Afferent regulation of A15 dopamine neurons in the ewe

Adrienne L. Bogusz

West Virginia University

Follow this and additional works at: https://researchrepository.wvu.edu/etd

Recommended Citation
https://researchrepository.wvu.edu/etd/2384

This Thesis is protected by copyright and/or related rights. It has been brought to you by the The Research Repository @ WVU with permission from the rights-holder(s). You are free to use this Thesis in any way that is permitted by the copyright and related rights legislation that applies to your use. For other uses you must obtain permission from the rights-holder(s) directly, unless additional rights are indicated by a Creative Commons license in the record and/or on the work itself. This Thesis has been accepted for inclusion in WVU Graduate Theses, Dissertations, and Problem Reports collection by an authorized administrator of The Research Repository @ WVU. For more information, please contact researchrepository@mail.wvu.edu.
Afferent Regulation of A15 Dopamine Neurons in the Ewe

Adrienne L. Bogusz

Thesis submitted to the
Davis College of Agriculture, Forestry and Consumer Sciences
at West Virginia University
in partial fulfillment of the requirements
for the degree of

Master of Science
in
Reproductive Physiology

Robert L. Goodman , Ph.D., Chair
Stanley M. Hileman, Ph.D.
Michael W. Vernon, Ph.D.

Department of Animal and Veterinary Sciences

Morgantown, West Virginia
2006

Keywords: Sheep, dopamine, LH, estradiol, GABA, seasonal reproduction, thyroid hormones
ABSTRACT

Afferent Regulation of A15 Dopamine Neurons in the Ewe

Adrienne L. Bogusz

Ovine reproduction is characterized by a breeding season and an anestrous season. Seasonal anestrus is induced by increased responsiveness of the GnRH neurosecretory system to estrogen (E) negative feedback. Previous studies have shown that inhibitory A15 dopamine neurons mediate this increased sensitivity to E, however, these neurons lack E receptors. In this thesis we performed three experiments. For experiment 1 we looked at the specific sites of actions that thyroid hormones act to cause neuronal plasticity of A15 DA neurons, utilizing dual-labeled immunocytochemistry. The results of this study demonstrated no change in dendrite morphology or the number of close contacts of A15 DA neurons. Because of the inconclusive data observed in Experiment 1, the final two experiments investigated the role of GABA as an estrogen-responsive afferent to A15 DA neurons. Studies have shown that GABA neurons express E receptors and local administration of a GABA\(_A\) receptor agonist in the A15 elevates LH levels in anestrous ewes. In experiment 2, we tested if this effect of the GABA\(_A\) agonist occurs directly on A15 neurons by determining whether A15 dopamine neurons express GABA\(_A\) receptors and if the expression of the GABA\(_A\) receptor increases in the breeding season, utilizing dual labeling immunocytochemistry. Approximately 30 percent of A15 DA neurons express GABA\(_A\) receptors, whereas 40 percent and 80 percent colocalization was seen in A14 DA neurons and A12 DA neurons respectively. There was no seasonal difference in receptor expression in any dopamine populations investigated. The third experiment investigated the whether administration of both GABA\(_A\) and GABA\(_B\) receptor antagonists into the A15 would cause a suppression in LH in OVX, anestrous season ewes and if administration of a dopamine receptor antagonist reverses this suppression. Administration of the antagonists locally into the A15 caused a significant decrease in LH pulse frequency and LH concentrations, however LH amplitude was unaffected. The dopamine receptor antagonist treatment caused a significant increase in mean LH concentrations. These data are consistent with a role for GABA in mediating estrogen negative feedback in anestrus.
ACKNOWLEDGMENTS

I would like to give my appreciation to all of the people who helped me during my time as a graduate student. First, and foremost, I would like to thank my advisor, Dr. Robert Goodman for his time, patience, teaching, and mentorship. Next, I want to thank the other members of my Master’s committee: Dr. Stanley Hileman and Dr. Michael Vernon for their valuable time and contributions. I am very grateful to Dr. Miroslav Valent, for none of the work would have been done without his dedication and help in the lab. I would like to thank other members of the lab for all of their help and support: Dr. Heather Billings for her help with the immunocytochemistry and intellectual input; Dr. Christina McManus for her unconditional support from the first day I stepped into the lab; Dr. Sushma Singh for help and friendship; and Mr. Casey Nestor and Ms. Britni Schoonover for their friendship and animal handling assistance. I would like to thank my close friends for their moral support, especially Mr. Jason Gentry. Finally, I would like to thank the members of my family for all of their unconditional love and support especially my mother, Ms. Laurie Bogusz, my father, Mr. Glenn Bogusz, and my grandmother and grandfather, Mr. and Mrs. Reid Koppler.
# TABLE OF CONTENTS

Title Page                  i
Abstract                    ii
Acknowledgments             iii
Table of Contents           vi
List of Figures             v

Chapter One: Literature Review  1
Chapter Two: Experiment 1     25
Chapter Three: Experiment 2   42
Chapter Four: Experiment 3    56
Chapter Five: Discussion      71
Chapter Six: Literature Cited 75
Curriculum Vita              86
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Representative image of a single Z-slice of A15 DA neurons</td>
<td>35</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Representative image of a single Z-slice of synapsin-positive staining in the RCH.</td>
<td>35</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Coexpression of A15 DA (TH-positive) neurons and synapsin-positive close contacts in the breeding season.</td>
<td>36</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Coexpression of A15 DA (TH-positive) neurons and synapsin-positive close contacts in the anestrus season.</td>
<td>36</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Representative image of TH staining for A15 DA neurons from the vmPOA treatment group.</td>
<td>37</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Neurolucida 3-D construction of an A15 DA neuron showing synapsin-positive close contacts.</td>
<td>37</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Number of primary dendrites on neurons.</td>
<td>38</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Mean surface area of dendrites.</td>
<td>38</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Mean length of dendrites on A15 DA neurons.</td>
<td>39</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Mean number of bifurcations per neuron.</td>
<td>39</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Cell body volume.</td>
<td>40</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Number of synapsin-positive close contacts on neurons.</td>
<td>40</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Mean number of synapsin-positive close contacts on dendrites.</td>
<td>41</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Mean number of synapsin-positive close contacts on cell bodies.</td>
<td>41</td>
</tr>
<tr>
<td>Figure 15</td>
<td>Coronal hypothalamic map depicting approximate location of the A15 dopaminergic population.</td>
<td>50</td>
</tr>
<tr>
<td>Figure 16</td>
<td>Coronal hypothalamic map depicting approximate location of the A14 dopaminergic population.</td>
<td>50</td>
</tr>
<tr>
<td>Figure 17</td>
<td>Coronal hypothalamic map depicting approximate location of the A12 dopaminergic population.</td>
<td>51</td>
</tr>
<tr>
<td>Figure 18</td>
<td>Representative images of a TH-ir neuron coexpressing GABA&lt;sub&gt;A&lt;/sub&gt; receptors in the A15.</td>
<td>51</td>
</tr>
<tr>
<td>Figure 19</td>
<td>Tracing of A15 TH-immunopositive cell bodies.</td>
<td>52</td>
</tr>
<tr>
<td>Figure 20</td>
<td>Percentage of A15, A14, and A12 TH-ir neurons coexpressing GABA&lt;sub&gt;A&lt;/sub&gt; receptors in breeding season and anestrous ewes.</td>
<td>52</td>
</tr>
<tr>
<td>Figure 21</td>
<td>Degrees of coexpression from the rostral through the caudal extent of A15 dopaminergic neurons.</td>
<td>53</td>
</tr>
<tr>
<td>Figure 22</td>
<td>Pixel count within the A15 and outside the A15 from the anestrus season and from the breeding season.</td>
<td>53</td>
</tr>
<tr>
<td>Figure 23</td>
<td>Dual immunofluorescence labeling of TH-ir neurons coexpressing GABA&lt;sub&gt;A&lt;/sub&gt; receptors in anestrous and breeding season.</td>
<td>54</td>
</tr>
<tr>
<td>Figure 24</td>
<td>Dual immunofluorescence labeling of TH-ir neurons coexpressing GABA&lt;sub&gt;A&lt;/sub&gt; receptors in anestrous and breeding season.</td>
<td>54</td>
</tr>
<tr>
<td>Figure 25</td>
<td>Pixel count of GABA&lt;sub&gt;A&lt;/sub&gt; receptor immunostaining in the A15 by season.</td>
<td>55</td>
</tr>
</tbody>
</table>
Figure 26. Comparison of pre- and post-treatment of mean LH in response to GABA antagonists in Trial 1.

Figure 27. Comparison of pre- and post-treatment of LH pulse frequency in response to GABA antagonists in Trial 1.

Figure 28. Comparison of pre and post-treatment of LH pulse frequency in response to GABA antagonists or vehicle and Sulpiride or vehicle in Trial 2.

Figure 29. Comparison of pre and post-treatment of mean LH in response to GABA antagonists or vehicle and Sulpiride or vehicle in Trial 2.

Figure 30. Comparison of pre and post-treatment of LH pulse amplitude in response to GABA antagonists or vehicle and Sulpiride or vehicle in Trial 2.

Figure 31. LH profile of ewes administered with GABA antagonists. Note that guide cannulae placement was rostral to the A15.

Figure 32. LH profile of ewes administered with GABA antagonists. Note that guide cannulae placement was caudal to the A15.

Figure 33. LH profile of ewes administered with GABA antagonists. Guide cannulae placement completely outside of A15.

Figure 34. LH profile of ewes administered with GABA antagonists. Note that guide cannulae placement was in the A15.

Figure 35. Serum LH profile from one representative ewe treated with GABA_A and GABA_B antagonists after sample 10 and sulpiride or vehicle after sample 26.
CHAPTER 1: Literature Review

In temperate zones, ovine reproduction is characterized by a breeding season in autumn and winter and a nonbreeding season during spring and summer. The mechanisms which control seasonal reproduction are currently being examined. This thesis will review literature about the control of the annual suppression of reproduction of the ewe, including a brief overview of the reproductive axis, the estrous cycle, tonic and surge secretion of GnRH/LH, and control of seasonal anestrus, specifically the role that thyroid hormones and GABA play in the seasonal changes in response to estrogen – negative feedback found in sheep.

Overview of Reproductive Axis

The primary neural regulator of reproduction in all mammals is the hypothalamic neuropeptide, gonadotropin-releasing hormone (GnRH). This decapeptide is released episodically into the hypophyseal-portal capillary system where it acts on the gonadotropes of the anterior pituitary gland to stimulate secretion of two gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). The gonadotropins then act directly on the ovary to promote gametogensis and steroidogenesis. Estrogen and progesterone, steroids produced by the ovary, then either negatively feedback or positively feedback on GnRH neurons to control the release of GnRH. Estrogen secreted in low levels and progesterone are responsible for negative feedback control of tonic GnRH and LH release, whereas estrogen secreted at high concentrations induces positive feedback responsible for the GnRH and LH surge required for ovulation. However, GnRH neurons are devoid of progesterone receptors and estrogen receptor alpha (Herbsion et al., 1993; Lehman and Karsch, 1993), but do contain estrogen receptor beta
(Skynner et al., 1999; Hrabovszky et al., 2000; Kallo et al., 2001). Although present, estrogen receptor beta does not appear to play a physiological role in estrogen-negative feedback on GnRH (Dorling et al., 2003). Collectively, this evidence supports the suggestion that other neural systems are involved in mediating steroid-feedback on GnRH neurons.

**Estrous Cycle**

The transition into the breeding season in ewes is characterized by an increase in ovarian activity and follicular development. The typical ovine estrous cycle includes a short, 2 to 3 day follicular phase and a 14-day luteal phase. As long as the animal does not become pregnant she will exhibit successive follicular and luteal phases until pregnancy occurs or until reproductive activity is suppressed because of inhibitory photoperiod signals that cause seasonal anestrus. During the follicular phase, tonic secretion of LH and FSH are under the control of estrogen and inhibin via a negative feedback loop. FSH is released from the pituitary to stimulate follicular growth, while LH stimulates estrogen secretion from the developing follicles. As the follicles grow they secrete estrogen, inhibiting GnRH amplitude, and therefore LH pulse amplitude. In ewes, one or two follicles become dominant, causing the remaining follicles to undergo atresia. The dominant follicle then begins to secrete increasing amounts of estrogen in response to LH, which shortly reaches a threshold to cause positive feedback on GnRH release and induce the LH surge. The action of the LH surge is to induce ovulation and luteinization of the follicular remnants. The growth of the corpus luteum leads to an increase in progesterone synthesis and release which inhibits tonic LH secretion. If
maternal recognition of pregnancy does not occur, then prostaglandin F$_2$$\alpha$ (PGF$_2$$\alpha$) is produced and secreted by the uterine endometrium and causes regression of the corpus luteum. Regression of the corpus luteum is followed by a dramatic decline in progesterone concentrations and the beginning of a new follicular phase.

**Tonic Secretion**

During the breeding season, tonic secretion of LH is regulated by negative feedback actions of both progesterone and estrogen (Malpaux, 2006; Goodman & Inskeep, 2006). Following the LH surge, LH concentrations begin to decrease as progesterone levels begin to rise (Hauger et al., 1977) because progesterone is the major controller of tonic LH secretion in the sheep. The rise in tonic LH before the onset of the LH surge is associated with the fall in progesterone concentrations that occur with the regression of the CL, although estrogen contributes to the increase in LH pulse frequency during the follicular phase (Karsch et al., 1983). In an ovariectomized (OVX) animal, the administration of estrogen and progesterone suppresses GnRH, and consequently pulsatile LH secretion. If estrogen or progesterone are administered to OVX ewes alone, partial inhibition of LH is observed, but not to the same extent seen in an intact ewe (Karsch et al., 1980). In the past, the functional relationship between estrogen, progesterone, and LH secretion was a topic of great debate among neuroendocrinologists. The controversy was whether estrogen or progesterone was the primary regulator of tonic LH secretion during the normal ovarian cycle. Studies provided answers to demonstrate how each steroid regulated the tonic release of LH, and that each steroid controlled LH secretion by different mechanisms. Progesterone was shown to act in the brain to
decrease GnRH and thus LH pulse frequency, while estrogen suppressed LH pulse amplitude by decreasing both the response of the anterior pituitary to GnRH and GnRH pulse amplitude (Goodman and Karcsh, 1980; Evans et al., 1994). Additional studies provided evidence that estrogen is required to prime the brain for progesterone-negative feedback (Goodman et al., 1981). The site of action of steroid-negative feedback within the hypothalamus is species specific. In the ewe, hypothalamic deafferentation provided evidence to demonstrate that both the retrochiasmatic region and the medial basal hypothalamus are sites of action that are necessary for the maintenance of tonic LH levels in OVX ewes (Jackson et al., 1978).

**Surge Secretion**

The LH surge that occurs in response to high circulating levels of estrogen happens once per estrous cycle. In artificially produced LH surges, injection of estrogen in OVX ewes during the breeding season causes an immediate decrease in GnRH pulse frequency and amplitude. After this initial decrease, there is an LH surge characteristic of the preovulatory surge of intact ewes (Caraty et al., 1989). This biphasic response to estrogen is due to resultant changes in the release of GnRH and a change in pituitary responsiveness to GnRH. Furthermore, Moenter et al (1990) has shown that the ability of GnRH neurons to produce a surge is not restricted to the breeding season. Anestrus, OVX ewes treated with follicular phase levels of estrogen exhibit a large and sudden GnRH surge concurrent with the onset of the LH surge, and this LH surge terminates even though GnRH secretion is still elevated in both the breeding and anestrus season.
In the ewe, the medial basal hypothalamus (MBH) is the primary site of action of estrogen in inducing the LH surge (Blache et al., 1991; Caraty A et al., 1998). In order to induce a surge, estrogen concentrations must be elevated for six to eight hours in the ewe. Dual immunostaining for Fos, a marker for cellular activation, and GnRH during an induced LH surge demonstrated that, in the sheep, several neuronal populations were activated during the surge in addition to GnRH neurons (Moenter et al., 1993). These data, along with the absence of ER-alpha in GnRH neurons, suggest that other neurotransmitters serve as intermediates for the positive-feedback response to estrogen just prior to the surge.

**Seasonal Reproduction**

Numerous mammals are seasonal breeders, including the hamster, horse, and sheep. The advantage of seasonal breeding is that birth(s) of offspring are timed to the optimal time of year for survival of the young and the availability of nutrients is maximized for the mother to support the great energy demands of lactation. Different seasons are most favorable depending on the species and its environment. Two types of seasonal breeders are recognized; long day breeders and short-day breeders. Mammals that are reproductively active during the spring and summer months like the hamster, (gestation length about one month) or the horse (gestation length about one year) are known as long day breeders. Conversely, short day breeders such as the sheep, which has a gestation length between five to six months, exhibit estrous cycles during the fall and winter months,
Seasonally breeding animals use ultimate and proximal cues to synchronize the time of fertility. These cues have to change with season in order to provide interpretable signals which can help time the onset and cessation of the breeding season. Ultimate cues are environmental factors that have led to the evolutionary development of seasonal breeding, such as food availability, water availability, temperature, and predator population. Proximal cues are environmental factors that occur about the same time every year such as photoperiod. Animals rely on proximal cues to time their reproductive cycles on a regular basis.

Seasonal Anestrus in the Ewe

During seasonal anestrus the pulsatile secretion of gonadotropin release is very infrequent (Scaramuzz and Baird, 1977; Barrel et al., 1992). Because of the low frequency of GnRH and LH pulses, follicular development cannot occur and hence estradiol does not rise sufficiently to induce an LH surge and ovulation. This depression in LH pulse frequency during anestrus is due largely to an increase in sensitivity of GnRH to steroid-negative feedback (Legan et al., 1977; Goodman & Karsch, 1981) as well as a steroid-independent control (Goodman & Karsch, 1981).

The steroid-dependent effect is primarily due to the change in responsiveness to estrogen. Estrogen suppresses LH pulse amplitude during the breeding season, but in anestrus estrogen causes an inhibition of LH pulse frequency (Goodman et al., 1980). This results in minimal concentrations of LH during this time of year. Progesterone is not a factor because progesterone concentrations are undetectable during anestrus (Yuthasastrakosol et al., 1975). The shift in LH response to estrogen during anestrus is
largely due to estrogen acting in the hypothalamus to suppress GnRH release, and is not due to a change in the response of the pituitary to GnRH (Goodman et al., 1982).

The steroid-independent decrease in LH pulse frequency is clearly demonstrated by comparing LH profiles of OVX ewes in the breeding season to OVX ewes in seasonal anestrus (Goodman et al., 1982). The seasonal decrease in LH pulse frequency and increase in pulse amplitude observed in anestrus OVX ewes, with no change in mean LH concentrations, are due to actions independent of steroid-feedback and may reflect a direct affect of photoperiod.

**Photoperiod**

Photoperiod is defined as the number of hours of light per day. The effects of photoperiod in mammals are two fold; limiting fertility by controlling secretion of GnRH and controlling the time between mating and delivery. Photoperiod is used extensively in temperate zone animals because it is consistent every year and therefore a reliable cue.

There is a complex interaction between photoperiod and the mechanisms timing reproduction. Although photoperiod is the primary signal used to time seasonal reproduction, some animals exhibit rhythmic breeding patterns in the absence of photoperiodic cues. For example, in constant environmental conditions, estrogen-treated, OVX sheep will continue to alternate between breeding and anestrus seasons (Goodman, 1999). In contrast, other species exhibit reproductive patterns that do not alternate when placed in constant photoperiod; however, they will undergo one spontaneous change (Goodman, 1999). The hamster is one example of a nonrhythmic animal that when placed in short days, reproductive activity is suppressed (Reiter, 1972). However, if kept in the
same inhibitory condition for four or five months, the hamster will become reproductively active. This response is known as photorefractoriness, which continues as long as the hamster remains in short days.

The location of photoreceptors necessary to respond to changes in day length varies depending on the species (Legan and Karsch, 1983). In birds, light gets through the skull to the hypothalamic photoreceptors, whereas the photoreceptors of mammals are located in the retina. Light input to the retina is converted by the photoreceptors to neural activity which is transmitted directly to the SCN. From the SCN it travels first to the paraventricular nucleus (PVN), and is then sent to the pineal gland via the superior cervical ganglion (SCG). The pineal gland, located on the posterior edge of the third ventricle, is required in mammals to synchronize the endogenous rhythm to the prevailing photoperiod. Melatonin is the hormone secreted from the pineal gland that is the endocrine indicator of photoperiod.

**Melatonin**

Melatonin is secreted in a circadian rhythm, with release occurring during the dark and ceasing with the presence of light. The most important parameter of melatonin secretion is the duration that it is elevated (Reiter, 1991; Barrell et al., 2000). The duration of the melatonin signal provides information about the length of darkness and is used to determine the time of year. Removal of the pineal gland in the sheep blocks the response to changing photoperiod. Pinealectomized (PNX) and OVX sheep implanted with estrogen will continue to exhibit endogenous rhythms of LH secretion of about 1 year but the rhythms are not synchronized with photoperiod (Woodfill et al., 1991).
However, replacement with the season-appropriate melatonin pattern allows them to exhibit normal synchronized rhythms. Similarly, pineal intact, seasonally anestrus ewes treated with subcutaneous melatonin implants will experience increased ovarian activity characteristic of the breeding season 40-60 days following treatment (Karsch et al., 1984). In contrast, melatonin is inhibitory to LH secretion in hamsters (Bartness et al., 1993). This is most likely because hamsters are long-day breeders, so that a long duration melatonin pattern produces the same inhibitory effects as short days.

Autoradiography studies using $^{125}$I-melatonin demonstrate high concentrations of melatonin receptors in the pars tuberalis of the sheep (DeReviers et al., 1989) and hamster (Duncan et al., 1993). Furthermore, these receptors have been located in the premammillary region (PMR) of the sheep (Malpeaux et al., 1998) and dorsal median nucleus (DMN) and suprachiasmatic nuclei (SCN) of the hamster (Duncan et al., 1993). In the sheep, melatonin acts on its receptors in the PMR to initiate the onset of breeding season (Malpeaux et al., 1998), while in the pars tuberalis melatonin acts to control seasonal changes in prolactin secretion (Lincoln and Clarke, 1994; Lincoln, 1994; Lincoln, 1999). In the Syrian hamster melatonin acts in the DMN to inhibit reproductive activity, and acts in the pars tuberalis to control prolactin secretion (Weaver et al., 1989; Lincoln, 1999). In addition to release in the blood, studies in sheep have demonstrated the release of melatonin in cerebrospinal fluid (CSF) (Tricoire et al., 2003a; Tricoire et al., 2003b), however it is unclear which pathway melatonin follows (blood or CSF) to arrive at its target tissue. In summary, photoperiod controls both the initiation of ovarian activity of the ewe in the fall and the transition into the anestrus season in the spring.
will next describe the steroid-dependent inhibitory neural systems that are activated during this transition into anestrus.

**Neural mediators of Estrogen – negative feedback**

**Dopamine neurons**

There is a great deal of evidence to support the hypothesis that dopaminergic (DA) neurons play a role in seasonal breeding. DA has been shown to act centrally in the hypothalamus to inhibit pulsatile GnRH secretion in ovary-intact, seasonally anestrous ewes (Meyer and Goodman, 1985). The specific location at which these DA neurons are acting seems to be in the MBH since pimozide (a dopamine receptor antagonist) implanted into the MBH, but not the POA, caused an increase in LH pulse frequency and mean LH concentrations in intact, anestrous ewes (Havern et al., 1991). Furthermore, studies illustrate DA axons synapsing on GnRH terminals in the median eminence, and administration of dopamine in this region inhibits GnRH release (Kuljis and Advis, 1989). Microdialysis of alpha methyl-p-tyrosine (a competitive inhibitor of tyrosine hydroxylase, the rate limiting enzyme for dopamine synthesis) into the median eminence of estrogen-treated, OVX ewes caused an increase in LH secretion during the anestrus season (Viguie et al., 1998; Bertrand et al., 1998), demonstrating the inhibitory role of DA in the median eminence during the anestrus season. To further examine the role of dopamine during the anestrous season, Pau et al. used hypothalamic deafferentation of the MBH or lesioning of the region just caudal to the optic chiasm to cause ovulatory
cycles in ovary-intact anestrous ewes and prevent the inhibitory action of DA on LH secretion in estrogen-treated OVX ewes (Pau et al., 1982).

**A15/A14 DA Neurons**

There are two dopaminergic cell populations that have been shown to have a role in controlling GnRH secretion during seasonal anestrus, namely the A14 and A15 dopaminergic neurons of the hypothalamus (Malpaux, 2006; Thiery et al., 1995). The cell bodies of the A14 group are located in the POA, with a small group clustered dorsal to the posterior portion of the optic chiasm, and others scattered in the periventricular region. The DA cell bodies of the A15 are confined to an area at the base of the brain, medial to the optic tracts, in the retrochiasmatic area (RCH) (Tillet et al., 1989; Tillet et al., 1990). Havern et al. investigated the role of the A14 and A15 DA cell populations in the seasonal control of LH secretion by lesioning these areas of the ovine hypothalamus. After doing so, the inhibitory actions of estradiol on LH secretion were reduced, as was the stimulatory effects of pimozide in anestrous ewes. To further test the hypothesis that these DA cell groups of the hypothalamus were important for the seasonal shift in estrogen negative feedback, Havern et al. (1994) showed that similar lesions placed in ewes during the breeding season did not affect estrogen’s ability to inhibit LH pulse amplitude.

DA neurons of the A15 and A14 hypothalamic region are activated, as measured by the expression of Fos, during the anestrous season by estrogen, but interestingly these neurons are not activated during the breeding season (Lehman et al., 1996). Additionally, estrogen treatment caused an increase in tyrosine-hydroxylase activity in the lateral RCH
but not the caudate nucleus (Gayrard et al., 1994) and the administration of 6 hydroxydopamine (to lesion DA neurons) into the A15 of estrogen-treated, OVX ewes caused an increase in LH pulse frequency (Thiery et al., 1989). Nevertheless, immunocytochemical staining revealed that A15 dopaminergic neurons are devoid of estrogen receptors (Lehman and Karsh, 1993; Skinner and Herbison, 1997), indicating that there may be mediation by other estrogen-responsive neurotransmitters upstream of this area.

**Estrogen Receptor alpha Neurons**

Immunocytochemical studies have provided detailed evidence to illustrate the location of estrogen-receptor alpha immunoreactive (ER-IR) cells in the sheep brain (Lehman, and Karsch, 1993). The majority of these ER-IR cells are located in the medial preoptic area (mPOA) and the mediobasal hypothalamus (MBH), as well as a few limbic structures. Furthermore, the greatest concentration of ER-IR cells in the medial preoptic area is at the level of the organum vasculosum of the lamina terminalis (OVLT) (Lehman and Karsch, 1993). To further investigate the role of these ER-IR neurons in seasonal reproduction, Stefanovic et al. (2000) utilized dual-labeled immunocytochemistry (ICC) for Fos, an indicator of cellular activation, and ER-alpha in hypothalamic tissue from estrogen-treated OVX ewes during the breeding and anestrus seasons. In seasonal anestrus, estrogen treatment induced a dramatic increase in fos expression in ER-alpha containing neurons that were primarily located in the ventromedial portion of the POA (vmPOA), but not in any other hypothalamic areas. In contrast, no increase in Fos expression was detected in ER-alpha neurons in the breeding season model (Stefanovic et al., 2000). Based on these observations, it was proposed that ER-IR neurons in the
vmPOA mediate the inhibitory effects of estrogen on LH secretion during seasonal anestrus.

Additional studies demonstrated that the RCH is another area of the hypothalamus that is involved in estrogen-negative feedback since administration of estrogen implants into the RCH of OVX anestrus ewes caused a significant increase in LH interpulse interval (Gallegos-Sanchez et al., 1997). In support of these results, Hardy et al. administered estrogen or genistein (ER-beta agonist) implants into the RCH of OVX ewes during seasonal anestrus or the breeding season. The results of this study demonstrated that estrogen implants administered during anestrus and not the breeding season caused a significant decrease in LH concentrations via a DA system, yet genistein was without effect in either season (Hardy et al., 2003). This experiment provided additional evidence to demonstrate that neurons in the RCH area of the hypothalamus are involved in estrogen-negative feedback on LH secretion. These results suggest that ER-alpha and not ER-beta containing neurons in the RCH contribute to estrogenic stimulation of A15 neurons during seasonal anestrus.

Tract tracing studies have shown projections from ER-alpha containing neurons of the vmPOA to the A15 dopaminergic region of the hypothalamus (Lehman et al., 2002; Gayard et al., 1995; Goubillion et al, 1999). Anderson et al. (2001) demonstrated that estrogen implants in the vmPOA of OVX ewes during the anestrous season decreased LH pulse frequency, whereas the same treatment during the breeding season had no effect. Furthermore, the ability of sulpiride (a dopamine receptor antagonist), to prevent the suppression of LH pulse frequency in anestrus ewes induced by estradiol when placed in the vmPOA suggests the involvement of DA neurons (Anderson et al.,
These data suggest a proposed model that involves estrogen–responsive afferents from the vmPOA mediating the increase in estrogen–negative feedback, by acting on DA neurons in the A15, to cause a seasonal suppression in LH.

Although the anatomical location of estrogen-responsive afferents to A15 neurons has been identified, the neurochemical identity of these neurons remains unknown. Likely candidate neurotransmitters, based on dual immunocytochemical studies (Goodman and Inskeep, 2006; Herbison et al., 1993, Pompolo et al., 2003), include gamma-amino butyric acid (GABA), glutamate, nitric oxide (NO), and dynorphin. The latter can be excluded because endogenous opioids are not involved in estrogen negative feedback in anestrus (Goodman and Inskeep, 2006) and previous work has not supported an important role for NO. Since my work focused on GABA, the next section will focus on this neurotransmitter.

**Phenotype of afferents to A15 DA neurons: GABA**

GABA is known as the major inhibitory neurotransmitters of the brain. GABA is found in numerous areas of the brain and expression is very high in the substantia nigra, hypothalamus, and hippocampus. GABA is synthesized from glutamate by the enzyme glutamic acid decarboxylase (GAD) and is involved in many neural systems, including those regulating neuroendocrine events controlling reproduction. There are three classes of GABA receptors, $\text{GABA}_{A}$, $\text{GABA}_{B}$, and $\text{GABA}_{C}$. $\text{GABA}_{C}$ receptors have not been investigated as heavily as the $\text{GABA}_{A}$ and $\text{GABA}_{B}$ and will not be discussed. The $\text{GABA}_{A}$ receptor is a ligand-gated chloride channel. GABA binds to and activates the $\text{GABA}_{A}$ receptor allowing chloride ions to flow out and hyperpolarize the neuron.
GABA\textsubscript{B} is a G-protein coupled receptor that also hyperpolarizes neurons. The GABA\textsubscript{A} receptor mediates post synaptic actions of GABA, while the GABA\textsubscript{B} receptor is found both presynaptically and post-synaptically, and the latter is known to act as an autoreceptor.

GABAergic neurons are located in the preoptic area (POA), the site where most GnRH cell bodies in the sheep are located. In view of the fact that GABA neurons have been shown to express estrogen receptors (Herbison et al., 1993; Sar et al., 1983; Flugge et al., 1986) and immunocytochemical studies have described synapses between GABAergic terminals and GnRH cell bodies in the POA (Leranth et al., 1985), many investigators have examined a potential role for GABA in mediating estrogen-negative feedback on GnRH neurons. Robinson et al. (1991) determined by microdialysis measurement that GABA release decreases in the POA of the ewe preceding an estrogen induced LH surge. Additionally, progesterone treatment in OVX ewes during the breeding season was associated with a decrease in LH and an increase in GABA release in the POA. Furthermore, an inhibition in the release of GABA in the POA was seen when an opioid antagonist was administered in addition to progesterone treatment. (Robinson and Kendrick, 1992). This response was not observed in the absence of steroids, providing evidence that GABA is an element of this steroid-negative feedback.

Conversely, studies have shown that GABA\textsubscript{A} receptor and not GABA\textsubscript{B} receptor agonists and antagonists microinjected into the mPOA of OVX ewes suppress LH concentrations regardless of steroid environment (Scott and Clarke, 1993a & 1993b) suggesting that GABA is not a component of estradiol-negative feedback. Similarly, the same effect on LH is seen when the GABA\textsubscript{A} agonist, muscimol, or the GABA\textsubscript{B}
antagonist, CGP 55845, are locally dialyzed into the mPOA (Ferreira et al., 1996; Ferreira et al., 1998) and the MBH (Jackson et al., 1998) of castrated rams. The fact that LH responds the same to both a GABA_A agonist and antagonist raises some confusion. One possible explanation for the similar results is that bicuculline (an antagonist) has been shown to act on other neural regulators of GnRH. Still, these results do show that GABA does influence GnRH secretion during the breeding season of gonadectomized ewes and rams.

There may be a seasonal shift in the control of GnRH release given that GABA_B agonists and antagonists given into the mPOA only have an effect on GnRH during seasonal anestrous. Specifically, the GABA_B agonist, baclofen, increased LH concentrations in estrogen-treated OVX ewes during anestrous, while the GABA_B antagonist, phaclofen, caused significant decreases in LH pulse amplitude in the same model (Scott and Clarke, 1993b). It’s important to note that these effects were not observed in the absence of steroids. These studies raise the possibility that there may be a seasonal change in GABA receptor subtype in control of GnRH secretion in the mPOA, however direct assessment of GABA_B expression in ovine GnRH neurons did not detect any seasonal differences (Sliwowska et al., 2002).

Besides the POA, the MBH is another potential site where GABA influences GnRH. Autoradiography studies have demonstrated high affinity uptake sites for GABA (Tappaz et al., 1977) and a thick plexus of GABAergic neurons (Vincent et al, 1982) in the MBH. Additionally, the administration of a GABA_B agonist inhibited the release of GABA in this area, suggesting that GABA_B autoreceptors are located here (Anderson and Mitchell, 1985).
Ferreira et al. (1996) dialyzed a GABA_A agonist into the MBH of testosterone–treated rams and saw a suppression in mean LH concentrations. In contrast, microinjection of a GABA_B agonist caused an increase in LH, specifically LH pulse amplitude (Ferreira et al., 1996), while the GABA_B antagonist phaclofen was without effect. This increase in LH release is mediated by an increase in GnRH output (Jackson et al., 2000) and is seen with a different GABA_B agonist, SKF97541 (Jackson and Kuehl, 2002). The fact that GABA_B agonists infused into the MBH causes an increase in LH is somewhat confusing, based on the notion that GABA is inhibitory. There are a couple possible explanations for this result. First, GABA_B receptors have been shown to be autoreceptors, thus administration of a GABA_B agonist could prevent GABA release and allow for elevated GnRH secretion. Second, GABAergic neurons could be inhibiting interneurons that are inhibitory to GnRH, also allowing for elevated GnRH release.

In contrast, administration of baclofen or SKF97541 into the MBH of estrogen-treated OVX ewes does not affect LH secretion during the breeding or anestrus season (Jackson and Kuehl, 2004). One possible reason for the difference between the studies is that there may be a sex difference in GABA_B receptor-mediated control of GnRH in the MBH. Also, there may be a difference in testosterone and estradiol effects on GABA release.

Recent studies have demonstrated a role for GABA in the control of LH release by A15 DA neurons of anestrous ewes. Microinjection of a GABA_A or GABA_B agonist into the A15 of anestrous ewes caused a marked increase in LH pulse frequency and mean LH concentrations (Goodman et al., 2004). Additionally, immunocytochemical studies have demonstrated GAD-positive close contacts (Hardy and Goodman, 2003) and GABA_B receptors (Sliwowska et al., 2002) on tyrosine-hydroxylase (TH) positive neurons.
in this area in both the breeding and anestrus seasons, suggesting that GABA is acting
directly at GABA\textsubscript{B} receptors on DA neurons in the A15. One aspect that hasn’t been
investigated is whether these DA neurons express GABA\textsubscript{A} receptors and if there is a
seasonal difference in GABA\textsubscript{A} receptor expression. Furthermore, the effects of
administration of GABA\textsubscript{A} and GABA\textsubscript{B} receptors antagonists into the A15 of OVX ewes
on LH secretion during the anestrus season remains to be investigated. There is clear
evidence to demonstrate that GABA modulates GnRH release in some way, however, the
regulation of this control is still unclear.

**Thyroid hormones and Neural Plasticity**

**Thyroid Hormones**

In the 1940s Woikewitsh was the first to demonstrate a relationship between
thyroid hormones and seasonal breeding in the starling (Woikewitsh, 1940). This has also
proven to be true in several mammalian species, including the sheep (Karsch et al., 1995).
Removal of the thyroid gland during the breeding or nonbreeding season prevents the
subsequent seasonal transition to anestrus, and thus, animals continue to exhibit ovulatory
cycles throughout the year (Nicholls et al., 1988; Webster et al., 1991a). These data
suggest that thyroid hormones are required for the endogenous transition from breeding
season to anestrus. To further support this conclusion, ovary-intact thyroidectomized
(THX) ewes that are housed in a fixed photoperiod continue to have estrous cycles
throughout the expected time for anestrus (Nicholls et al., 1988). Removal of the thyroid
gland prevents the seasonal increase in response to estradiol-negative feedback on LH
pulse frequency that terminates the breeding season (Moenter et al., 1991). Replacement
of thyroxine to THX ewes allows the ewes to transition into anestrus season like thyroid-intact ewes, indicating that thyroid hormones are an essential requirement for the transition into anestrus. Even though thyroid hormones are necessary for this transition, removal of the thyroid hormones does not affect other feedback actions of gonadal steroids (Webster et al., 1991b).

Interestingly, thyroid hormones display a seasonal change in concentrations with elevated concentrations of thyroxine occurring just prior to the transition to anestrus (Webster et al., 1991a). This led to the hypothesis that an increase in T4 concentrations contributes to the decrease in LH pulse frequency. To address this, Dahl et al. (Dahl et al., 1995) manipulated thyroxine concentrations in THX ewes and demonstrated that low T4 replacement was sufficient for the transition to anestrus. Animals in the study that had incomplete thyroidectomies, with barely detectable thyroxine concentrations, transitioned into anestrus at the appropriate time (Dahl et al., 1995). They thus concluded that thyroid hormones play a permissive role in the transition to anestrus. This permissive effect of thyroid hormones is mediated via estrogen-negative feedback on the GnRH system since pulsatile GnRH secretion, measured in hypophyseal portal blood, is elevated in THX ewes in anestrus (Webster et al., 1991a). Furthermore, central administration of thyroxine to THX ewes allows for seasonal suppression of GnRH and LH secretion at the normal termination of the breeding season, whereas the same dose of T4 administered peripherally to THX ewes does not (Viguie et al, 1999). To investigate the site of action of thyroid hormones in the hypothalamus, Anderson et al. (2003) administered T4 implants to the POA, vmPOA, MBH, RCH, and the PMR to find the specific site of action of this hormone. Of the five regions that were explored, seasonal anestrus
occurred only when T₄ was placed within the vmPOA and the PMR (Anderson et al., 2003).

Given that the seasonal reduction in GnRH secretion is a result of a thyroid-dependent increase in the response to estrogen-negative feedback, Thrun et al. (1997a) wanted to determine if thyroid hormones were required to maintain the increased response to estrogen during anestrus. Ewes that were thyroidectomized during seasonal anestrus remained anestrus and transitioned into the breeding season at the same time as thyroid-intact ewes. From these results, Thrun et al. concluded that thyroxine is not necessary to maintain anestrus, nor is it required for initiating the breeding season (Thrun et al., 1997a). Obviously, thyroid hormones are required at a specific time for the termination of the breeding season. Specifically, there is a critical period of responsiveness to T₄ of about 60 to 90 days late in the breeding season that results in the seasonal suppression of the reproductive axis (Thrun et al., 1997b). Not only are thyroid hormones a part of the steroid-dependent increase in response to estrogen-negative feedback, but thyroid hormones also are important in the steroid-independent seasonal suppression of reproduction (Anderson et al., 2002). In summary, thyroid hormones are necessary for changes in hypothalamic function that produce the increased response to estrogen-negative feedback that is responsible for the seasonal transition into anestrus.

Seasonal plasticity

Seasonal influences on neuronal plasticity in the control of GnRH release has been recently studied and was first demonstrated utilizing electron microscopy. This study showed significantly more synaptic contacts on GnRH cell bodies in the breeding
season when compared to anestrus (Xiong et al., 1997). These results were confirmed utilizing immunocytochemistry and confocal microscopy (Jansen et al., 2003). One explanation of these results is that these changes in the number of synaptic contacts indicated an increase in stimulatory inputs on GnRH neurons during the breeding season, and then removal of this stimulatory input, perhaps by an increase in response to estradiol-negative feedback, during seasonal anestrus. Since A15 DA neurons are an integral part of this increased response to estrogen-negative feedback, Adams et al, investigated if there was a seasonal difference in synaptic input on these DA neurons. The results demonstrated an increase in close contacts on DA dendrites as well as an increase in dendritic length of these neurons during anestrus. However, in ewes that were THX in December, this increase in dendrite length was not observed, suggesting that neural plasticity of A15 DA neurons may contribute to their mediation of estrogen-negative feedback in the anestrus season (Adams et al., 2006).
Summary

Thyroid hormones act in both the vmPOA and the PMR to permit the transition into anestrus (Fig 1), and these hormones are required for DA neurons to exhibit seasonal plasticity at that time. However, the specific site that thyroid hormones target to cause this plasticity is unknown.

It is known that estrogen acts within the vmPOA and RCH to inhibit LH secretion during the anestrus season (Fig 1). ER-positive afferents from the vmPOA projecting to A15 DA neurons potentially mediate the increased response to estrogen-negative feedback. Recent studies have demonstrated a possible role for GABA as this estrogen-responsive afferent. Immunocytochemistry shows that GABAergic close contacts and GABA$_B$ receptors have been observed on DA neurons in the A15. However, the expression of GABA$_A$ receptors on these neurons has not been investigated.

According to the proposed model (Fig 1); estrogen inhibits GABA release in the A15 during anestrus season, thus, increasing inhibition of LH by dopamine. Evidence that administration of a GABA$_A$ or GABA$_B$ agonist into the A15 of anestrus ewes causes an increase in LH concentrations further strengthens this model. Following that logic, administration of a GABA$_A$ or GABA$_B$ antagonist to OVX ewes should inhibit LH, however, this effect was not observed. Thus, there is a need to investigate simultaneous inhibition of both receptor subtypes in anestrus, OVX ewes.
Objectives

The first objective of this thesis is to further investigate the role that thyroid hormones have on neural plasticity of the A15 DA neurons in the hypothalamus. Specifically, dual labeled immunocytochemistry is utilized to quantify the number of close contacts on A15 DA neurons, and morphology of these DA neurons were analyzed in THX ewes treated with T4 implants in the vmPOA and PMR.

The second and third objective of this thesis is to expand on GABA’s role in mediating the seasonal suppression of GnRH via the A15 DA neurons. The first of these experiments was to determine if GABA acts directly on DA neurons by investigating the expression of GABA_A receptors on DA neurons and if this expression varied seasonally, via dual labeled immunocytochemistry. The final objective investigated the effects of microinjection of a combination of both GABA_A and GABA_B receptor antagonists into the A15 region of OVX, anestrus ewes. Additionally, sulpiride (D2 receptor dopamine
antagonist) was administered to confirm that DA neurons are responsible for the inhibition of LH following GABA antagonist treatment.
CHAPTER 2: Experiment 1

Introduction

The ewe is a seasonal breeder with an endogenous rhythm that controls the transitions between the breeding season and seasonal anestrus (Karsch et al., 1989; Malpaux et al., 1988). One of the many elements that control this endogenous rhythm is thyroid hormones. Thyroid hormones are required for ewes to transition into anestrus. This was first shown by Nicholls and Follett who demonstrated that thyroidectomy (THX) of ewes results in the continued expression of estrous cycles throughout the year (Nicholls et al., 1988). Moenter et al. then demonstrated that THX prevented the seasonal increase in response to estrogen – negative feedback (Moenter et al., 1991). Thyroxine (T\textsubscript{4}) replacement in THX ewes can restore the expression of seasonal anestrus (Webster et al., 1991b). Additionally, thyroid hormones facilitate the steroid-independent seasonal shift in pulsatile LH secretion (Anderson et al., 2002).

Thyroid hormones act permissively (Dahl et al., 1995) to allow the decrease in GnRH secretion that drives the transition into anestrus. This was demonstrated by an increase in GnRH pulse frequency in THX ewes (Webster et al., 1991a). This permissive effect of thyroid hormones occurs centrally on the GnRH neural system (Viguie et al., 1999). Since Viguie et al. demonstrated that ICV administration of T\textsubscript{4} to OVX ewes caused suppression of LH pulse frequency, Anderson et al. subsequently investigated the specific site of action of this hormone. Specifically, T\textsubscript{4} implants were administered into the vmPOA, mPOA, A15 area, PMR, and MBH of THX, estrogen – treated ewes. Of the five regions of the hypothalamus that were investigated, only the ewes that were given microimplants in the vmPOA and PMR exhibited the normal annual suppression of LH,
leading Anderson et al. (2003) to suggest that these regions are the specific sites of action for thyroid hormones.

One possible mechanism for annual changes in the activity of the reproductive axis is synaptic plasticity in the neural circuitry inhibiting GnRH during anestrus. Plasticity in this case is defined as a change or a disruption in this circuit at the transition to the breeding season and reorganization of this circuit at the transition into anestrus. During seasonal anestrus in the ewe, synaptic input on GnRH cell bodies and dendrites is significantly less than during breeding season (Xiong et al., 1997; Jansen et al., 2003). Furthermore, neural plasticity has been investigated in the A15 DA cell population (Adams et al. 2006). This population of DA neurons is activated by estrogen during seasonal anestrus, but not during the breeding season (Lehman et al., 1996) and inhibits LH concentrations, suggesting that the DA neurons of the A15 are involved in the mediation of estrogen – negative feedback in the anestrus season. One caveat to this hypothesis is that these dopamine neurons do not express receptors for estrogen (Lehman and Karsh, 1993; Skinner and Herbison, 1997), providing evidence to propose that there must be estrogen – responsive afferents to this region.

The number of close contacts on dendrites of A15 DA neurons are significantly greater in the anestrus season as compared to breeding season (Adams et al. 2006). In addition to a greater number of contacts, the dendrites of these DA neurons are longer during seasonal anestrus (Adams et al, 2006). Removal of the thyroid gland prevented this change in dendritic length, suggesting that the neural plasticity of A15 DA neurons that is associated with the annual suppression of the reproductive axis during the nonbreeding season is dependent on thyroid hormones.
The objective of this study was to determine if thyroid hormones act in a specific area to allow synaptic plasticity in the A15 associated with seasonal anestrus. To achieve this objective we quantified the number of close contacts on A15 DA neurons and analyzed the morphology of these neurons in THX animals with no T4 replacement, thyroid-intact ewes, or THX ewes treated with T4 microimplants in the vmPOA and PMR regions of the hypothalamus.

**Methods**

**Animals**

All ewes were housed indoors starting at least three days prior to surgery, with lighting adjusted to simulate natural photoperiod. Each ewe received free access to water and a daily ration of silage and grain. All surgeries and tissue collections were conducted indoors. All procedures involving animals were approved by the West Virginia University Animal Care and Use Committee.

**Experimental Design**

The tissue for this experiment was collected from an earlier study that examined the specific sites of action of thyroid hormones necessary for the transition into the anestrous season (Anderson et al., 2003). Because this study has already been published, I will only briefly describe it here. The experiment was conducted between December and May, spanning the time of the normal transition to seasonal anestrus in the Northern Hemisphere. OVX animals who were given a 3 cm-long sc estrogen implant were subjected to one of four treatments: thyroid-intact animals serving as positive controls
(n=5), THX ewes serving as negative controls (n=5), THX animals receiving T₄ implants in the vmPOA (n=5), and THX animals receiving T₄ implants in the PMR (n=5). All T₄ treatments began on January 5th. Blood samples were collected 3 times per week and LH was assessed to determine when animals entered anestrus. Estrogen implants were removed in late March, replaced 3 weeks later, and then ewes were killed and tissue collected in April.

**Tissue Collection**

Animals were injected i.v. with sodium heparin (25000 IU) 10 minutes prior to, and again immediately before the injection of an overdose of sodium pentobarbital (3000 mg i.v.). Their heads were removed and perfused via both internal carotid arteries with 4% paraformaldehyde in 0.1 M phosphate buffer containing 2.5 IU of sodium heparin/mL and 0.1% NaNO₃. The brains were removed and tissue blocks containing the hypothalamus, from the rostral preoptic area to the mammillary body, were dissected for verification of microimplant locations. The tissue blocks were post-fixed at 4°C for twenty-four hours then infiltrated with 0.1 M phosphate buffer containing 30% sucrose. Tissue blocks from the intact, THX, and PMR treatment groups, were then frozen and coronal sections (50 µm thick) were cut on a freezing microtome and stored in cryopreservative. The tissue block from the vmPOA treatment group was embedded in a warm egg-yolk solution (to hold the tissue together while sectioning) which was allowed to set in ice and then placed back in fixative overnight. The embedded tissue blocks were frozen and coronal sections were cut on a freezing microtome and stored in cryopreservative. Any ewe that did not have correct microimplant placement were
removed from the experiment. For each ewe, two sections per A15 region were selected for analysis.

**Dual label immunocytochemistry:**

Immunocytochemical staining procedures were utilized to identify A15 DA neurons by staining tissue sections using a mouse anti-tyrosine hydroxylase (the rate limiting enzyme for dopamine biosynthesis) monoclonal antibody (Chemicon International, Temecula, CA) labeled with a green fluorescence tag (Alexa Fluor 488 donkey anti-mouse IGG, Molecular Probes, Carlsbad, CA; 488 \( \lambda \)). Synaptic boutons were identified by using a rabbit polyclonal antibody against bovine brain synapsin I (Molecular Probes), a marker for synaptic vesicles (Mandell et al., 1992), tagged with a red fluorescence (Alexa Fluor streptavidin, Molecular Probes, 555 \( \lambda \)).

Free-floating sections were rinsed in 0.1M phosphate buffer solution (PBS) three successive times, fifteen minutes per rinse, then rinsed in PBS overnight at 4°C. Sections were then rinsed in PBS four times (fifteen minutes per rinse) followed by incubation in 4% normal donkey serum (NDS) in PBS for 1 hour to minimize non-specific binding. Next, sections were incubated in 1:500 rabbit anti-synapsin in 4% NDS overnight at 4°C. After the overnight incubation, sections were then rinsed in PBS four times (fifteen minutes per rinse) followed by incubation in 1:200 biotinylated donkey anti-rabbit IGG in 4% NDS (the biotin is required for the streptavidin binding in next step) for 1 hour at room temperature. Then sections were rinsed again four times (five minutes per rinse) and incubated in 1:1000 Alexafluor 555-conjugated streptavidin in PBS for 30 minutes and then rinsed four times (five minutes per rinse). Sections were then blocked in 4%
NDS for 1 hour and incubated in 1:200 mouse anti-tyrosine hydroxylase in 4% NDS overnight at room temperature. The following day sections were rinsed in PBS four times at five minutes per rinse then incubated in 1:400 Alexafluor 488 – conjugated to donkey anti-mouse in PBS for 30 min, followed by four rinses in PBS (five minutes per rinse). Sections were then mounted onto slides with 50% phosphate buffer and 50% glycerol. Slides were cover slipped and then sealed using clear nail polish and stored at 4ºC until analysis.

**Confocal Microscopy:**

To determine the number of close contacts on TH-immunopositive cells, sections were scanned using a LSM-510 laser scanning microscope (CarlZeiss) equipped with Ar and He/Ne lasers. The confocal microscope allowed for visualization of both green TH positive (Fig 1) staining and red synapsin positive (Fig 2) staining in the same section using different filters (Figs 3, 4). Tissue collected from the vmPOA treatment group had very few synapsin-positive staining (Fig. 5), presumably due to the fact that this group was imbedded in egg-yolk gelatin and fixed in paraformaldehyde twice. Therefore these data were not included in the analysis. Ten neurons per ewe were scanned at 40X and 63X to count the number of synapsin positive close contacts and to evaluate neuron morphology. A stack of scanned images were taken along the z-plane (z-stack) to create a 3-D representation of each neuron. The stack of images consisted of 22-24 images slicing the neuron from the top to bottom of the Z-plane at 1 µm intervals.

The z-stacks of images were then reconstructed using MicroBrightfield’s Neurolucida software. First, the cell body of each neuron was traced by tracing each
layer from top to bottom, then the cursor was used to trace over each dendrite by adjusting the width of the cursor to fit to the specific width of each dendrite through the stack. This tracing created a 3-D construction of each neuron. Next, synapsin-positive close-contacts were marked on the 3-D construction. The 3-D construction was rotated 360° (Fig. 6) to ensure that each close contact was identified and that contacts were not counted twice. Once reconstruction of the neuron and close contacts was complete the computer software counted number of marks indicating synapsin-positive close-contacts and calculated mean dendrite length, number of primary dendrites, surface area of dendrites, volume of the cell body, and number of bifurcations.

**Statistical Analysis**

The results from all neurons were averaged together for each animal and used for statistical analysis. Differences between groups were analyzed by one-way ANOVA. As mentioned above, brain tissue from the group implanted with T₄ into the vmPOA was eliminated from statistical analysis because this group was embedded in an egg yolk gelatin and then fixed in paraformaldehyde twice, and this presumably prevented appropriate staining of the tissue. Statistical significance was set at P < 0.05 and values in each figure represent mean ± SEM.

**Results:**

**Morphology**

There were no significant differences in: the number of primary dendrites, dendrite surface area, dendrite length, the number of bifurcations, and cell body volume.
(Figures 7–11; P > 0.05).

Contacts

There were no significant differences in: total number of contacts, contacts on dendrites, and contacts on cell body (Figures 12–14; P > 0.05).

Discussion

The morphological results of this study are unexpected since an earlier experiment similar to this one (Adams et al., 2006) demonstrated a significant increase in dendrite length of A15 dopaminergic neurons in thyroid intact and T₄–treated compared to THX ewes. Additionally in that study, mean surface area of dendrites was greater in T₄–treated animals as compared to THX ewes. However, the lack of effects of THX on synaptic inputs observed here is consistent with the data of Adams et al.

The results of the current experiment were that no differences in dendrite length or dendrite surface area were found in any of the treatment groups. However, a few differences can be noted between the previous study and this one. In the present study egg yolk was used during the processing of the tissue of ewes receiving vmPOA implants and tissue was in fixative for a longer amount of time. This most likely accounts for the lack of synaptic staining in this group. However, this cannot account for the lack of differences observed in our study in other tissues. Additionally, the present study utilized a different TH antibody and fluorescent tags for the visualization of A15 DA neurons than Adams et al. To confirm that a difference in antibody and fluorescent tag for TH could account for the differences between the present study and Adams et al. on dendrite morphology, tissue sections from the same animals used by Adams et al. were processed
with the same ICC protocol used in the present study. Results from this analysis showed no difference in dendrite morphology in T₄–treated animals as compared to THX ewes, confirming that a difference in the ICC protocol is a good candidate for the differing results of the two studies. Most likely, the difference in the type of fluorescence used is responsible for the contrasting morphological results. This is because Adams et al. utilized a fluorescent tag with a larger and less specific wavelength than the fluorescent tag used in the present study. This may have allowed Adams et al. to detect TH further out in the dendrite providing a significant difference.

Additionally, ewes used in Adams et al. had estrogen implants removed for six weeks whereas ewes from the present experiment had estrogen implants removed for only three weeks and were therefore exposed to estrogen longer than ewes from Adams et al. The rationale for discussing this difference is that Beccavin et al. demonstrated an increase in tyrosine hydroxylase mRNA in the A15 in ewes treated with estrogen implants as compared to OVX ewes during the breeding season (Beccavin et al., 1998). Thus it is possible that the lack of shorter dendrites in the THX group (breeding season animals) observed in the present study was due to a longer duration of exposure to estrogen and increase in TH expression.

The present experiment demonstrated that there were no differences in close contacts between any of the treatment groups. These results are not surprising since the same results were seen in Adams et al. when comparing number of contacts between T₄–treated and THX ewes. However, the morphological data from the present study did not confirm Adams et al. These results suggest that thyroid hormones are not responsible for
synaptic plasticity to occur, but we cannot not rule out the possibility that estrogen or other neurotransmitters may play a role in seasonally occurring neural plasticity.
Figure 1. Representative image of a single Z-slice of A15 DA neurons (tyrosine hydroxylase) at 40X magnification.

Figure 2. Representative image of a single Z-slice of synapsin-positive staining in the RCH at 40X magnification.
Figure 3: Coexpression of A15 DA (TH-positive) neurons and Synapsin-positive close contacts in the breeding season at 63X magnification.

Figure 4: Coexpression of A15 DA (TH-positive) neurons and Synapsin-positive close contacts in the anestrus season at 63X magnification.
Fig 5. Representative image of TH staining for A15 DA neurons from the vmPOA treatment group.

Figure 6. Neurolucida 3-D construction of an A15 DA neuron showing synapsin-positive close contacts.
Figure 7: Number of primary dendrites on neurons, $P > 0.05$.

Figure 8: Mean surface area of dendrites, $P > 0.05$. 
Figure 9. Mean length of dendrites on A15 DA neurons, P > 0.05.

Figure 10. Mean number of bifurcations per neuron, P > 0.05.
Figure 11. Cell body volume, P > 0.05.

Figure 12. Number of synapsin-positive close contacts on neurons, P > 0.05.
Figure 13. Mean number of synapsin-positive close contacts on dendrites, $P > 0.05$.

Figure 14. Mean number of synapsin-positive close contacts on cell bodies, $P > 0.05$. 
CHAPTER 3: Experiment 2

Introduction:

The breeding season of the ewe begins in late August and terminates in February. The transition into anestrus is caused by an increase in responsiveness of GnRH neurons to estrogen negative feedback. Previous studies have determined that this suppression occurs due to an enhancement of dopaminergic activity, specifically dopaminergic neurons of the A14 and A15 region of the hypothalamus (Lehman et al., 1996). These neurons are activated by estradiol at this time of year and inhibit GnRH, and therefore LH, secretion. However, A14 and A15 DA neurons do not express estrogen receptors (Lehman and Karsh, 1993; Skinner and Herbison, 1997), so it is likely that the activity of these neurons is controlled by estrogen-responsive afferents.

One likely candidate for this role are neurons containing γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter of the central nervous system. GABA neurons contain estrogen receptors, and previous studies have shown that, during the breeding season, injection of a GABA_A receptor or a GABA_B receptor agonist into the region of the hypothalamus containing DA neurons increases LH secretion (Goodman et al., 2004). This suggests that GABAergic neurons may mediate the enhanced responsiveness of the reproductive axis to estradiol negative feedback during the anestrous season. Furthermore, dual labeled immunocytochemistry has demonstrated the expression of GABA_B receptors (Sliwowska JH et al., 2002) and glutamic acid decarboxylase (the rate limiting enzyme in the synthesis of GABA) immuno-positive close contacts on A15 DA neurons. Thus, GABA may directly inhibit A15 DA neurons via GABA_B receptors, but whether it can also do so via GABA_A receptors is unknown.
Therefore, the first objective of this experiment is to determine if A15 DA neurons contain GABA<sub>A</sub> receptors. If GABAergic neurons directly inhibit A14 or A15 DA neurons, a mechanism for inhibiting the activity of the DA neurons during the breeding season would be to increase their responsiveness to GABAergic inhibition, thus allowing the release of GnRH. To test this possible mechanism, the second objective was to determine if expression of the GABA<sub>A</sub> receptor increases during the breeding season.

**Methods**

**Animals**

Animals were ovariectomized in the breeding season (n=4) or during anestrus (n=4) using sterile procedures via midventral laparotomy under halothane anesthesia. Approximately two weeks after ovariectomy, a single 0.5 cm capsule containing estradiol was inserted in the submaxillary region for 7 days.

Additional animals were ovariectomized and given estrogen at the time of OVX in the breeding season (n=2) and during anestrus (n=2) for Objective 2.

**Tissue Collection:**

Animals were injected i.v. with sodium heparin (25000 IU) 10 minutes prior to, and again immediately before the injection of an overdose of sodium pentobarbital (3000mg i.v.). Their heads were removed and perfused via both internal carotid arteries with 4% paraformaldehyde in 0.1 M phosphate buffer containing 2.5 IU of sodium heparin/mL and 0.1% NaNO<sub>3</sub>. The brains were removed and tissue blocks containing the hypothalamus, from the rostral POA to the mammillary body were dissected. The tissue blocks were post-fixed at 4°C for twenty-four hours then infiltrated with 0.1 M phosphate buffer.
buffer containing 30% sucrose. The tissue block was frozen and coronal sections (50µm thick) were cut on a microtome and stored in cryopreservative.

A series of sections at 250 µm intervals through the RCH region were utilized for this experiment. Three sections per A15, A14, and A12 dopaminergic region per ewe were selected for analysis (Figs. 15, 16, 17).

**Dual label immunocytochemistry:**

Dopamine neurons were stained using antibodies against tyrosine hydroxylase and tagged with a green fluorescence as described in the previous chapter. GABA\textsubscript{A} receptors were stained using antibodies against the GABA\textsubscript{A} receptor alpha 1 subunit and tagged with red fluorescence. Free floating sections were rinsed in phosphate buffer solution (PBS) three successive times (fifteen minutes each) on a shaker table, and then incubated overnight in PBS at room temperature. On day 2 sections were rinsed in PBS four times at five minute intervals followed in 1% H\textsubscript{2}O\textsubscript{2} in PBS for 10 minutes, rinsed in PBS four times at five minute intervals and incubated in 4% normal donkey serum (NDS) for 1 hour to minimize non-specific binding. After blocking in 4% NDS, sections were incubated in 1:55,000 mouse anti-GABA\textsubscript{A} receptor alpha 1 subtype monoclonal antibody (Chemicon International, Temecula, CA) in 4% NDS overnight at room temperature. Sections were rinsed in PBS four times at five minute intervals and incubated in 1:200 biotinylated donkey anti-mouse IGG (Jackson Immunoresearch Laboratories Inc., West Grove, PA) in 4% NDS for 1 hour then rinsed in PBS four times at five minutes per rinse. Then sections were incubated in 1:200 Avidin: biotinylated enzyme complex (Vector Labs, Burlingame, CA) in PBS for 1 hour for double-bridging, followed by four rinses in
PBS at five minutes per rinse. After rinsing, sections were incubated in 1:250 biotinylated tyramide (Perker Elmer, Boston, MA) in 4% NDS containing 1µl/ml of 3% H₂O₂ for 10 minutes for amplification, followed by four rinses in PBS at five minutes per rinse, and incubated in 1:400 Cy-3 conjugated streptavidin (Jackson Immunoresearch Laboratories) in PBS for 30 min. Sections were rinsed in PBS four times at five minute intervals, blocked in 4% normal goat serum (NGS) for 1 hour, then incubated in 1:5,000 mouse anti-tyrosine hydroxylase monoclonal antibody (Chemicon Int.) in 4% NGS overnight at room temperature. On the final day, sections were rinsed in PBS four times at five minute intervals, incubated in 1:200 Alexa Flour 488 goat-anti mouse IGG antiserum (Molecular Probes, Invitrogen, Carlsbad, CA), and rinsed again in PBS four times at five minute intervals. Finally, sections were mounted in PBS and gelvatol, coverslipped and stored at 4°C.

**Confocal microscopy:**

To determine the coexpression of GABAₐ receptor on A12, A14, and A15 TH-immunopositive cells (180-200 neurons/ewe), sections were scanned using a Carl Zeis laser scanning microscope. TH-immunopositive neurons were identified by green fluorescence (Fig. 18.A; 488 λ) and GABAₐ receptor containing neurons were identified by a red fluorescence (Fig. 18.B; 555 λ). Colocalization of GABAₐ receptors on TH positive neurons was determined by red fluorescence overlapping green fluorescence, producing a yellow signal (Fig 18.C). The investigator was unaware of the treatment groups during analysis.
Computer Imaging (Pixel Count)

To determine if an increase in GABA<sub>A</sub> receptor expression in the area of A15 DA neurons was found in hypothalamic tissue obtained during the breeding season as compared to that collected during seasonal anestrus, images were taken at 10X of the A15 region of the hypothalamus for each ewe. The red images were then converted to a jpeg and the Optimas software package was then utilized to count the number of pixels in each image as well as within the A15 region and just outside of it. To determine the number of pixels within the A15 region, a 200cm<sup>2</sup> ellipse was made around the cell bodies of the A15 DA neurons and this ellipse was then transposed on the red image (Fig 19). To determine the number of pixels outside the A15 region, a 200cm<sup>2</sup> ellipse was made dorsal to the A15 DA cell bodies and then transposed on the red image.

Statistical Analysis:

The number of TH-ir neurons coexpressing GABA<sub>A</sub> receptors was expressed as the percentage of total number of TH-ir neurons in each section. Percentages were transformed by taking the arcsine of the square root of the percentage. Transformed mean percentages of TH-ir neurons coexpressing GABA<sub>A</sub> receptors were compared for differences between seasons and among hypothalamic regions using two-way ANOVA.

Because of heterogeneity of variance the number of red pixels within the A15 region and outside the A15 region were transformed by taking the natural log of the data. Transformed mean data were compared for differences between seasons and between the inside of the A15 and the outside of the A15 using two-way repeated measures ANOVA. Statistical significance was set at P < 0.05.
Results

Expression of GABA$_A$ receptors

All dopaminergic regions exhibited coexpression of GABA$_A$ receptors, albeit to varying degrees. More than 80% of TH-ir neurons in the A12 coexpressed GABA$_A$ receptors, while 60% of TH-ir neurons in the A14 coexpressed GABA$_A$ receptors. In contrast, less than 30% of TH-ir neurons in the A15 coexpressed GABA$_A$ receptors. The difference in the percentage of TH-ir neurons coexpressing GABA$_A$ receptors was statistically significant ($P < 0.05$) when the A12, A14, and A15 dopaminergic regions were compared (Fig. 20). Within the A15 region the coexpression varied directionally with approximately 20% coexpression in the rostral area and approximately 40% in the caudal A15 (Fig. 21). The GABA$_A$ receptor expression in the periphery of the A15 was localized to primarily fibers, whereas GABA$_A$ receptor expression within the A15 was mostly on cell bodies. Additionally, there was a significantly lower percentage of red pixels within the A15 as compared to outside the A15 (Fig. 22).

Seasonal comparison

There was no difference in the percentage of TH-ir neurons coexpressing GABA$_A$ receptors between the breeding season and anestrus (Figs 20, 23, 24; $P > 0.05$) in any cell populations investigated. The varied coexpression of GABA$_A$ receptors within the A15 region was consistent between seasons (Fig. 21). Additionally, no difference in the percentage of pixel count was observed between seasons, within the A15, nor outside of the neuron population (Figs. 22,24,25). The significant difference of percentage of red pixels within the A15 as compared to outside the A15 was consistent between seasons (Fig. 22).
Discussion

The present study demonstrates that GABA can directly inhibit some dopaminergic A14 and A15 neurons via the GABA\textsubscript{A} receptor during the non breeding season. That only 30 percent of A15 DA neurons expressed GABA\textsubscript{A} receptors together with the strong response to the GABA\textsubscript{A} raises the possibility that a subset of A15 DA neurons project to GnRH neurons. However, there is no data to support or refute this proposed mechanism and techniques to determine this have not been validated.

There are differences among the A15, A14, and A12 dopaminergic groups in the percentage of neurons containing GABA\textsubscript{A} receptors. There appears to be heterogeneity within the A15 area in expression of GABA\textsubscript{A} receptors. Approximately eighty percent of A12 DA neurons express GABA\textsubscript{A} receptors. These results further support a role for GABA in regulating prolactin secretion reported by Ferreira et al. (1998). In that study, microdialysis infusion of GABA\textsubscript{A} and GABA\textsubscript{B} agonists as well as a GABA\textsubscript{A} antagonist caused a significant increase in prolactin secretion (Ferreira et al., 1998).

The observation that there is an increase in the percentage of GABA\textsubscript{A} receptors on the periphery of the A15 as compared to within the A15 raises the possibility that local interneurons that are responsive to GABA may be innervating the A15 indicating that a multisynaptic pathway rather than a monosynaptic pathway is being utilized. GABA\textsubscript{A} receptors on the periphery of the A15 could be located on interneurons that synapse on the dendritic trees of A15 DA neurons. Evidence to suggest a multisynaptic pathway is that when a GABA\textsubscript{A} or GABA\textsubscript{B} agonist is administered to the RCh there is a delay in response to the treatment (Goodman et al., 2004). This delay in response to the GABA
drugs may be due to a multisynaptic pathway, since one would think the response would be more immediate if a monosynaptic pathway was present. Glutamate is one possible candidate in this multisynaptic pathway, since vGlut close contacts have been identified on A15 DA neurons (Singh et al., 2006).

There was no seasonal difference in expression of GABA\textsubscript{A} receptors in any parameters of the A15 investigated. These results are similar to Hardy et al., who reported no seasonal difference in GABAergic close contacts on A15 DA neurons (Hardy and Goodman, 2003). These data do not rule out the possibility that the A15 DA neurons are more sensitive to GABA during a particular season. One scenario is that there are seasonal changes in the release of GABA under the control of estrogen. At the present time, our laboratory is measuring the release of GABA in the A15, via microdialysis, in estrogen – treated OVX ewes and vehicle-treated OVX ewes during the anestrus season. The results of that study will provide more insight into whether the seasonal release of GABA exists and the present working model.
Figure 15. Coronal hypothalamic map depicting approximate location of the A15 dopaminergic population.

Figure 16. Coronal hypothalamic map depicting approximate location of the A14 dopaminergic population.
Figure 17. Coronal hypothalamic map depicting approximate location of the A12 dopaminergic population.

Figure 18. Representative images of a TH-ir neuron coexpressing GABAA receptors in the A15. A: TH neuron  B: GABAA receptors C: Overlapping images of TH-ir neuron and GABAA receptors
Figure 19. Tracing of cell bodies in the A15.

Figure 20. Mean percentage of TH-ir neurons coexpressing GABAA receptors in A15, A14, and A12 hypothalamic areas of breeding season and anestrous ewes.
Figure 21. Degrees of coexpression from the rostral through the caudal extent of A15 dopaminergic neurons.

Figure 22. Pixel count within the A15 and outside the A15 from the anestrus season, left, and from the breeding season, right. * P < 0.01
Figure 23. Dual immunofluorescence labeling of TH-ir neurons coexpressing GABAA receptors in anestrous (A) and breeding season (B).

Figure 24. Dual immunofluorescence labeling of TH-ir neurons coexpressing GABA<sub>A</sub> receptors in anestrous (A) and breeding season (B).
Figure 25. Pixel count of GABA_A receptor immunostaining in the A15 by season.
CHAPTER 4: Experiment 3

Introduction

Most temperate zone mammals are seasonal breeders, including the ewe. The ewe exhibits ovulatory cycles during the autumn and winter months and is in seasonal anestrus during the spring and summer months. The transition into seasonal anestrus is characterized by an increased responsiveness of the GnRH neural system to estrogen-negative feedback that causes suppression in GnRH and LH pulse frequency (Goodman and Inskeep, 2006).

Lesion studies have demonstrated that A15 dopaminergic (DA) neurons mediate estrogen-negative feedback during seasonal anestrus (Havern et al, 1994). Additionally, it has been shown that these DA neurons are activated by estrogen during seasonal anestrus but not during the breeding season (Lehman et al., 1996). These data led to the hypothesis that these DA neurons are responsible for the increased response to estrogen-negative feedback in anestrus. However, estrogen receptors have not been found in the A15 neurons, suggesting that estrogen – responsive afferents to this area provide the response to estrogen that influences the A15. As evidence for this hypothesis, tract tracing studies demonstrated anatomical connections between estrogen – receptor containing neurons of the vmPOA and the A15 DA neurons (Lehman et al., 2002). The vmPOA contains ER alpha – positive neurons that show increased activation, as indicated by an increase in Fos expression, in response to estrogen – treatment only during the anestrus season (Stefanovic et al., 2000). Moreover, estrogen implants in the vmPOA of OVX ewes inhibit LH pulse frequency during seasonal anestrus, but not during breeding season.
One neurotransmitter which may act in the neural pathway afferent to the A15 is gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter of the central nervous system. GABA neurons are located in the vmPOA and express estrogen receptors (Herbison et al., 1993). According to this model, GABA inhibits the A15 DA neurons, preventing them from inhibiting GnRH during the breeding season. During seasonal anestrus, estrogen suppresses the release of GABA, thus removing the inhibition on the DA neurons in the A15 and allowing them to suppress GnRH release.

Recent studies demonstrated that microinjection of either a GABA_A or a GABA_B receptor agonist into the A15 region caused a significant increase in LH pulse frequency and mean LH concentrations in ovary-intact, anestrous ewes (Goodman et al., 2004). However, when these agonists were administered to OVX ewes, LH levels remained unchanged. The simplest explanation for these data is that the fall in estrogen allows endogenous GABA to increase so that GABA receptors are occupied and can no longer respond to GABA agonists. Furthermore, DA neurons in this region are known to express both GABA_A (Experiment 2) and GABA_B receptors (Sliwowska et al., 2002) and receive GABAergic close contacts (Hardy and Goodman, 2003). Thus, GABA may act directly on A15 DA neurons to mediate estradiol negative feedback during the nonbreeding season.

Based on the above model, administration of either a GABA_A or a GABA_B antagonist into the A15 of OVX, anestrous ewes should cause a suppression of LH by blocking the inhibition of A15 neurons by endogenous GABA. However, microinjection of either antagonist did not alter LH (Goodman et al., 2004). Since A15 dopamine neurons contain both GABA_A and GABA_B receptor subtypes, we hypothesize that
simultaneous inhibition of both receptor subtypes is required to inhibit DA release and thereby cause LH levels to decline. The objective of this experiment was to determine if microinjection of a combination of GABA$_A$ and GABA$_B$ receptor antagonists would cause suppression in LH release in OVX ewes during the nonbreeding season and if a subsequent administration of a dopamine receptor antagonist reverses this suppression.

**Methods**

**Animals**

All ewes were housed indoors beginning at least three days prior to surgery, with lighting adjusted to simulate natural photoperiod. Each ewe received free access to water, and a daily ration of alfalfa pellets and corn. All procedures involving animals were approved by the West Virginia University Animal Care and Use Committee.

**Surgery:**

Bilateral ovariectomies were performed via midventral laparotomy under halothane anesthesia, using sterile procedures at the time of neurosurgeries.

*Implantation of Chronic Guide Tubes into the RCh*

Neurosurgeries were performed using sterile techniques with ewes under halothane (2%) anesthesia in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) as described previously (Anderson et al. 2001). After the skull was exposed, a 20 mm wide X 30 mm long hole, centered about 10 mm rostral to the bregma, was drilled through the skull using a Weck arbor bone drill (George Tiemann & Co., Hauppauge, NY) and expanded using a Schlesinger bone punch (George Tiemann & Co.). After
exposing the dorsal surface of the brain, the superior sagittal sinus was ligated and cut. A lateral ventricle was temporarily cannulated by lowering a sharpened 18-G stainless steel tube just rostral to bregma and 4 mm lateral to midline, and radio-opaque dye (Iohexol, Omnipaque 350; Winthrop, New York, NY) was injected (1.5 ml over a period of 90 seconds) to visualize the third ventricle. Using lateral and frontal X-ray pictures, bilateral 18-G sharpened stainless steel guide tubes were lowered to a point 2 mm dorsal to the A15 for the microinjections. After positioning, guide tubes were blocked with 22-gauge wire stylets, the lateral cannula was removed, and the exposed brain was covered with gelfoam and a fine nylon mesh. Dental acrylic was applied over the fine mesh and around cranial screws for anchorage. The upper portion of a 20-cc plastic vial with a screwable lid was cemented in place with dental acrylic to protect the protruding guide tubes and the skin was sutured around the apparatus, (Anderson et al., 2001).

Dexamethasone was administered i.m. in decreasing daily doses, beginning with 20 mg on the day prior to surgery and ending 3 days afterwards with 2 mg. Penicillin (8 mg) was also injected daily during this time period and atropine (15 mg) was given immediately prior to surgery. A postsurgical analgesic (flunixin meglumine; 100mg) was administered while the animals were recovering from anesthesia. Each ewe was allowed at least two weeks to recover before beginning the experiments. Animals were treated with an antibiotic (gentamicin sulfate; 2 – 3 mg) following all treatment periods.

**Drugs**

Bicuculline methochloride (GABA$_A$ receptor antagonist) and CGP 52432 (GABA$_B$ receptor antagonist) were purchased from Tocris Cookson Inc. (Ellisville, MO). These ligands were dissolved in sterile water. The concentrations were 5 µg/µl for both
bicuculline methochloride and CGP 52432. However, since the drugs were combined just before administration, the final concentrations were 2.5 µg/µl for each. Each ewe received bilateral microinjections of either 300nl or 400 nl of GABA antagonists via a 22-G blunt-ended, 1 µl Hamilton syringe. Control ewes received bilateral microinjections of sterile water. The DA antagonist sulpiride (Sigma-Aldrich Co.) was injected intramuscularly in 0.1M tartaric acid at a dose of 1.2mg/kg, a dose that increases LH pulse frequency in anestrous ewes (Anderson et al., 2001; Hardy et al., 2003).

**Experimental Design**

Two replicates of this experiment were carried out to validate site specific results. The first replicate (n=5) was carried out in the summer of 2005 and included two treatment groups: 1) bilateral microinjections of vehicle (300nl sterile water) to serve as control; 2) bilateral microinjections of a combination of GABA_A and GABA_B receptor antagonist in 300nl. Each ewe (n = 5) received each treatment once in a crossover design. Frequent blood samples (every 12 min) were taken for two hours before and 3 hours following microinjections.

The second replicate (n=8) was carried out in the summer of 2006 and included three treatment groups: 1) bilateral microinjections of vehicle (400 nl of sterile water) to serve as control; 2) bilateral microinjections (400nl) of both receptor antagonists followed by an im injection of sulpiride; 3) bilateral microinjections (400nl) of both receptor antagonists followed by an im injection of the vehicle for sulpiride, tartaric acid. Each ewe (n = 8) received each treatment once, in a latin-square design, with a 5 day recovery period between each replicate.
Frequent blood samples (every 12 min) were taken for two hours for assessment of pretreatment LH pulse patterns and concentrations. Following the two hours, bilateral microinjections of both GABA antagonists or vehicle were administered. Blood samples were collected for three hours after microinjections and sulpiride or tartaric acid was administered i.m. to ewes receiving GABA antagonists. Finally, blood samples were collected for three more hours.

Blood Collection

On all days of blood sample collection, peripheral blood samples (4 ml) were collected via jugular venipuncture at 12-minute intervals for 8 hours. This frequency of blood collection allows for easy identification of LH pulses (Goodman et al., 1980; Anderson et al. 2001). Serum was harvested and stored at -20°C until analysis of LH concentrations by radioimmunoassay.

Tissue Collection

Animals were injected i.v. with sodium heparin (25000 IU) 10 minutes prior to, and again immediately before the injection of an overdose of sodium pentobarbital (3000 mg i.v.). Their heads were removed and perfused via both internal carotid arteries with 4% paraformaldehyde in 0.1 M phosphate buffer containing 2.5 IU of sodium heparin/mL and 0.1% NaNO₃. The brains were removed and tissue blocks containing the hypothalamus, from the rostral preoptic area to the mammillary body were dissected for verification of guide tube locations. The tissue blocks were post-fixed at 4°C for twenty-four hours then infiltrated with 0.1 M phosphate buffer containing 30% sucrose.
Hypothalami were sectioned into 50µm coronal slices using a freezing microtome for histological analysis of guide cannulae positions. After cresyl violet staining, every fifth section was mounted onto microscope slides to visualize guide cannulae placements. Each guide cannulae placement was evaluating by comparing its location to previously known locations of the A15 DA neurons in the RCh (Tillet and Thibault, 1989).

**Radioimmunoassay Analysis**

Concentration of LH was determined in 100-µl aliquots by radioimmunoassay, using a modification of a previously described method (Anderson et al., 2001). Values are expressed in terms of the ovine standard, NIH S24. Radioiodinated ovine LH (AFP-8614B, courtesy of A.F. Parlow, NIDDK) was used as trace and primary aniserum was AFP-192279 (courtesy of A.F. Parlow, NIDDK; dilution 1:2,000,000). The sensitivity (95% confidence interval at 0 ng/ml) averaged 0.16 ng/tube. Intra-assay coefficients of variation (CV) averaged 13.2 % and 14.1% respectively, for serum pools displacing radiolabeled LH to approximately 42.9% and 65.2% of the total bound, and interassay CVs were 14.1% and 21.2% for the same serum pools.

**Statistical Analysis**

Mean LH, LH pulse frequency, and LH pulse amplitude were calculated for: 0-2 hours, 2-5 hours, and 5-8 hours (2nd replicate). These data were compared (0-2 hrs versus 2-5 hours and 2-5 hours versus 5-8 hours) by paired student’s T-test. Statistical significance was set at P < 0.05. Ewes with completely misplaced guide cannulae were removed from statistical analysis.
Results

Trial 1

Mean LH concentrations were significantly reduced in response to treatment with GABA antagonists (Fig. 26; P < 0.05), however, LH pulse frequency was not significantly altered (Fig 27; P > 0.05). LH pulse amplitude could not be calculated in trial 1 due to the absence of pulses post-treatment in two animals. Four out of five ewes received accurately placed guide cannulae into the A15. Ewes that had guide cannulae rostral and caudal to the A15 had modest inhibition of LH. Guide cannulae placed outside of the A15 resulted in no significant change in LH. However, guide cannulae placements within the medial A15 exhibited a dramatic decrease in LH.

Since a dramatic effect was only seen in 2 animals, when guide cannulae were placed directly in the medial aspect of the A15, we tested the effects of a higher dose to ensure optimal activation of this area.

Trial 2

Mean pulse frequency and mean LH concentrations were significantly reduced in response to treatment with GABA antagonists (Figs 28, 29; P < 0.05). However, LH pulse amplitude was unaffected by treatment (Fig. 30; P > 0.05). Mean LH concentrations were significantly increased in response to treatment with sulpiride (Fig. 29; P < 0.05), but LH pulse frequency and LH pulse amplitude were unaffected (Fig 28, 30; P > 0.05). Guide cannulae placement verification using histology showed that only five out of eight ewes had accurate placements. Ewes that had guide cannulae rostral and caudal to the A15 had modest inhibition of LH (Figs 31, 32), whereas ewes with guide cannulae outside of the A15 resulted in no significant change in LH (Fig 33). However,
guide cannulae placement within the medial A15 exhibited a dramatic decrease in LH (Fig 34).

Discussion

The results of this study demonstrate that administration of a combination of a GABA_A and GABA_B receptor antagonists into the A15 of seasonally anestrus, OVX ewes causes suppression in LH. These data are consistent with the hypothesis that endogenous release of GABA in OVX ewes is inhibiting the activity of the A15 DA neurons, which allows high frequency release of GnRH and LH pulses. Furthermore, this data demonstrates that the GABA_B agonist is most likely acting post-synaptically when injected in the A15.

These results, however, are contingent upon the specific site of administration of GABA antagonists. In both trials, administration of the GABA antagonists to the rostral or caudal portions of the A15 caused a modest depression in LH, whereas, administration of the GABA antagonists into the medial A15 caused a marked decrease. One possible explanation for these site-specific results is that administration of the drugs in the distal edges of the A15 activates fewer dopamine neurons than administration to the medial A15. Therefore, if fewer dopamine neurons are activated, insufficient suppression is observed. One way to overcome this would be to administer higher concentrations of the antagonists. However, due to the low solubility of CGP, it would not be possible to administer the same volume at a higher concentration. Additionally, one animal was treated with a 500nl bilateral microinjection of the GABA antagonists and adverse behavioral effects were observed. However, when a 400nl bilateral microinjection was
given to the same animal, the same affects were seen, so we may be able to use larger volumes in future work.

That sulpiride caused an increase in LH concentrations was not surprising. However, at this time it cannot be concluded that sulpiride actually had an effect on LH concentrations because when looking at the LH profiles (Fig. 35), it looks as if LH is escaping the GABA-antagonist treatment before the sulpiride is given. To determine if this was the case, an alternative experimental design could be used. Since the present experiment demonstrated a decrease in LH pulse frequency and LH concentrations for 3 hours as a result of administration of GABA antagonists, one could administer the GABA antagonists and the sulpiride at the same time, expecting LH frequency and concentrations to remain unchanged in response to the GABA antagonists. These data together with previous experiments showing that administration of GABA agonists in to the A15 increase LH in ovary intact, but not OVX, anestrus ewes, support the hypothesis that estrogen inhibits release of GABA from neurons afferent to the A15, thus stimulating DA neurons to suppress GnRH release during anestrus.

One limitation of this interpretation is the inference that estrogen inhibits GABA release in the A15 during the anestrus season. Estrogen has been shown to increase GABA release in the POA. However, prior to the surge, when estrogen levels are highly elevated, GABA release is extremely low, supporting the hypothesis that estrogen can inhibit GABA release. To test this hypothesis, our laboratory is presently measuring the release of GABA in the A15, via microdialysis, in estrogen – treated OVX ewes and vehicle treated OVX ewes during the anestrus season.
Figure 26. Comparison of pre- and post-treatment of mean LH in response to GABA antagonists in Trial 1. * P < 0.05.

Figure 27. Comparison of pre- and post-treatment of LH pulse frequency in response to GABA antagonists in Trial 1.
Figure 28. Comparison of pre and post-treatment of LH pulse frequency in response to GABA antagonists or vehicle and Sulpiride or vehicle in Trial 2. * P < 0.05.

Figure 29. Comparison of pre and post-treatment of mean LH in response to GABA antagonists or vehicle and Sulpiride or vehicle in Trial 2. * P < 0.05; ** P < 0.01.
Figure 30. Comparison of pre and post-treatment of LH pulse amplitude in response to GABA antagonists or vehicle and Sulpiride or vehicle in Trial 2. * P < 0.05.

Figure 31. LH profile of ewes administered with GABA antagonists. Note that guide cannulae placement was rostral to the A15. Trial 1: Ewe # 44. Trial 2: Ewe # 123
Figure 32. LH profile of ewes administered with GABA antagonists. Note that guide cannulae placement was caudal to the A15. Trial 1: Ewe # 42. Trial 2: Ewe # 122, 125

Figure 33. LH profile of ewes administered with GABA antagonists. Guide cannulae placement completely outside of A15. Trial 1: Ewe # 48. Trial 2: Ewe # 121, 135, 136
Figure 34. LH profile of ewes administered with GABA antagonists. Note that guide cannulae placement was in the A15. Trial 1: Ewe # 45,49. Trial 2: Ewe # 118,120

Figure 35. Serum LH profile from one representative ewe treated with GABA_A and GABA_B antagonists after sample 10 and sulpiride or vehicle after sample 26.
CHAPTER 5: Discussion

The primary goal of this thesis was to investigate the degree to which the increase in sensitivity to estrogen negative-feedback, during seasonal anestrus, involves the dopaminergic A15 cell population of the hypothalamus. Specifically, the role of the specific site of action of thyroid hormones on neural plasticity of the A15 DA neurons was investigated by utilizing dual labeling immunocytochemistry. The results of this experiment were somewhat unclear based on the fact that no differences were observed in neuron morphology or close contacts in any treatment groups. These results are somewhat inconclusive because Adams et al. observed an increase in dendrite length in T4-treated animals as compared to THX ewes (Adams, Endo, 2006). One explanation for the differences observed is that different protocols were used to stain the dopamine neurons.

Since the results of the first experiment were inconclusive, the focus of the second and third experiment was based on recent evidence from our laboratory demonstrating a role for GABA acting within the A15 region of the hypothalamus. Specifically, previous studies suggest that during the anestrus season, estrogen prevents GABAergic inhibition (by restricting the release of GABA) of A15 DA neurons, thus allowing the dopaminergic inhibition of GnRH and therefore LH (fig 1 from lit. review). The objective of the second experiment was to investigate the expression of GABA\_A receptors on these A15 DA neurons during the anestrus season, via immunocytochemistry. GABA\_A receptors were found on DA neurons indicating that GABA can act directly on these neurons via their receptors. An interesting observation that was made during the analysis was that there was more expression of GABA\_A receptors on the periphery of the A15 as compared
to within the cell population. This observation suggests that GABA may be acting on other neurons located in this area and therefore the role of GABA in the regulation of the activity of A15 DA neurons may be both direct and indirect.

The final objective of this thesis further investigated the role of GABA in the regulation of A15 neurons during seasonal anestrus in an in vivo model. Specifically, the administration of a combination of GABA$_A$ and GABA$_B$ receptor antagonists into the A15 of OVX, seasonal anestrus ewes was hypothesized to cause a suppression of LH. The results of this study confirmed the hypothesis, and further validated the proposed model seen in fig 1 from lit. review, that GABA acts on DA neurons to inhibit the action of the dopaminergic inhibition of LH. This model proposes that GABA exhibits an increased response to estrogen during the anestrus season, causing a decrease in GABA release within the A15. At the present time, the effects of estrogen on GABA and glutamate release in the A15 during the anestrus season are being investigated via microdialysis. The results from that study will provide more insight into the proposed model. The next step would then be to investigate the possibility for a seasonal change in response of GABA neurons to estrogen by making a seasonal comparison in the expression of ER-alpha on GABA neurons.

An additional objective of experiment 2 was to make a seasonal comparison of GABA$_A$ receptor expression, however, no seasonal difference was observed. These results suggest that the inhibition of DA by GABA is not due to a seasonal change in receptor expression. These results suggest an indirect role for GABA in this neural pathway.
Glutamate is another likely candidate for mediating estrogen-negative feedback of A15 DA neurons during the anestrus season. Glutamate neurons in the ewe express ER-alpha (Pompolo et al., 2003) and recent studies have demonstrated significantly more vGLUT close contacts on A15 DA neurons during the anestrus season as compared to the breeding season (Singh et al., 2006). Additionally, administration of glutamate receptor antagonists into the A15 of ovary-intact, seasonally anestrus ewes caused an increase in LH pulse frequency (Goodman et al., 2006). These results suggest that estrogen is stimulating the release of glutamate and that glutamate is directly activating the A15 DA neurons during the anestrus season, and thus causing suppression of LH at this time.

From these results and the observation that GABA<sub>A</sub> receptor expression on the periphery of the A15 is greater than within the A15 itself, an alternative explanation is that during the breeding season, GABA is acting indirectly on the A15 by directly inhibiting glutamate release, thus preventing the stimulation of DA, and therefore allowing the release of GnRH and LH (Fig 1, see below). To further test this hypothesis, anatomical studies need to be done to investigate the expression of GABAergic close contacts and GABA receptors on glutamate neurons during the breeding and nonbreeding seasons.

Since administration of GABA antagonists and agonists into the A15 alters LH levels, an additional model could be that GABA acts directly and indirectly on the A15. (Fig 2, see below).
Fig 1. Model for neural circuit mediating $E_2$ negative feedback in anestrous ewes

Fig 2. Model for neural circuit mediating $E_2$ negative feedback in anestrous ewes
CHAPTER 6  Literature Cited


Scott CJ, Clarke IJ. 1993a. Inhibition of luteinizing hormone secretion in ovariectomized ewes during the breeding season by gamma-aminobutyric acid (GABA) is mediated by GABA-A receptors, but not GABA-B receptors. Endocrinology. 132: 1789-1796.

Scott CJ, Clarke IJ. 1993b. Evidence that changes in the function of the subtypes of the receptors for gamma-amino butyric acid may be involved in the seasonal changes in the negative-feedback effects of estrogen on gonadotropin-releasing hormone secretion and plasma luteinizing hormone levels in the ewe. Endocrinology. 133: 2904-2912.


Stefanovic I, Adrian B, Jansen HT, Lehman MN, Goodman RL. 2000. The ability of estradiol to induce Fos expression in a subset of estrogen receptor-α containing neurons in the preoptic area of the ewe depends on reproductive status. Endocrinology. 141: 190-196.


Curriculum Vita

Adrienne Lynne Bogusz

Education

<table>
<thead>
<tr>
<th>Institution</th>
<th>Degree</th>
<th>Dates</th>
</tr>
</thead>
<tbody>
<tr>
<td>WVU</td>
<td>M.S. in Reproductive Physiology</td>
<td>Aug. 2004-present</td>
</tr>
<tr>
<td>Wingate Univ.</td>
<td>B.S. in Biology</td>
<td>Aug 2002-May 2004</td>
</tr>
</tbody>
</table>

Work Experience

- Research Assistant Aug. 2004-present
- Anatomy and Physiology Teaching Assistant Aug. 2002-May 2004
- Anatomy and Physiology Tutor and Biology Tutor Aug. 2002-May 2004

Abstracts

- Scocco EA, Bogusz AL, Odom CB. 2004. Diversity of RAPD-genetic markers within a colony of the red imported fire ant; Solenopsis invicta (Buren) in Union County, NC. Association of Southeastern Biologists. Abstract

Honors/Awards

- Sigma Xi National Honor Society Research Competition April 2006
  - Awarded 3rd place – General Science Category
- Davis College of Agriculture and Consumer Science Research Day April 2006
  - Awarded 2nd place – Master’s poster
- Gamma Sigma Delta Honor Society March 2006
- Order of Omega October 2002
  - Greek Honor Society

Organizations

- Sigma Xi, The Scientific Research Society June 2006 - present
  - Associate Member
- Sigma Sigma Sigma sorority Aug 2000-May 2004