights represent mean GnRH (ng/mg protein/ml/hr) released, and vertical lines above columns denote the S.E.M. N=6-8 animals for all treatments, *, significantly different from all other treatment values (P<0.001).
**Figure 3-15.** Effect of alpha-1 adrenergic antagonism on the stimulation of GnRH from proestrous BH by NE and/or OT

*In vitro* release of GnRH from basal hypothalamus (BH) explants obtained at 1500hrs from normally cycling proestrous female Sprague-Dawley rats. GnRH release is in response to buffer alone (control), or buffer containing norepinephrine (NE, $10^{-8}$M), oxytocin (OT, $10^{-11}$M), and/or the alpha-1 adrenergic receptor antagonist prazosin, (PRZ, $10^{-6}$M). Column heights represent mean GnRH (ng/mg protein/ml/hr) released, and vertical lines above columns denote the S.E.M. N=8 animals for all treatments. *, significantly different from basal control values ($P<0.01$).
In vitro release of GnRH from basal hypothalamus (BH) explants obtained at 1500hrs from normally cycling proestrous female Sprague-Dawley rats. GnRH release is in response to buffer alone (control), or buffer containing norepinephrine (NE, $10^{-8}$M), oxytocin (OT, $10^{-11}$M), and/or the alpha-2 adrenergic receptor antagonist yohimbine (YO, $10^{-6}$M). Column heights represent mean GnRH (ng/mg protein/ml/hr) released, and vertical lines above columns denote the S.E.M. N=9 animals for all treatments. *, significantly different from basal control values ($P<0.05$).
OT and NE Stimulation of Nitric Oxide: Mechanisms of Action and Similarities to GnRH Stimulation

NO has been implicated in the control of GnRH secretion in males following its stimulation by NE. In order to determine whether NO synthesis and secretion was stimulated from the same proestrous BH explants as were used in the GnRH studies presented here, nitrate/nitrite levels were measured using a modified Greiss reaction. GnRH secretion levels were also measured in these experiments. In addition to basal secretion levels, stimulated levels of NO and GnRH secretion were determined following treatment with NE and/or OT. The ability of an OT receptor antagonist to inhibit OT/NE-induced secretion of NO from proestrous BH explants was also tested.

In combination, NE/OT stimulated NO release more than either neurotransmitter by itself, and stimulated GnRH in a synergistic manner (Figure 3-17). OT receptor antagonism attenuated both GnRH and NO release in response to combined NE/OT (Figure 3-18). Figure 3-19 shows a comparison of the effect of NE, OT, and NE/OT on both GnRH and NO in the same animals. These results support the hypothesis that NE/OT utilizes NO and an OT receptor-mediated mechanism in order to stimulate GnRH release on the afternoon of proestrus.
**Figure 3-17.** Effect of NE and/or OT on NO synthesis in proestrous BH

*In vitro* release of nitric oxide (NO), as measured by percent change in nitrite/nitrate levels versus basal (control), from proestrous basal hypothalamus (BH) explants obtained at 1500hrs from normally cycling female Sprague-Dawley rats. NO release is in response to buffer alone (control), or buffer containing norepinephrine (NE, $10^{-8}$M), and/or oxytocin (OT, $10^{-11}$M). Column heights represent mean percent change from basal secretion levels (1.57 ug/ml ± 0.41), and vertical lines above columns indicate the S.E.M. N=6-8 animals for all treatments, ***, significantly different from basal control (P<0.01) using raw data.
Figure 3-18. Effect of addition of an OT-receptor antagonist on stimulation of NO synthesis by NE, and/or OT from proestrous BH

In vitro release of nitric oxide (NO), as measured by percent change in nitrite/nitrate levels vs basal (control), from proestrous basal hypothalamus (BH) explants obtained at 1500hrs from normally cycling female Sprague-Dawley rats. NO release is in response to buffer alone (control), or buffer containing norepinephrine (NE, $10^{-8}$M), oxytocin (OT, $10^{-11}$M), and/or an oxytocin receptor antagonist (OTA, $10^{-6}$M). Column heights represent mean percent change from basal secretion levels ($1.6 \pm 0.41$ ug/ml), and vertical lines above columns indicate the S.E.M. N=6-8 animals for all treatments, **, significant compared to basal (P<0.01) using raw data.
Figure 3-19. Comparison of NO and GnRH Stimulation in Proestrous BH

Comparison of *in vitro* stimulation of GnRH and nitrate/nitrite (NO) synthesis from proestrous basal hypothalamus (BH) harvested at 1500hrs from normally cycling female Sprague-Dawley rats.
Effect Of OT, NE, and NE/OT On GnRH and NO Release From Diestrous and Estrous BH Explants

Whether there is a difference between the ability of OT and/or NE to stimulate GnRH and NO release from BH explants harvested on the afternoon estrus and diestrus versus proestrus was tested. As shown in Figure 3-20, OT and/or NE, stimulated GnRH release from diestrous and estrous BH in vitro, but in a different manner than seen on proestrus. On estrus (Figure 3-20A), NE and OT stimulated GnRH at levels near those seen on proestrous, but the combination of NE/OT stimulated GnRH significantly less than seen on proestrus. On diestrous, NE stimulated GnRH more than on either proestrus or estrus. OT stimulated GnRH on diestrous, but less than on proestrus or estrus. The combination of NE/OT stimulated GnRH on diestrous to about the same level as NE did, and to a significantly lesser degree than on proestrus. Thus, though NE and OT are capable of stimulating GnRH release on diestrous, estrus, or proestrus, the magnitude of these responses is altered the and synergism seen on proestrus is absent during other phases of the estrous cycle.

Figure 3-21 depicts OT-, NE-, and OT/NE- induced stimulation of NO on estrus or diestrous versus proestrus. NE stimulated NO synthesis significantly more on estrus than on diestrous or proestrus. OT had no effect on NO stimulation on estrus, and NE/OT combined stimulated NO to about the same level as NE. On diestrous, neither NE, OT, nor a combination of the two significantly stimulated NO synthesis. Table 3-1 summarizes the data for both GnRH and NO stimulation by NE and/or OT on proestrus, estrus, and diestrus.
These results indicate that NE, OT, and their combination differ in their ability to stimulate GnRH release and NO synthesis on each day of the estrous cycle. The most obvious differences are that the combination of NE/OT stimulates much more GnRH release and NO synthesis on proestrus than diestrus or estrus, that NE stimulates GnRH release more on diestrus than on any other day of the cycle, and that OT is capable of stimulating GnRH release from the BH to some extent on all days of the cycle. Although OT’s greatest influence, and its synergism with NE, is seen only on the afternoon of proestrus, the ability of OT and/or NE to stimulate NO and GnRH release appears to be modulated by other factors, which may include plasma estradiol and progesterone levels.
Figure 3-20. Comparison of the ability of oxytocin (OT, $10^{-11}$M), norepinephrine (NE, $10^{-8}$M), and OT/NE combined to stimulate GnRH from estrous, diestrous, and proestrous basal hypothalamus (BH) explants harvested at 1500hrs from normally cycling Sprague-Dawley rats. Column heights represent mean GnRH, bars above columns represents the SEM. N=6-8 animals for each treatment group. *, significantly different all other treatment groups.
A. Estrous NO Synthesis

B. Diestrous NO Synthesis

C. Proestrous NO synthesis

Figure 3-21. A comparison of the stimulation of nitrate/nitrite (NO) synthesis and release from BH explants by oxytocin (OT, 10^{-11}M) and/or norepinephrine (NE, 10^{-8}M), on the afternoons of estrus, diestrus, or proestrus.
Table 3-1. GnRH and NO stimulation from BH explants by NE and/or OT on the afternoons of proestrus, estrus, or diestrus.

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<th>Proestrus</th>
<th>Estrus</th>
<th>Diestru s</th>
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<tr>
<td><strong>GnRH (ng/mg protein/ml/hour)</strong></td>
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<tr>
<td>Control</td>
<td>1.90 ± 0.47</td>
<td>1.76 ± 0.36</td>
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<td>4.24 ± 0.68</td>
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<tr>
<td>OT</td>
<td>5.92 ± 0.71</td>
<td>5.58 ± 0.39</td>
<td>5.29 ± 1.45</td>
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<tr>
<td>NE/OT</td>
<td>14.84 ± 3.20</td>
<td>7.42 ± 2.14</td>
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</tbody>
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<th>Proestrus</th>
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<tr>
<td><strong>Nitrites/Nitrates (ug/ml)</strong></td>
<td></td>
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<tr>
<td>Control</td>
<td>1.57 ± 0.41</td>
<td>1.7 ± 0.52</td>
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<tr>
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<tr>
<td>NE/OT</td>
<td>3.65 ± 0.98</td>
<td>2.2 ± 0.21</td>
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Chapter 4: Discussion

Although many important factors involved in the generation of the proestrous GnRH surge and ovulation have been elucidated, the exact neural mechanisms controlling this phenomenon remain undetermined. For instance, it has been known for some time that in mammals, the ovaries communicate the readiness of the peripheral reproductive system for ovulation to the brain through the secretion of estradiol and progesterone. The CNS then responds to this hormonal information by releasing a large pulse of GnRH on the afternoon of proestrus. This in turn causes LH to be secreted from the pituitary into the peripheral circulation, ultimately resulting in ovulation at the ovaries. However, it has proven difficult to localize estradiol and progesterone receptors on GnRH neurons, indicating that there is involvement of other neural systems in “sensing” the hormonal messages from the ovaries. Indeed, many neurotransmitter systems have been implicated in the CNS control of female fertility cycles (see Table 1-1). However, no single neurotransmitter system, or combination of transmitter systems, has been shown to entirely account for the initiation and propagation of the large preovulatory, proestrous GnRH surge. Thus, despite decades of effort by researchers and clinicians, the CNS mechanisms involved in female reproductive cycles remain unelucidated.

As outlined in the introduction section, the research for this dissertation focused primarily on three neurotransmitter systems that have been previously implicated in stimulating the preovulatory GnRH surge: NE, OT, and NO. The results of these studies
are discussed below, and a model of the mechanisms of interaction between the three systems is proposed, along with ideas for future work in this field.

**Stimulation Of Proestrous GnRH and NO Release**

Previous research indicated a role for central OT in the preovulatory, proestrous LH surge (Johnston et al. 1992). However, whether this effect was due to stimulation of the hypothalamic hormone responsible for stimulating LH release, GnRH, or because of some other, non-physiological effect central OT might have on other neuroendocrine regulators of LH secretion was unknown. The present results demonstrate that OT stimulated GnRH release from proestrous BH explants, but not ME fragments, where GnRH nerve terminals reside. This stimulation occurred at concentrations as low as $10^{-13}$ M, and appeared to peak by $10^{-9}$ M. Thus, the data suggest that the neuroanatomical and neurochemical connectivity present within the intact BH, which includes the ME (where GnRH nerve terminals reside), are both necessary and sufficient for very low doses of OT to stimulate GnRH release on the afternoon of proestrus.

These initial findings lead to a variety of mechanistic hypotheses concerning OT's stimulatory influence on proestrous GnRH release. One possibility is that OT may directly stimulate GnRH release from proestrous BH by stimulating OT receptors located somewhere on the GnRH neuron, itself. However, OT- receptors have not been clearly demonstrated on GnRH neurons, leaving the likelihood that the stimulatory effect of OT on proestrous GnRH release is mediated through some other neural mechanism. As the BH contains a very large number of neurotransmitter phenotypes, there are a variety of
possible intermediaries through which OT may work to stimulate proestrous GnRH release. The most obvious candidates include other central neurotransmitters implicated in the stimulation of proestrous GnRH release, especially NE, which has been shown to be involved in both the tonic and pulsatile regulation of GnRH secretion (Legan and Callahan 1999; Gallo 1980). Further, NE and OT have been demonstrated to interact in the stimulation of GnRH release in males (Rettori et al. 1977), so the possibility that they also do so in females should be considered. Other possible neurotransmitters OT might work through to stimulate GnRH from proestrous BH explants include glutamate, which has been shown to stimulate GnRH release from the ME (Mahesh and Brand 1998), and NO, which also has been shown to be involved in the stimulation of proestrous GnRH release (Bonavera, Kalra, and Kalra 1996), as well as OT- mediated GnRH release from male MBH tissues (Rettori et al. 1997).

The hypotheses outlined above were addressed in the present research. In order to confirm that the exogenously applied OT was working through OT receptors and not related receptors such as those for AVP, the ability of a specific OTA to block the OT-induced stimulation of GnRH from proestrous BH explants was examined. OTA administration completely blocked the OT- induced stimulation of GnRH release from proestrous BH explants. These results agree with in vivo data demonstrating a selective ability of OT antagonists, but not AVP antagonists to block the proestrous LH surge (Johnston and Negro-Vilar 1988). Thus, OT appears to stimulate proestrous GnRH and subsequent LH release through an OT- receptor mediated mechanism.

The ability of NOS inhibition to block the OT- induced stimulation of proestrous GnRH was also investigated. Administration of the NOS inhibitor, LNAME, blocked the
OT- induced stimulation of proestrous GnRH release from BH explants. This result was somewhat expected, as OT has been shown to stimulate GnRH release through an NO mediated mechanism in males (McCann et al. 1996). Further, NO-ergic neurons have been observed near GnRH neurons in the BH (Herbison et al., 1997), and NO has been implicated in the release of GnRH at the level of the SON and the PVN, where OT neuron cell bodies are found (Grossman et al. 1994), as well as at the level of the ME (Rettori et al. 1997).

Whether OT stimulates proestrous GnRH release by activation of the noradrenergic system was examined by testing the ability of selective pharmacological antagonists of alpha- adrenergic receptors to block OT- induced GnRH release from proestrous BH explants. The results demonstrated that selective antagonists of alpha-1 and alpha-2 adrenergic receptors (PRZ and YO) failed to block the OT- induced stimulation of GnRH release from proestrous BH, indicating that if there is any interplay between NE and OT on GnRH release, it is not due to OT stimulating NE neurons to release NE and activate alpha- adrenergic receptors. The ability of OT to stimulate GnRH release in the presence of a glutamate antagonist, NBQX (a specific kainate receptor antagonist), was also tested. Kainate receptor antagonism did not block the OT- induced GnRH stimulation from proestrous BH explants. This indicates that kainate receptors also do not mediate OT's stimulatory effect on proestrous GnRH release.

In summary, the data from these initial OT studies support the hypothesis that OT stimulates GnRH release from the BH on the afternoon of proestrus. This event required the connectivity found within the entire BH, which contains both GnRH terminals and cell bodies, and did not occur at the level of the ME, where only terminals of GnRH
neurons reside. Further, the OT-induced release of GnRH was mediated through an OT receptor mechanism, as well as through a NOS-mediated mechanism. Kainate, alpha-1 and alpha-2 adrenergic receptor antagonism did not affect OT-induced stimulation of proestrous GnRH release, indicating that OT does not induce GnRH release by stimulating glutamate or NE release, and subsequent activation of kainatae or alpha-adrenergic receptors.

Having determined that OT stimulates GnRH release from BH explants harvested on the afternoon of proestrus, studies to determine the ability of NE to stimulate GnRH release from proestrous BH explants, and the mechanisms involved in this effect, were performed. NE (10^-6 M to 10^-10 M) stimulated GnRH release from proestrous BH explants in vitro in a manner similar to that seen by Nowack and Swerdloff (1985), who found that NE stimulated GnRH release effectively in doses higher than 10^-10 M. The data here also confirm that increasing doses of NE beyond 10^-8 M only modestly increase the release of GnRH from proestrous BH explants, indicating the system is nearly saturated by NE levels in this range.

Whether NE-induced stimulation of proestrous GnRH release was mediated by alpha-adrenergic receptors, or by downstream stimulation of OT-receptor mediated mechanisms was tested. Alpha-1 adrenergic receptor antagonism by PRZ blocked the ability of NE to stimulate GnRH inform proestrous BH explants. Alpha-2 adrenergic receptor blockade by YO did not influence the NE-induced increase in proestrous GnRH release. Finally, OT receptor antagonism attenuated the ability of NE to stimulate GnRH release from proestrous BH explants. These results confirm the observation by others that NE stimulates GnRH release on the afternoon of proestrus, and that this phenomenon is
mediated through alpha-1, and not alpha-2, adrenergic receptors (Lee et al, 2000). Whether this is a direct effect of NE on GnRH neurons was not proven by these studies. Although NE projections have been shown in close proximity to GnRH neurons (Gallo 1980), alpha-1 adrenergic receptors have not yet been conclusively shown on GnRH neurons. Thus, it would appear that NE, like OT, might be working through some downstream mechanism to stimulate GnRH release from the BH on proestrus. The data here suggest this mechanism could utilize the OT neurotransmitter system, as OT-receptor antagonism attenuated the ability of NE to stimulate GnRH release from proestrous BH explants. This hypothesized NE-induced OT release and subsequent OT receptor activation could occur at the level of the PVN or SON, where NE projections are sent and OT cell bodies are found (Daftary et al. 1998; Sawyer and Clifton 1980). The result that OT receptor antagonism attenuated but did not entirely block NE-induced GnRH release was not surprising, as NE has been shown to directly stimulate GnRH release from ME explants in males in vitro (Contijoch, Johnson and Advis 1990), and the ME is target of an adrenergic projection originating in the locus ceruleus (Adler et al. 1983).

Because recent literature has indicated that there might be interactions between the NE- and OT-ergic systems that could play an important role in the generation of the preovulatory, proestrous GnRH surge, the interplay between these two neurotransmitter systems in the stimulation of GnRH from proestrous BH explants was examined. In combination, NE/OT stimulated GnRH release from proestrous BH explants synergistically rather than additively. This represents the first demonstration of this synergistic effect between OT and NE on GnRH release.
The mechanisms of this synergism were investigated. Alpha-1 adrenergic receptor antagonism by PRZ reduced NE/OT- induced proestrous GnRH release to a level similar to that seen with OT alone. Alpha-2 adrenergic receptor antagonism by YO did not affect NE/OT- stimulation of GnRH release from proestrous BH. Administration of a selective OTA blocked proestrous GnRH stimulation by NE/OT. Finally, the NE/OT- induced release of GnRH from proestrous BH explants also was blocked by inhibition of NOS via administration of the NOS antagonist, LNAME.

Since the synergistic effect of NE/OT on stimulation of GnRH release from proestrous BH explants was blocked by both an OT receptor antagonist, and a NOS inhibitor, the most important conclusion to be drawn from the present data is that NE and OT work through alpha-1 adrenergic and OT- receptor mediated mechanisms, respectively, as well as through a NOS mediated mechanism, in order to synergistically stimulate proestrous GnRH release. Taking into account the effect of an OTA on NE-induced stimulation of GnRH release from proestrous BH, it seems that part of this synergism might be due to NE facilitating OT release, and subsequent stimulation of OT receptors in the BH in order to stimulate GnRH release. Whether this is a direct effect of NE on OT neurons, or if another mediating factor is involved cannot be concluded from the present data. However, the data does confirm the hypothesis that at least part of the synergism by OT and NE in stimulating GnRH release is through an alpha-1 adrenergic receptor mediated mechanism, and not an alpha-2 adrenergic receptor mediated mechanism.

That the doses of NE and OT which synergistically stimulated GnRH release from proestrous BH explants were near the maximal effective dose for each transmitter alone
(as determined by the dose-response curves) suggests that another neural intermediary must be involved in the effect. That NO might be this intermediary responsible for the synergistic effect is not surprising. Both NE and OT have been implicated in hypothalamic NO stimulation (Rettori et al. 1997, Canteros et al. 1996), and NO has been shown to be involved in the preovulatory, proestrous GnRH surge (McCaan et al. 1999). NO is a diffusible gas neurotransmitter with a potentially wide target area. This fact, when viewed in combination with the sparse numbers and wide distribution of GnRH neurons, gives rise to the intriguing possibility that NO may be the chief signaling agent for the stimulation of proestrous GnRH release. Further, NOS activity is stimulated by both NE and OT, and has been linked to GnRH stimulation on proestrus (McCann et al. 1998). A plausible hypothesis supported by the current data is that following stimulation of NOS by NE and OT, the resultant NO diffuses and recruits the widely scattered, and few in number, GnRH neurons to release GnRH in a coordinated fashion, ultimately causing in the large preovulatory GnRH surge which results in the LH surge late in proestrus, causing ovulation. Previous studies which have concentrated on single neurotransmitter systems, including NE or OT, have been unable to reproduce the magnitude of GnRH or LH surges observed on proestrus in the normally cycling female rat. The present results suggest that this may have been the result of missing the synergism demonstrated here for OT/NE-induced stimulation of GnRH release.

To summarize the data regarding proestrous GnRH release, OT and NE, alone, stimulate proestrous GnRH from proestrous BH explants. When used in combination, this stimulation of GnRH from proestrous BH explants is synergistic. This effect is blocked by OT receptor antagonism, as well as by inhibition of NOS. Alpha-1 adrenergic
receptor antagonism decreased the NE/OT- induced stimulation of GnRH somewhat, indicating a removal of the NE contribution in stimulating GnRH, whereas alpha-2 adrenergic receptor antagonism had no effect. NE and OT synergistically stimulate GnRH via a NO mediated mechanism, which is activated in a synergistic manner by an alpha- adrenergic and OT receptor mediated mechanism, respectively.

The ability of NE and OT to stimulate NO from the same BH tissues used in GnRH experiments was tested. Both NE and OT also stimulated NO from proestrous BH explants beyond basal control levels in vitro. In combination, they stimulated NO in a manner which appeared additive. Stimulation of NO by NE/OT was attenuated by addition of an OT receptor antagonist. Due to limitation of the assay used, it was not possible to accurately test whether NOS inhibition by LNAME blocked the effect of NE/OT on NO stimulation, nor whether NE alpha- adrenergic antagonism blocked the effect. Unfortunately, the assay records these compounds as false positives.

The stimulation of NO synthesis and release in response to OT and/or NE reported here could occur in a variety of hypothalamic areas, including the ME, where there is a rich plexus of NOS-containing neurons (Canteros et al 1996), in other hypothalamic areas where NOS- positive and GnRH neurons are found, or in OT neurons and NE projections. The two main areas where this overlap occurs are the PVN and SON (Hatakeyama et al. 1996). Future studies will be designed to determine where this NOS activation occurs in response to NE and/or OT, and on what neuronal processes these effects are mediated.
Stimulation of GnRH and NO on Estrus and Diestrus

The ability of OT and/or NE to stimulate GnRH and NO synthesis and release from BH explants harvested on the afternoon of estrus and diestrous was also examined. On estrus, OT or NE alone stimulated GnRH release in a manner similar to that seen on proestrus. However, OT/NE- induced stimulation of GnRH release from estrous BH was additive, and the level of GnRH released was only about half of that seen on proestrus. Estrous NO secretion was stimulated somewhat by NE, but not OT. NE/OT- induced NO synthesis was equal to that by NE alone, indicating that OT does not stimulate NOS-containing neurons to produce NO on estrus.

These results were not surprising, as estradiol and progesterone levels remain somewhat elevated throughout the earlier part of estrus, and this hormonal input could stimulate the NE and OT systems to be partially primed for inducing GnRH release. That the synergistic effect of NE/OT on GnRH release from BH explants was not seen on estrus suggests that in order to stimulate GnRH release, all three neurotransmitter systems need to be primed for this event, as they are only on the afternoon of proestrus. That OT did not stimulate NO from BH explants harvested on estrus, and did not contribute to the stimulation of NO when used in combination with NE, suggests that the key to the synergism between NE and OT on stimulation of proestrous GnRH release lies in their ability to jointly stimulate proestrous NO secretion. This hypothesis is supported by the results from diestrous BH explants as well (below).

On diestrous, NE- induced stimulation of GnRH release that was, on average, greater than on estrus or proestrus. The reason for this probably lies in the fact that NE is
involved in the tonic regulation of GnRH secretion, and thus the noradrenergic receptor system remains primed to stimulate GnRH release at any time during the estrous cycle if enough NE is available, as it is when the neurotransmitter is exogenously applied as in these experiments. OT also stimulated some GnRH release on diestrus, but not as significantly above basal levels as it did on estrus or proestrus. In combination, NE/OT stimulated GnRH release from diestrous BH explants to an even lesser extent than NE alone (although it should be noted that there was a large amount of variability in levels of NE- induced GnRH release on diestrus), indicating again that a central component involved in the stimulation of GnRH release on proestrus is not at work on other days of the cycle. This component appears to be NO, as NE and/or OT did not significantly stimulate NO release from diestrous BH explants. This finding provides further proof that the key to the synergism between NE and OT in stimulating GnRH release from proestrous BH explants is the stimulation of NO.

**Model of Mechanisms of Action of NE and OT on the Stimulation of GnRH**

Since receptors for neither OT nor NE have been conclusively localized directly on GnRH neurons, the research presented here examined the intermediary mechanisms by which NE and/or OT stimulate GnRH release from BH explants harvested on the afternoon of proestrous. The present data indicate that NE- and OT- stimulation of proestrous GnRH is at least in part due to stimulation of NO. Further, NE alpha-1 adrenergic receptor activation was shown to be integral for the NE- induced stimulation
of GnRH. OT receptor activation is also necessary for both NE- and OT- induced stimulation of GnRH release from proestrous BH explants.

A possible model of the mechanism(s) by which NE and OT synergistically stimulate GnRH release has been developed based upon both the present results and previous studies, and is presented in Figure 4-1. In this model, both NE and OT influence proestrous GnRH release by directly stimulating NO- ergic neurons through activation of alpha- adrenergic and OT receptors, respectively. This, in turn, causes the release NO. This NO then diffuses throughout the BH and reaches GnRH neuron processes. Following activation of guanylyl cyclase and the subsequent formation of cyclic GMP, GnRH neurons in the BH are stimulated to release their contents in a coordinated, synchronized fashion from the ME. Other contingencies touched upon in the model include a noradrenergic stimulation of OT neurotransmission on the afternoon of proestrous, when endogenous hypothalamic OT levels are at their peak (Sarkar, Frautschy and Mitsugi 1992). This phenomenon would thereby increase the amount of OT- stimulated NO. OT levels are then postulated to be further increased by stimulation of a positive feedback loop, whereby OT stimulates it’s own release even further to cause an additional positive influence on the system at the proper time of the reproductive cycle. Evidence for such an OT- positive feedback system exists (Moos et al. 1998; Chevaleyre et al. 2000), but its relevance to proestrous GnRH release has not been examined.
**Future Directions**

It is said that valuable research creates more questions than it answers. There are several questions this research has created regarding the roles of NE, OT, and NO in the induction of the preovulatory, proestrous GnRH surge. Among these is whether OT and NE would have the same effect on GnRH and LH release *in vivo* as they do *in vitro*. Further, the data does not address, beyond eliminating the ME as the site of action of OT on GnRH release, the anatomical loci of the events described by the biochemical data presented here. Thus, to further examine the way these central neurotransmitters interact to affect GnRH release on the afternoon of proestrus, a number of different avenues of research could be pursued. These should include a variety of *in vivo* and *in vitro* anatomical and biochemical studies.

*In vivo* studies of interest would include determining whether the synergistic effect of OT/NE on GnRH release seen *in vitro* also caused a synergistic effect on LH release from live, normally cycling female rats. To perform these studies, animals would be fitted with ICV cannulae, and OT, NE, or a combination thereof injected into the third ventricle on the afternoon of proestrus, prior to the endogenous GnRH and LH surges. Subsequent LH release would then be measured from blood plasma taken during a time course following the injection. Further, the ability of centrally administered OTA and LNAME to attenuated the synergistic effect of OT and NE on LH release could be determined using a similar protocol. Such a study could help prove whether OT and NE exert a similar effect on reproductive-specific hormonal release *in vivo* as they do *in vitro*, with the endpoint to be measured being LH release rather than GnRH. Of course,
as with any study where agents with more than one potential set of targets are injected into the intact brain, other neural mechanisms that might indirectly, and non-physiologically effect, LH release might be stimulated by NE and/or OT, and these would have to be considered when analyzing the results.

In order to determine the neuroanatomical loci involved in the OT and/or NE effects on proestrous GnRH release, ICC studies could be performed to determine where in the BH NE and OT might stimulate GnRH neurons on the afternoon of proestrus, and what other neurotransmitters or relevant gene transcription products are associated with the phenomenon. Since GnRH synthesis and release in the female rat is linked to increased transcription of IEGs such as Fos and Jun (Fin, Steiner and Clifton, 1998), induction of these IEGs on proestrus by centrally administered NE and/or OT could be visualized using ICC techniques and used as markers of cell activity in order to determine whether the combination of NE/OT stimulate Fos and/or Jun in a manner similar to how they stimulate proestrous GnRH and NO synthesis and release. The results of these studies would tell both whether OT and NE synergistically stimulate Fos in the same fashion as GnRH, and, perhaps more importantly in an anatomical sense, where in the brain this stimulation occurs. If significantly increased levels of Fos induction were found, the ability of NOS inhibition, alpha-1 adrenergic and/or OT receptor antagonism on its induction could then be ascertained to discover whether there is an easily visualized anatomical basis for the biochemical results presented here.

Several other anatomical localization studies could also be performed in order to further examine the roles of OT, NE, and NO in the generation of the preovulatory GnRH surge. For instance, co-localization studies could be performed in order to ascertain...
whether alpha-1 adrenergic receptors, and/or OT receptors are found on neurons expressing NOS in hypothalamic areas known to contain GnRH neurons, as well as examining where in the BH the increases in NO production occur in response to NE and/or OT. Studies to determine whether neurons that demonstrated a 15-fold increase in OT-receptor message expression on the afternoon of proestrus (Bale et al. 1996) also express GnRH, OT or NE neurons could also be performed. Also of interest would be whether alpha adrenergic receptors are found on OT-ergic neurons, since the data presented here suggests that part of NE effect on proestrous GnRH release is mediated via NE stimulation of OT, which in turn stimulates the OT receptor. Another important question to be addressed is which of the subtypes of estrogen receptors, estrogen alpha or estrogen beta (Laflamme et al. 1998), mediate the changes seen in NE concentrations, as well as OT and OT-receptor synthesis, on proestrus in the BH. This could potentially help delineate how OT and NE neurons sense the hormonal messages sent by the ovaries that indicate time is right for ovulation.

Another question that could be addressed by future experimentation is whether NE/OT stimulation of proestrous GnRH release is accompanied by increases in 2nd messenger systems associated with NO and/or other agents implicated in the release of GnRH. For example, the question of whether cGMP levels are increased by treatment with NE/OT to proestrous BH tissues could be examined via biochemical assay. Also, whether inhibitors of the formation of cGMP can block the OT/NE effect on proestrous GnRH release could be examined. Finally, as GnRH synthesis and release is linked to increases in intracellular calcium levels following stimulation of the IP3/DAG system, the effect of inhibitors of this system on the NE and/or OT-induced stimulation of GnRH
from proestrous explants and the effect of OT/NE administration on its formation could be assessed.

Summary and Conclusions

In summary, the data demonstrated that OT (10⁻⁹M to 10⁻¹³M) stimulated GnRH release in vitro from BH, and not the ME explants, on the afternoon of proestrus in normally cycling female rats. This effect was mediated by an OT- receptor mediated mechanism, and likely used NO synthesis and release to affect GnRH release. The effect of OT on GnRH did not appear to be mediated by kainate or adrenergic receptor-mediated mechanisms. NE (10⁻⁶M to 10⁻¹⁰M) also stimulated the release of GnRH from proestrous BH explants, through an alpha-1 adrenergic receptor mediated mechanism. In combination, NE and OT stimulated GnRH release from proestrous BH explants in a synergistic manner. This synergism was mediated by an NO synthesis mechanism and involved the stimulation of both OT and alpha-1 adrenergic receptors. Both NE and OT also stimulated NO synthesis, as measured by nitrate/nitrite production, on the afternoon of proestrus in normally cycling female rats. Together, NE and OT stimulated proestrous NO release in an additive manner. This stimulation was mediated, at least in part, through an OT- receptor mediated mechanism.

In conclusion, the synergistic stimulation of GnRH release from proestrous BH explants by OT and NE was due to OT and alpha-1 adrenergic receptor stimulation, respectively, and the consequent production of NO. Together, these events may explain
the role that OT plays in the generation of the proestrous GnRH and LH surges that result in ovulation on the afternoon of proestrous in the cycling female rat.
Figure 4-1. Model of the events hypothesized to be involved in the generation of the proestrous GnRH surge. In the model, increasing estradiol and progesterone (E/P) levels being secreted from the ovaries are sensed by norepinephrine (NE) and oxytocin (OT) neurons in the brain. This results in increased secretion of OT and NE. NE secretion further increases the amount of OT produced, as well as stimulating gonadotropin-releasing hormone (GnRH) at the median eminence. Both NE and OT stimulate nitric oxide production, which further stimulates the release of GnRH and coordinates that secretion to produce the larve preovulatory surge of GnRH. This ultimately results in the release of the preovulatory surge of luteinizing hormone (LH) from the pituitary, causing ovulation.

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