The Role of Somatostatin in the Regulation of Gonadotropin Secretion in Sheep

Richard B. McCosh

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The Role of Somatostatin in the Regulation of Gonadotropin Secretion in Sheep

Richard B. McCosh

Dissertation submitted to the School of Medicine at West Virginia University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Biomedical Science

Cellular and Integrative Physiology

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2017

Key Words: gonadotropin releasing hormone, luteinizing hormone, somatostatin, kisspeptin, sheep
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ABSTRACT

The Role of Somatostatin in the Regulation of Gonadotropin Secretion in Sheep

Two modes of gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) secretion are necessary for female fertility: surge and episodic secretion. However, the neural systems that regulate these GnRH secretion patterns are still under investigation. The neuropeptide somatostatin (SST) inhibits episodic luteinizing hormone (LH) secretion in humans and sheep, and several lines of evidence suggest SST may regulate secretion during the LH surge. Neither a SST receptor 2 (SSTR2) agonist (octreotide) or antagonist (CYN154806; CYN) altered the amplitude or timing of the LH surge. Administration of CYN to intact ewes during either the breeding season or anestrus increased LH secretion and increased c-Fos in a subset of GnRH and kisspeptin cells during anestrus. To determine if these stimulatory effects are steroid-dependent or -independent, we administered CYN to ovariectomized ewes. This SSTR2 antagonist increased LH pulse frequency in ovariectomized ewes during anestrus, but not during the breeding season. The results demonstrate that SST, acting through SSTR2, inhibits episodic LH secretion, likely acting in the mediobasal hypothalamus, but action at this receptor does not alter LH surge secretion. Additionally, these data provide evidence that SST contributes to the steroid-independent suppression of LH pulse frequency during anestrus.

Potential sites for SST action in the ovine hypothalamus were investigated using immunohistochemistry to determine whether cells that produce kisspeptin (KNDy cells in the arcuate nucleus) or GnRH receive direct synaptic contact from SST fibers, and whether the amount of these inputs changes throughout the estrous cycle or by season. The majority of KNDy cells receive synaptic input (evident by presynaptic synaptophysin immunostaining) from
SST cells, but the amount of these inputs did not differ among groups. A subset of GnRH cells in both the preoptic area (POA) and mediobasal hypothalamus also receive synaptic input from SST cells. A greater percentage of POA GnRH cells had SST synapses during the surge than in anestrus, the luteal or early follicular phase of the estrus cycle. The total number of synaptic inputs onto GnRH and KNDy cells was altered by phase of the estrous cycle and season, extending the hypothesis that changes in the GnRH and KNDy cell synaptic connectivity may contribute to altered gonadotropin secretion throughout the estrous cycle and between seasons.

Several lines of evidence support the hypothesis that somatostatin (SST) cells in the ventral medial nucleus (VMN) may be involved in the generation of the LH surge in sheep. In this study, we confirmed that SST cells in the VMN are activated during the LH surge using c-Fos as a marker for cellular activation. One explanation for the discrepancy between the observations of SST cell activation during the LH surge, but no effect of pharmacological manipulation of SSTR2, is that the cells that contain SST in the VMN also produce additional signaling molecules, such as nitric oxide. We used immunohistochemistry to determine that a high percentage (70-80%) of these SST cells also contain neuronal nitric oxide synthase (nNOS). Triple-label immunohistochemistry was used to determine that a greater percentage of the dual labeled SST and nNOS cells contain c-Fos during the LH surge compared to the early follicular phase. In contrast, the percentage of single labeled nNOS or SST cells that contained c-Fos did not differ between the LH surge and early follicular phase. Thus we propose that this population of SST cells in the VMN also release nitric oxide and that this transmitter contributes to the generation of the LH surge in sheep.
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# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>GnRH</td>
<td>Gonadotropin- Releasing Hormone</td>
</tr>
<tr>
<td>LH</td>
<td>Lutienizing Hormone</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle Stimulating hormone</td>
</tr>
<tr>
<td>hCG</td>
<td>human Chronic Gonadotropin</td>
</tr>
<tr>
<td>PMSG</td>
<td>Pregnant Mare Serum Gonadotropin</td>
</tr>
<tr>
<td>SST</td>
<td>Somatostatin</td>
</tr>
<tr>
<td>SSTR2</td>
<td>Somatostatin receptor 2</td>
</tr>
<tr>
<td>CYN</td>
<td>CYN154806, SSTR2 antagonist</td>
</tr>
<tr>
<td>NKB</td>
<td>Neurokinin B</td>
</tr>
<tr>
<td>Dyn</td>
<td>Dynorphin</td>
</tr>
<tr>
<td>KNDy</td>
<td>Kisspeptin, Neurokinin B, Dynorphin</td>
</tr>
<tr>
<td>E₂</td>
<td>Estradiol</td>
</tr>
<tr>
<td>P₄</td>
<td>Progesterone</td>
</tr>
<tr>
<td>ERα</td>
<td>Estrogen Receptor-alpha</td>
</tr>
<tr>
<td>OVX</td>
<td>Ovariectomized</td>
</tr>
<tr>
<td>PGF₂α</td>
<td>Prostaglandin F₂α</td>
</tr>
<tr>
<td>CIDR</td>
<td>Controlled internal release device</td>
</tr>
<tr>
<td>CL</td>
<td>Corpus luteum</td>
</tr>
<tr>
<td>MBH</td>
<td>Mediobasal hypothalamus</td>
</tr>
<tr>
<td>ARC</td>
<td>Arcuate Nucleus</td>
</tr>
<tr>
<td>VMN</td>
<td>Ventral Medial Nucleus</td>
</tr>
<tr>
<td>AHA</td>
<td>Anterior hypothalamic Area</td>
</tr>
<tr>
<td>RCh</td>
<td>Retrochiasmatic area</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>POA</td>
<td>Preoptic area</td>
</tr>
<tr>
<td>DBB</td>
<td>Diagonal Band of Broca</td>
</tr>
<tr>
<td>OCh</td>
<td>Optic chiasm</td>
</tr>
<tr>
<td>ME</td>
<td>Median eminence</td>
</tr>
<tr>
<td>icv</td>
<td>intracerebroventricular</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>PB</td>
<td>Phosphate Buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate Buffered Saline with Triton X-100</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IPI</td>
<td>Interpulse interval</td>
</tr>
<tr>
<td>AMPL</td>
<td>Pulse amplitude</td>
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</table>
# TABLE OF CONTENTS

THE ROLE OF SOMATOSTATIN IN THE REGULATION OF GONADOTROPIN SECRETION IN SHEEP

ABSTRACT

ACKNOWLEDGEMENTS

ABBREVIATIONS

CHAPTER 1: LITERATURE REVIEW ......................................................... 1

REPRODUCTIVE CYCLES ................................................................. 2

OVARIAN FUNCTION THROUGHOUT THE ESTROUS CYCLE ..................... 4

HYPOTHALAMIC REGULATION OF THE ESTROUS CYCLE ......................... 7

BRIEF COMPARISONS TO THE HUMAN MENSTRAUL CYCLE ...................... 16

BRIEF COMPARISONS TO THE RODENT ESTROUS CYCLE .......................... 16

SEASONAL BREEDING IN SHEEP ....................................................... 18

STEROID-DEPENDENT SUPPRESSION OF GONADOTROPIN SECRETION ........ 18

STEROID INDEPENDENT SUPPRESSION OF GONADOTROPIN SECRETION ....... 19

SOMATOSTATIN ................................................................................. 19

SPECIFIC AIMS .............................................................................. 21

REFERENCES .................................................................................. 23

CHAPTER 2: EVIDENCE THAT ENDOGENOUS SOMATOSTATIN INHIBITS

EPISODIC, BUT NOT SURGE, SECRETION OF LH IN FEMALE SHEEP .......... 36

ABSTRACT .................................................................................... 37

INTRODUCTION ................................................................................ 38

MATERIALS & METHODS .................................................................. 41

RESULTS ....................................................................................... 49

DISCUSSION .................................................................................... 52

REFERENCES .................................................................................. 58

FIGURE LEGENDS: ........................................................................... 64

CHAPTER 3: HYPOTHALAMIC SITES OF ACTION FOR SOMATOSTATIN IN THE

REGULATION OF GONADOTROPIN SECRETION IN SHEEP ..................... 75
THE ROLE OF SOMATOSTATIN IN THE REGULATION OF GONADOTROPIN SECRETION IN SHEEP

CHAPTER 1

LITERATURE REVIEW
**REPRODUCTIVE CYCLES**

The potential for female fertility is manifest through reproductive cyclicity. Menstrual or estrous cycles are neural and endocrine phenomena that synchronize the necessary processes for fertility: 1) production and release of ova, 2) preparation of the reproductive tract for fertilization and implantation, and 3) reproductive behavior. Reproductive cycles are named based on observable signs. In female humans and primates the inner layers of the endometrium, are sloughed off and flows out through the vagina as 35mL (on average) of thick blood and cellular mixture, in a process termed menstruation and thus the reproductive cycles in these species are termed “menstrual cycles.” In most other species, however, menstruation does not occur, instead the outward sign is altered behavior, which varies by species but is generally aimed at attracting and/or receiving a mate and only occurs around the time of ovulation, termed estrous behavior, and thus in these species, reproductive cycles are termed “estrous cycles.”

The term gonadotropin will be used extensively; this term refers to hormones that affect change in the gonads, which are endocrine glands. The endogenous gonadotropins in sheep are luteinizing hormone (LH) and follicle stimulating hormone (FSH). Two additional molecules isolated from other species are experimentally relevant: human chorionic gonadotropin (hCG) and pregnant mare serum gonadotropin (PMSG), which have LH-like and FSH-like activities, respectively.

The current model for the estrous cycle in sheep posits that follicular development requires sequential treatment with FSH and then with LH. The growing follicle(s) produce low amounts of estradiol (E₂) which exerts a negative feedback effect to maintain low gonadotropin releasing hormone (GnRH) and LH secretion from the hypothalamus and pituitary gland,
respectively. Increased LH pulse frequency stimulates further E₂ production and final stages of follicular development. When the viable follicle(s) secrete enough E₂, positive feedback action at the hypothalamus and pituitary ensues stimulating a surge secretion of GnRH and LH. The GnRH and LH surge is characterized by very high and sustained release of these hormones. The LH surge stimulates resumption of meiosis, final development of the ovum and ovulation. The high concentrations of E₂ also causes estrous behavior. The ovulated ovum and attached granulosa cells, together called the cumulus oocyte complex, are ejected from the ovary, collected by the infundibulum and transported through the oviducts toward the uterus. The remaining granulosa and theca cells from the ovulated follicle undergo luteinization (also induced by the LH surge) to transform into luteal cells which form the corpus luteum (CL), a transient structure on the ovary that produces progesterone (P₄), and in some species, also E₂. Progesterone potently inhibits GnRH, and LH release, and also acts on the reproductive tract to suppress myometrial contractions and stimulate fluid secretion from the endometrium. In the absence of a pregnancy, the CL functionally (based on P₄ secretion) and structurally regresses though a combination of apoptosis and necrosis. In the absence of inhibitory actions of P₄, gonadotropin concentrations again rise, stimulating follicular growth and allow the cycle to repeat.

Reproductive cycles can be divided in two phases based on the dominant structure on the ovary: follicle(s) (follicular phase) or CL (luteal phase). The average estrous cycle length in sheep is 17 days, consisting of a 3 day follicular phase and a 14 day luteal phase.
OVARIAN FUNCTION THROUGHOUT THE ESTROUS CYCLE

In sheep, follicular development occurs throughout the estrous cycle. Primordial follicles that are formed during gestation and are maintained in a quiescent state until recruitment (1). Primordial follicles consist of single oocyte surrounded by a single layer of approximately 4 flattened granulosa cells (2). Almost all of the 100,000-250,000 follicles present at birth will regress by atresia continuously throughout life (3). Follicular development can be divided into stages based on structure of the growing follicle. The first stage of follicular growth is the formation of primary follicles from the primordial pool. These follicles grow into primary follicles by granulosa cell proliferation (~19 cells per follicle) and transition to cuboidal cell morphology (2). Follicular growth in the preantral stage continues by proliferation of granulosa cells, emergence and proliferation of theca interna cells, and development of the zona pellucida (2). The next observable stage in follicle development is formation of a fluid filled antrum, which will grow in size as the follicle matures into a preovulatory follicle.

Follicular growth can also be divided into stages based on signals that stimulate follicular growth. Hypophysectomized ewes developed follicles up to 2 mm in diameter, indicating an initial phase of gonadotropin-independent follicular growth (4). Growth of follicles larger than 2 mm in hypophysectomized ewes has been achieved by administration of PMSG and growth hormone (5), indicating a later gonadotropin sensitive phase of growth. Granulosa cells contain mRNA for FSH receptor (6) and bind radio-labeled FSH (7) beginning in the early preantral stage (as soon as two layers of cuboidal cells appear), and continuing throughout follicular development or advanced atresia. As follicles continue to grow, one to three follicles are selected for continued growth and the remaining follicles become atretic. Follicles, particularly granulosa cells produce the protein hormone inhibin (8,9) that accumulates in follicular fluid (10) and
circulates in concentrations proportional to follicular growth (11). Inhibin acts synergistically with E₂ on the gonadotropes of the anterior pituitary gland to suppress FSH secretion (8,12) but not LH secretion (13–16). FSH deprivation induces atresia in the smaller follicles, which can be reversed with administration of PMSG (17). The larger follicle(s) are instead supported by LH, because there is a robust increase in LH receptor content in granulosa cells in follicles 4-6mm in diameter compared to smaller follicles as detected by radio-ligand binding (7). In sheep, there is weak evidence for follicular dominance as described in other species (18). Instead the cohort of growing follicles is likely thinned to the ovulatable follicle(s) by atresia in smaller follicles that do not contain LH receptor. Early follicular growth is dependent on FSH, but after achieving some growth these follicles require high frequency LH pulses to grow to into ovulatory follicles (19).

Some, but not all reports have described the pattern of follicular development as wave-like, such that 2-3 ‘waves’ of follicle development, each 5-6 days long, occur each cycle (3,20–23). During each wave a cohort of follicles is recruited, from this pool a few (one to three) growing follicles are selected with the remaining follicles regressing through atresia (24). During the terminal follicular wave of the estrous cycle, the dominant follicle(s) will continue to develop until ovulation, during other waves, these follicles will become atretic (25).

An important function of the growing follicles is E₂ production. Androgens are produced in the theca cells which diffuse into the granulosa cells that contain aromatase, which is necessary for production of E₂. Synthesis of E₂ is stimulated by pulsatile LH secretion (26); pulses of E₂ are observable following pulses of LH (27). Steroid synthesis likely commences just prior to antrum formation with the induction of the essential enzymes for androgen production (steroid acute regulatory protein, P450 side chain cleavage, 17α-hydroxylase and 3β
hydroxysteroid dehydrogenase) in the theca cells and aromatase in granulosa cells (28). Synthesis of E$_2$ increases with follicle development and follicle diameter is correlated with follicular fluid E$_2$ concentrations (20).

The ovulatory cascade is initiated by increased cAMP in theca and granulosa cells induced by surge secretion of LH. Ejection of the oocyte is accomplished by enzymatic degradation of the ovarian stroma involving matrix metalloproteases and collagenases (29) and an increase in follicular pressure caused by hyperemia and smooth muscle contraction (30). The preovulatory rise in LH also stimulates urokinase-type plasminogen activator in the ovarian epithelium which targets enzymatic degradation to the apical follicular wall resulting in stigma formation and ultimately rupture (31,32). In oocytes, the LH surge reduces cAMP which allows the resumption of meiosis in preparation for fertilization (33).

Following ovulation, theca and granulosa cells are transformed into the small and large luteal cells that form the CL (34). Development of the CL is associated with marked changes in cell function and vascularization of the tissue. In ruminants, large luteal cells constitutively produce P$_4$, whereas LH stimulates P$_4$ secretion from small luteal cells via activating phosphorylation of steroid acute regulatory protein (35). In sheep, the CL is maintained by LH and growth hormone (36).

The luteal phase is terminated with the regression of the CL, which is caused by uterine derived prostaglandin F2α secretion in sheep (37–39). The luteolytic properties of prostaglandin F2α are mediated by multiple pathways including intra-luteal production of oxytocin (40), reduced blood flow (41), reduced P$_4$ synthesis (42), and induction of apoptosis (43). Ultimately
functional (cessation of P₄ production) and structural (remodeling of the CL tissue) demise of the CL occurs.

HYPOTHALAMIC REGULATION OF THE ESTROUS CYCLE

HYPOTHALAMIC ANATOMY

The major neuro-circuits that regulate steroid feedback mechanisms necessary for the estrous cycle reside in the hypothalamus. Other brain regions also participate in regulation of reproduction and sensing or mediating extrinsic and intrinsic signals that are ultimately integrated in the hypothalamus, including the brainstem, amygdala, and pineal gland. The hypothalamus is located in the basal portion of the diencephalon, above the pituitary gland and below the thalamus. The hypothalamus consists of numerous nuclei, which are clusters of neuronal soma, that are typically named for their location. The major regions important for regulation of reproduction include the arcuate nucleus (ARC), the ventral medial nucleus (VMN), the retrochiasmatic area (RCh), and the preoptic area (POA). The mediobasal hypothalamus (MBH) is a larger region encompassing the ARC, VMN, RCh and surrounding areas; as the name implies it is located in the ventral medial region of the hypothalamus. The median eminence is located beneath the third cerebral ventricle (3V) at midline and is the site of the primary capillary bed of the hypothalamic-pituitary portal system, which carries neurohormones produced in the hypothalamus to their site of action in the anterior pituitary (Figure 1).

The ARC is located in the ventral medial portion of the hypothalamus near the 3V and above the median eminence. This nucleus extends in the rostral-caudal direction a considerable distance and is often divided into rostral, middle and caudal portions. In sheep, common criteria
for the division between the anterior and middle ARC is the rostral aspect of the tubero-infundibular sulcus. The middle and caudal ARC are often separated at the rostral portion of the mammillary recess. The VMN is located dorsal to the ARC and medial to the fornix (a large fiber tract connecting the hippocampus and mammary bodies). The RCh is located just caudal to the optic chiasm at the ventral aspect of the hypothalamus. The POA is located in the brain directly above the optic chiasm, surrounding the rostral portion of the 3V.

In sheep, neurons that produce GnRH are distributed throughout the hypothalamus, the four major populations are in diagonal band of Broca, POA, anterior hypothalamic area (located between the POA and MBH) and in the MBH (44). The external zone of the median eminence contains a dense plexus of GnRH containing fibers where GnRH is released into the portal blood.

Figure 1: Sagital section of the ovine hypothalamus. POA, preoptic area; OCh, optic chiasm; RCh, retrochiasmatic area; VMN, ventral medial nucleus; ARC, arcuate nucleus; ME, median eminence; MB, mammary bodies.
PULSATILE SECRETION OF LH

Throughout much of the estrous cycle (and in OVX ewes) LH is secreted from the anterior pituitary in a pulsatile pattern (45) in response to pulsatile secretion of GnRH from the hypothalamus (46). During the breeding season, $E_2$ suppresses LH pulse amplitude whereas $P_4$ suppresses LH pulse frequency (47). Suppression of pulse amplitude by $E_2$ is mediated within the hypothalamus, evident by a decrease in GnRH pulse amplitude (48), and in the pituitary gland by reducing the sensitivity to GnRH (47). The ability of $P_4$ to suppress pulse frequency requires recent $E_2$ exposure (49), which increases $P_4$ receptor content within the hypothalamus in numerous species (50–54). Throughout most of the estrous cycle, $E_2$ and $P_4$ suppress LH secretion and is referred to as ‘negative feedback.’

The organized episodic pattern of GnRH and LH release has led to the concept of a pulse generator to develop and synchronize discrete pulses of GnRH release. Several lines of evidence support the hypothesis that the GnRH pulse generator is located in the MBH in sheep. Neural deafferentiation immediately caudal to the optic chiasm or POA did not disturb pulsatile pattern of LH secretion (55). However, cuts through the ARC did ablate the pulsatile pattern of LH secretion indicating that pulse generation does not require input from rostral areas but neuronal connections within the MBH are essential for generation of pulses. Further evidence that the GnRH pulse generator is located in the MBH comes from the report that bursts in multi-unit activity recorded in the MBH are highly correlated to LH secretion events (56).

a. THE KNDy HYPOTHESIS FOR GnRH PULSE GENERATION

The current prevailing model for generation of GnRH pulses involves paracrine and autocrine actions of neurons in the ARC that contain the peptides: kisspeptin, neurokinin B
(NKB), and dynorphin (Dyn) and the steroid receptors: ERα (57) and progesterone receptor (58).

A role for kisspeptin signaling in the regulation of reproduction was identified by genetic screening in humans with hypogonadotropic hypogonadism. Some of these patients were found to harbor mutations in the gene encoding the receptor for kisspeptin (GPR54 or Kiss1R) (59,60). Subsequent studies have identified mutations in the kiss1 gene (which encodes kisspeptin) that cause hypogonadotropic hypogonadism (61) and activating mutations in the Kiss1R gene that are associated with precocious puberty (62). There are two populations of hypothalamic kisspeptin containing cells: a large population in the ARC and a smaller population in the POA. In sheep and primates, the kisspeptin cells are scattered in the preoptic area, whereas in rodents these cells are more densely arranged in the rostral periventricular region of the third ventricle (RP3V). Kisspeptin or Kiss1R knock-out mice have small gonads and low gonadotropin concentrations (59,63). In sheep, iv (64,65) or icv (66) kisspeptin injection increased GnRH and LH secretion and icv infusion of a Kiss1R antagonist inhibited pulsatile LH secretion (65). Neurokinin B is expressed in the mediobasal hypothalamus and icv injection of NKB (67,68) or neurokinin receptor 3 (NK3R) agonist (sentkide) (66,67,69) also stimulated LH secretion in sheep. Conversely, antagonists to NK3R inhibited pulsatile LH secretion in sheep when administered into the lateral ventricle (66) or placed directly into the ARC (70). Similar to kisspeptin, mutations in the genes that encode NKB and NK3R cause hypogonadotropic hypogonadism in humans (71). The endogenous opioid peptide Dyn acts via the κ-opioid receptor (KOR) to inhibit LH secretion and is critical for P₄ negative feedback (72).

Several groups independently proposed a model for pulse generation in which NKB is first released and acts on KNDy cells to stimulate kisspeptin release, dynorphin is subsequently released and acts to inhibit kisspeptin release. In this model kisspeptin is the output signal from
The KNDy neurons which acts on GnRH neurons to stimulate GnRH and LH release during a pulse (73–77). Kisspeptin is released in the median eminence in a pulsatile pattern that is highly correlated with GnRH release in monkeys (78). Administration of NKB or an antagonist to KOR increased, and dyn inhibited volleys of multi-unit activity recorded in the ARC and LH pulses in goats (74). Kisspeptin administration increased LH secretion, but did not alter multi-unit activity, which was interpreted to indicate that kisspeptin acts downstream of the pulse generator to stimulate GnRH release. GnRH cells in both the POA and MBH as well as GnRH fibers in the median eminence receive inputs from kisspeptin containing fibers (77,79) furthermore GnRH cells in the POA contain mRNA for Kiss1R in sheep (65).

KNDy cells are part of a highly interconnected network in which KNDy cell soma receive synaptic input from KNDy peptide containing fibers in sheep and rodents (77,79–81). Additionally KNDy cells contain NK3R (82) and KOR (83), but not Kiss1R mRNA (65). Administration of receptor agonists and antagonists directly into the ARC in sheep further support the model where NKB induces LH pulses and Dyn inhibits LH pulses. Micro-implants containing NKB or a KOR antagonist increased LH pulse frequency and NK3R antagonist containing implants decreased LH pulse frequency (70). Senktide or NKB administration increased LH concentrations and c-Fos in KNDy cells in sheep (84), goats (85), and rats (86). NKB or senktide increased spike firing in KNDy cells, but did not directly stimulate GnRH cells (87,88). The stimulatory effects of NKB on LH secretion in mice (89), rats (90), and primates (91) is dependent on functional Kiss1R, further illustrating that NKB acts via KNDy cells to cause kisspeptin release which stimulates LH secretion.

Progesterone and E2 alter GnRH secretion in indirect ways because GnRH neurons do not contain estrogen receptor alpha (ERα) or progesterone receptor (92,93). Although some GnRH
neurons contain ERβ (94), there is no evidence that activation of ERβ alters gonadotropin secretion in sheep (95,96). Additionally, ERα but not ERβ, knock-out mice have impaired gonadotropin responses to E2 treatment (97). Thus it is likely that effects of steroids on GnRH secretion are conveyed via action on neuronal afferents to GnRH cells that contain ERα.

Central to their role in steroid negative feedback, the expression of KNDy peptides and activation of KNDy cells is regulated by steroids. Cells containing mRNA for NKB are hypertrophied in post-menopausal woman (a natural condition of estradiol withdrawal) (98). In sheep, acute treatment with E2 decreased mRNA for NKB in P4 primed OVX+E2 ewes (99). Gonadectomy increased the number of NKB –immunoreactive (-ir) cells in the ARC in pre and post pubertal sheep (100). The number of cells containing kiss1 mRNA and the amount of kiss1 mRNA per cell is increased by OVX and reduced by E2 or P4 (101). Similarly, the number of kisspeptin-ir cells is increased by OVX in pre-pubertal (100) and adult sheep (102) and was reduced with E2 treatment (103). In the caudal ARC, kiss1 mRNA is reduced by E2 or E2 plus P4 treatment in OVX ewes, and is correlated with mean LH secretion and LH pulse amplitude (96). In sheep, OVX increases the percentage of kisspeptin cells that contain c-Fos in the ARC (102). Deletion of ERα specifically from NKB containing neurons (KNDy cells) in mice disrupts estrous cyclicity and causes elevated LH concentrations (104). Together these data support the theory that steroid negative feedback is mediated by altered expression of NKB and kisspeptin, and KNDy cell activation, such that steroids inhibit expression of peptides that stimulate LH secretion. Contrary to an abundance of evidence supporting steroid regulation of KNDy cells, one study reported no difference in firing frequency of ARC kisspeptin cells from OVX, diestrus or proestrus mice (105).
Thus, there is functional evidence and an anatomical framework by which three neuropeptides contained in a single population of neurons in the ARC generate pulsatile LH secretion. Moreover, these cells are regulated by ovarian steroids (E$_2$ and P$_4$) to facilitate negative feedback actions on gonadotropin secretion. However, these studies do not preclude the possibility that other neuropeptides or transmitters contribute to steroid negative feedback or regulation of pulsatile LH secretion.

**SURGE SECRETION OF LH IN SHEEP**

Surge secretion of LH occurs once per estrous or menstrual cycle and causes resumption of meiosis and ovulation. An LH surge is characterized by very high and prolonged secretion of LH, often over 100ng/mL and lasting hours in sheep. The LH surge is accompanied by a GnRH surge detectable in the hypothalamic-pituitary portal blood, that lasts several hours longer than the LH surge detected in the peripheral circulation (106). The GnRH and LH surge (hereafter referred to as LH surge) is caused by high and sustained E$_2$ concentrations (106,107). This condition of E$_2$ stimulating LH secretion is often referred to as ‘positive feedback.’ The LH surge is blocked by P$_4$ (108) acting via classical progesterone receptor (109). Although E$_2$ can stimulate an LH surge without prior P$_4$ exposure, normal amplitude GnRH surge requires prior exposure to P$_4$ (110). Additionally, depending on the duration and concentration of P$_4$ exposure, P$_4$ pretreatment delays the occurrence of the LH surge (111). Thus P$_4$ has an important role in the preparation of neural circuits for an LH surge, though P$_4$ itself bocks the LH surge.

a. **SITES OF ACTION OF E$_2$**

The generation of a surge in GnRH release implies a central site of action for E$_2$ to elicit the LH surge. Implants containing E$_2$ placed into the VMN, and not other places in the
hypothalamus, induced LH surge secretion in ewes (112). In one study, deafferentation of rostral hypothalamic areas prevented, or greatly reduced, LH surge secretion in OVX ewes treated with estradiol benzoate and blocked ovulation in intact ewes (55). Reciprocal tract-tracing experiments have demonstrated that soma in the VMN project to the POA (113,114). During the LH surge, GnRH neurons throughout the hypothalamus (including both POA and MBH) are activated (as determined by c-Fos expression) (115). Together, these studies provide a framework in which E2 acts in the MBH and that signal is transmitted though rostral hypothalamic circuits to ultimately cause GnRH release. Additionally, E2 potentiates gonadotropin release during the LH surge by increasing the sensitivity of gonadotropes to GnRH (116). In one study, ewes with surgical disconnection of the hypothalamus and pituitary treated with invariant pulses of GnRH are able to elicit negative and positive feedback effects of E2 (117). Though estradiol benzoate injection did increase LH secretion in this model, the magnitude of this effect was much lower than a typical LH surge, illustrating involvement of hypothalamic circuits in generation of an LH surge.

b. KISSPEPTIN AND NKB

Since its discovery, discussed above, the role of kisspeptin in regulation of LH surge generation has been actively studied in numerous species. In sheep, kisspeptin infusion induced an LH surge in anestrus and hastened and synchronized the occurrence of an LH surge during the breeding season (64). Kisspeptin receptor blockade blunted (~50%) an estradiol benzoate induced LH surge in anestrus ewes (65).

Three studies have examined c-Fos expression in kisspeptin cells during the LH surge. One hour after a bolus injection of E2, known to induce an LH surge in OVX ewes, there was an
increase in c-Fos only in ARC kisspeptin cells, similarly there was a trend for an increase in the percentage of kisspeptin cells that contained c-Fos during the LH surge (118). A second report identified an increase in c-Fos only within POA kisspeptin cells during an LH surge in intact ewes synchronized with E\textsubscript{2} and P\textsubscript{4} (these animals had also undergone neurosurgery for other experiments) (119). A third report identified an increase in c-Fos in both ARC and POA kisspeptin cells in intact ewes killed during behavioral estrus with elevated LH concentrations (120). Thus, there is evidence for activation of both populations of kisspeptin neurons during the LH surge in sheep.

Neurokinin B has also been implicated in the LH surge in ewes. Senktide injection into the 3V induces surge-like secretion of LH evident by the very high (greater than 5 fold increase) and sustained release of LH. Micro implants (22 gauge tubing) containing crystalline senktide placed into the RCh or POA, but not ARC or periventricular region caused a similar surge-like release of LH (69). Application of an NK3R antagonist to the RCh, but not POA, blunted (~50%) the amplitude of an E\textsubscript{2} induced LH surge in OVX ewes (69). Activation of NK3R containing cells in the RCh induced c-Fos expression in the ARC kisspeptin cells and the stimulatory effects on LH secretion has been blocked by icv application of a Kiss1R antagonist (84). Neurons in the RCh that presumably contain NK3R project onto KNDy cells in the ARC, however, the phenotype of these cells is unknown (84). Together, these studies demonstrate a role for kisspeptin and NKB in the generation of the full LH surge. Of note, is that application of either the Kiss1R or NK3R antagonist blunted the LH surge by only about half. Though pharmacological limitations could account for this, other signaling molecules likely contribute to the generation of the LH surge.
BRIEF COMPARISONS TO THE HUMAN MENSTRAUL CYCLE

Compared to sheep (and other domestic livestock species), humans have a relatively short follicular phase lasting three days compared to approximately 14 in humans. The primary cause of this difference is likely that the CL in humans/primates, unlike sheep, secrete E$_2$ and inhibin which act to potently suppress FSH secretion and therefore follicular development (17). As a result, follicles in humans are relatively smaller at the end of the luteal phase compared to sheep (121), and therefore require extended gonadotropin support to achieve growth sufficient to stimulate ovulation. The surge generation mechanisms also differ between sheep and humans: unlike sheep, complete neural deafferentation of the MBH does not prevent ovulation in monkeys (122).

BRIEF COMPARISONS TO THE RODENT ESTROUS CYCLE

The length of estrous cycles differ markedly in sheep (17 days) and rodents (4-5 days). The rodent estrous cycle consists generally of one day of proestrus, one day of estrus and 2 days of diestrus. Ovulation occurs on the evening of proestrus and CL form, however these structures do not secrete sufficient P$_4$ to support a decidua reaction. Coitus or mechanical stimulation of the cervix stimulates secretion of prolactin (the luteotropin in rodents) to maintain the CL and stimulate P$_4$ secretion for 12 days which allows for decidua reaction and implantation (123).

The relative importance of NKB signaling in rodents appears to differ from other species. In humans, mutations in the genes that encode NKB or its receptor cause hypogonadotrophic hypogonadism (71). But in mice, genetic deletion of tac2 (encodes NKB) or tacr2 (encodes receptor for NKB) yielded modest effects only in females (124) or no reported effect on reproduction (125), respectively. In OVX rats, pulsatile LH secretion was inhibited by
administration of a cocktail of NK1R, NK2R and NK3R receptor antagonists, but not by any one antagonist alone. Similarly, ARC kisspeptin cell firing induced by NKB was only inhibited by a cocktail of all three receptor antagonists and not any single antagonist alone in mice (126). Therefore it is possible that in rodents the other tachykinins and tachykinin receptors can provide compensation to maintain gonadotropin secretion. In sheep, high doses of Neurokinin A (relative to NKB) or higher doses of substance P stimulate LH secretion. The relative potency of these molecules matches the relative affinity of these molecules for NK3R, which likely means NKB-NK3R signaling is more important for LH secretion than the other tachykinins in sheep (68).

There are also critical differences in the LH surge generating mechanisms in rodents compared to sheep. First, E2 implants placed into the POA, and not elsewhere, stimulate surge LH secretion (127). Several lines of evidence support the hypothesis that the LH surge is mediated by the rostral population of kisspeptin neurons. Estradiol increases kiss1 mRNA within the rostral population of kisspeptin cells in OVX rats (128) and kiss1mRNA abundance is highest during the evening of proestrus (129), while similar changes were not observed in the ARC. Additionally, c-Fos expression was increased in this kisspeptin population during the evening of proestrus in mice and rats (129–131). Full expression of the LH surge in OVX and E2-primed rats requires P4 and E2 (132). Whereas in sheep, E2 alone is sufficient the stimulate an LH surge of normal amplitude (133). Additionally, circadian inputs from the suprachiasmatic nucleus (134) are critical in generating the LH surge in rats which only occur in the afternoon and at night (135).
SEASONAL BREEDING IN SHEEP

Sheep, like most mammals, are seasonal breeders (humans and laboratory rodents are notable exceptions). Seasonal breeding is an effective strategy for synchronizing the birth of young with seasonal variations in resources necessary for their survival. Ewes are fertile in the fall, when day length is shortening and are referred to as short day breeders. Photoperiod is the environmental factor used to time reproduction in sheep. Melatonin is secreted from the pineal gland at night (136). Photoperiod (technically length of night) is encoded by the duration of melatonin secretion which acts to synchronize circannual rhythms of fertility and sensitivity to E2 negative feedback in sheep (137,138). The inhibition of gonadotropin secretion during the anestrus season is mediated by steroid-dependent and a more modest steroid-independent mechanism evident in OVX ewes.

a. STEROID-DEPENDENT SUPPRESSION OF GONADOTROPIN SECRETION

During seasonal anestrus, but not the breeding season, low concentrations of E2 inhibit LH pulse frequency (139). This heightened sensitivity to E2 negative feedback occurs during anestrus (140) due, at least in part, to activation of an inhibitory A15 dopaminergic neurons with cell bodies in the RCh (141–145) and project to the MBH (143). Moreover, KNDy cells receive direct input from dopaminergic terminals (146) and contain the relevant dopamine receptor (D2R) (147). Administration of D2R stimulates LH in a kisspeptin dependent manner (147). In anestrus, E2 acts in the RCh (95) and the POA (148) to suppress LH via afferents to A15 dopaminergic neurons, as A15 neurons do not contain ERα (93,149).
b. **STEROID INDEPENDENT SUPPRESSION OF GONADOTROPIN SECRETION**

Evidence for steroid-independent suppression of gonadotropin secretion stems from the observation that OVX ewes during anestrus have a lower LH pulse frequency than OVX ewes during the breeding season (139). The only signaling molecule that has been implicated in this phenomenon is serotonin. Serotonin receptor blockade (methysergide or cyprohetadine) increased LH pulse frequency in OVX ewes in anestrus but not during the breeding season (150,151). Serotonin depletion during anestrus also increased LH pulse frequency in OVX ewes, but had no effect in OVX ewes treated with E₂ (151). However a 5-HT receptor antagonist (cyprohetadine) increased LH pulse frequency in E₂ treated OVX ewes (152), but not in intact ewes (153), during anestrus. Thus, it is likely that the steroid-independent effects of serotonin to suppress LH are present in E₂ treated ewes, in which LH is suppressed by a combination of E₂ and these other steroid-independent factors. Furthermore, selective (ketanserin and methysergide) and non-selective (cyprohetadine) 5-HT receptor antagonists all increased LH pulse frequency, data that are consistent with action at the 5-HT₂ receptors (154).

**SOMATOSTATIN**

Somatostatin (SST) is a 14 or 28 amino acid peptide cleaved from preproSST protein. SST is produced in diverse tissues throughout the body, including many portions of the brain. SST immunoreactive neurons have been detected throughout the ovine hypothalamus, including populations in the ARC, VMN, POA and paraventricular region (155). The diverse effects of SST are mediated by five isoforms of the somatostatin receptor (SSTR1-5). All isoforms are G-alpha inhibitory type G-protein coupled receptors. Activation of these receptors inhibits
adenylate cyclase activity and causes membrane hyperpolarization by increasing potassium channel conductance (156).

The best known effects of SST are to suppress growth hormone secretion from the anterior pituitary, opposing the actions of growth hormone releasing hormone (157). Additionally, SST suppresses thyroid stimulating hormone pulse frequency and amplitude (158). In rats, only SST cells in the periventricular nucleus project to the median eminence (159).

Somatostatin has been demonstrated to suppress LH secretion in humans, sheep and rodents. In humans, iv SST infusion (158) and subcutaneous injections of the SST receptor 2 (SSTR2) agonist octreotide (160) suppressed LH secretion. In rodents, SST inhibited GnRH release from hypothalamic explants (161) and GnRH spike firing from hypothalamic slices in mice (162) and rats (163). Additionally, a SSTR2 agonist blunted the LH surge and prevented the typical increase in the percentage of GnRH cells expressing c-Fos during the LH surge (164). Thus there is both in vitro and in vivo data for action of SST within the hypothalamus of rodents to inhibit LH secretion.

The primary site of action for SST in rodents is likely the POA, as SST has direct actions on GnRH cells and GnRH cells contain mRNA for SST receptors (162,163). In mice, SST fibers have been observed in close apposition to GnRH soma (162). However in rats, SST fibers have not been observed in close apposition to GnRH soma and only rarely in apposition to GnRH fibers, which raises the possibility of SST acting via volume transmission (163).

In sheep, icv infusion of SST inhibits pulsatile LH secretion (165), however most work has focused on the LH surge. Several lines of evidence suggest that SST may be involved in surge secretion of LH. First, approximately 30% of the SST cells in the VMN (site of E2 positive
feedback discussed above) contain ERα (155,166). Second, ewes treated with E₂ implants known to cause an LH surge had more preproSST per cell within the VMN than control treated ewes. However, the increase in preproSST mRNA was detected at a time when E₂ inhibits LH secretion (before the LH surge) (165). Third, the percentage of SST cells that contained c-Fos within the VMN was greater in ewes with naturally occurring LH surges (167) or anestrus ewes treated with estradiol benzoate (155) compared to early follicular phase ewes or oil treated anestrus ewes, respectively. Finally, prenatally androgenized ewes, which do not display surge type LH secretion, had a lower percentage of SST cells that contained c-Fos following E₂ implant insertion compared to normal ewes (168).

SST may act in the POA of sheep as SST fibers have been observed in close apposition to GnRH cells in the POA (168) and a preliminary report indicate that GnRH cells in the POA contain SSTR2 (but not the other SST receptors) as detected by immunohistochemistry (169). Thus, the hypothalamic site of action for SST may be direct action at the GnRH neurons within the hypothalamus.

**SPECIFIC AIMS**

As review above, several studies have shown that exogenous SST inhibits LH secretion in sheep, rodents and humans. However, there is no clear evidence that endogenous release of SST plays an important role in control of LH. Therefore, we propose 3 aims to investigate the role of endogenous SST in the regulation of gonadotropin secretion in sheep and to study the neuroanatomical substrates that may mediate the effects of SST on LH secretion.

**Specific Aim 1:** To determine if endogenous SST alters pulsatile or surge secretion of LH. We hypothesize that endogenous SST inhibits pulsatile and surge secretion of LH in sheep. To determine if SST contributes to LH surge secretion, we administered a SSTR2 agonist and
antagonist to ewes during the LH surge. To determine if SST contributes to pulsatile LH secretion, we administered an SSTR2 antagonist to ovary intact ewes during anestrus and luteal phase of the estrous cycle. Additionally, we measured c-Fos expression in GnRH and kisspeptin cells in ovary-intact anestrous ewes following SSTR2 blockade. To determine if the stimulatory effects of SSTR2 blockade on LH secretion was dependent on steroids, we applied the antagonist to OVX ewes during both the breeding season and anestrus.

**Specific Aim 2:** To determine whether SST could act directly on GnRH or KNDy cells, we analyzed SST synapses onto GnRH or KNDy cells in ewes from throughout the estrous cycle and anestrus.

**Specific Aim 3:** To determine whether SST cells in the VMN are activated during the LH surge. First we confirmed that c-Fos is increased in SST cells in the VMN during the LH surge. Because neither a SSTR2 agonist nor antagonist altered LH surge secretion in Chapter 2 (Aim 1), we hypothesized that SST cells in the VMN produce additional signaling molecules that may contribute to the LH surge. To test this possibility, we examined SST and nNOS colocalization in the VMN. We analyzed c-Fos expression during the surge in the subset of neurons that contain both SST and nNOS. We also determined whether c-Fos expression in SST cells was increased during surge-like secretion of LH caused by senktide administration to the RCh or POA.
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CHAPTER 2

EVIDENCE THAT ENDOGENOUS SOMATOSTATIN INHIBITS EPISODIC, BUT NOT SURGE, SECRETION OF LH IN FEMALE SHEEP


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ABSTRACT

Two modes of gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) secretion are necessary for female fertility: surge and episodic secretion, however the neural systems that regulate these GnRH secretion patterns are still under investigation. The neuropeptide somatostatin (SST) inhibits episodic luteinizing hormone (LH) secretion in humans and sheep, and several lines of evidence suggest SST may regulate secretion during the LH surge. In this study, we examined whether SST alters the LH surge in ewes by administering a SST receptor 2 (SSTR2) agonist (octreotide) or antagonist (CYN154806; CYN) into the third ventricle during an estrogen-induced LH surge, and whether endogenous SST alters episodic LH secretion. Neither octreotide nor CYN altered the amplitude or timing of the LH surge. Administration of CYN to intact ewes during the breeding season or anestrus increased LH secretion and increased c-Fos in a subset GnRH and kisspeptin cells during anestrus. To determine if these stimulatory effects are steroid-dependent or -independent, we administered CYN to ovariectomized ewes. This SSTR2 antagonist increased LH pulse frequency in ovariectomized ewes during anestrus, but not during the breeding season. This is the first evidence in any species that endogenous SST contributes to the control of LH secretion. The results demonstrate that SST, acting through SSTR2, inhibits episodic LH secretion, likely acting in the mediobasal hypothalamus, but action at this receptor does not alter surge secretion. Additionally, these data provide evidence that SST contributes to the steroid-independent suppression of LH pulse frequency during anestrus.
Précis

Neither a SSTR2 agonist nor antagonist altered the LH surge but the latter stimulated episodic LH secretion by increasing mean LH in intact ewes and increasing LH pulse frequency in ovariectomized anestrous, but not breeding season ewes.

INTRODUCTION

Ovarian function, and therefore fertility in females, is dependent on cyclical changes in two modes of gonadotropin secretion. Surge secretion of LH occurs once per estrous or menstrual cycle at the end of the follicular phase in response to positive feedback effects of $E_2$ and induces ovulation and luteinization. Pulsatile secretion of luteinizing hormone (LH) is necessary for folliculogenesis and steroidogenesis, and is regulated by negative feedback effects of estradiol ($E_2$) and progesterone, with progesterone inhibiting LH pulse frequency and $E_2$ inhibiting pulse amplitude (1). These actions result in changes in LH pulse patterns during the normal ovarian cycle that are important for the control of ovulation because the increasing episodes of LH secretion during the follicular phase drive the pre-ovulatory rise in circulating $E_2$.

Increased LH pulse frequency also underlies the transition from infertility to reproductive competence at the onset of puberty and the transition into the breeding season. In several species, these changes are controlled by both steroid-dependent (changes in response to the negative feedback actions of $E_2$ or testosterone) and steroid-independent (evident in gonadectomized animals) inhibition of LH pulse frequency, but the importance of each varies with species (2). In sheep, the steroid-dependent suppression, which reflects an increased ability of $E_2$ to inhibit gonadotropin-releasing hormone (GnRH) and LH pulse frequency prior to puberty and during the non-breeding (anestrous), is more dramatic than the modest decrease in...
GnRH and LH pulse frequency seen in ovariectomized (OVX) animals (3). In contrast, steroid-independent suppression of LH is evident and critical for prepubertal suppression of LH in the agonadal monkey (4,5) and children (6,7).

The neurons that produce GnRH do not contain estrogen receptor alpha (ERα) or progesterone receptor (8–10), implicating a role for neuronal afferents to GnRH cells that convey the negative and positive feedback signals. The neuropeptide somatostatin (SST) is produced throughout the CNS, including the hypothalamus and preoptic area (POA), and may contribute to the regulation of both episodic and surge secretion of LH. SST exerts its effects via five different G\_i/o protein-coupled receptors (SSTR1-5). Activation of these receptors inhibits adenylate cyclase activity and opens potassium channels, resulting in membrane hyperpolarization and therefore reduced responsiveness to stimulatory input (11). Consistent with a generally inhibitory effect, SST inhibited GnRH release from rat hypothalamic explants (12) and spike firing in GnRH cells in mice (13) and rats (14). Furthermore, these actions are likely mediated, primarily through SSTR2 because the SSTR2 agonist, seglitide, produced similar inhibition of spike firing and membrane hyperpolarization as SST in mice (13).

SST neurons in the ventromedial nucleus (VMN) of the ovine hypothalamus contain ERα (15,16) and several lines of evidence suggest that they may be involved in the positive feedback actions of E\_2 in ewes (female sheep). First, the VMN is considered the site of E\_2 positive feedback in ewes because E\_2 microimplants placed into this area, but not elsewhere, induced an LH and GnRH surge (17). Second, ewes treated with E\_2 implants known to induce an LH surge 24 hrs later had more preproSST mRNA per cell (in the VMN) than ewes that did not receive E\_2.
implants (18). It should be noted that the increase in preproSST mRNA was observed shortly (4 hrs) after E₂ implants were inserted, a time when E₂ inhibits LH secretion. Third, c-Fos expression was higher in SST neurons within the VMN of ewes at the time of naturally occurring LH surges (19) and in anestrous ewes treated with estradiol benzoate than in controls (16). Finally, intracerebroventricular (icv) administration of the SSTR2 agonist, octreotide, blunted the LH surge and prevented the typical increase in c-Fos within GnRH cells, in rats (20). More direct evidence exists for a possible role for SST in regulation of pulsatile LH secretion. Infusion of SST into the third ventricle using an early follicular phase model in ewes inhibited mean LH concentrations and the number of LH pulses (18). Similarly, intravenous (iv) SST infusion into healthy humans (women in the follicular phase of menstrual cycle and men) suppressed mean LH concentrations (21) and octreotide suppressed pulse amplitude and mean LH concentrations in women with polycystic ovarian syndrome (22).

In this study, we tested the hypothesis that SST, acting via SSTR2, acts in the hypothalamus to suppress both the LH surge and episodic LH secretion in ewes. We targeted SSTR2 based on evidence that this receptor mediates the actions of SST in rodents (13,20) and that GnRH cells in the preoptic area of sheep contain SSTR2, but not the other SST receptor subtypes, as determined by immunohistochemistry (23). To determine the role (if any) of SST on the generation of the LH surge, we administered either a SSTR2 agonist or antagonist to ewes during an E₂-induced LH surge. To determine if endogenous SST alters episodic LH secretion, we administered a SSTR2 antagonist to ovary-intact breeding season ewes during the luteal phase (when progesterone is the dominant suppressor of LH), to ovary-intact ewes during anestrus (when E₂ is the dominant suppressor of LH) and to OVX ewes during both the breeding and anestrous season. Previous work in sheep had provided no evidence on the neural sites of
action of SST. Therefore, we administered the SSTR2 agonist and antagonist into the third ventricle because this approach has the advantage of testing a functional role for SST signaling without a priori information of sites of action.

MATERIALS & METHODS

Animals

Multiparous mature ewes (>3 years old) of mixed breeding were maintained in a light and temperature controlled research building. Lighting conditions were adjusted every other week to mimic local natural lighting conditions. Ewes were fed a maintenance ration of cubed alfalfa hay and had free access to water and mineral blocks. Breeding season experiments were done in October and November and anestrous studies were done in May and June. All procedures were approved by the West Virginia University Animal Care and Use Committee and were performed in accordance with the NIH guidelines for the care and use of research animals.

General Methods

Surgical procedures: All surgeries were performed using aseptic techniques with anesthesia maintained with 2-5% isoflurane in oxygen. Wool was removed from the surgical site and skin was scrubbed with betadine solution. For OVX, the reproductive tract was externalized by mid-ventral laparotomy, the ovarian blood supply was ligated with suture and ovaries were removed. The remaining reproductive tract was rinsed with sterile saline to minimize adhesions and returned to the abdominal cavity. Peritoneum, subcutaneous layers and skin were closed with suture. For neurosurgery, an 18 gauge needle was prepared and placed in the caudal, basal portion of the third ventricle using a stereotaxic procedure as previously described (24). The cannula was cemented in place with dental acrylic, the needle hub was
plugged and covered with a plastic cap. Animals were treated pre- and post-operatively with analgesics (carprofen for both surgery types and gabapentin for neurosurgeries) and antibiotics (ampicillin for both surgery types and gentamycin for neurosurgeries). All veterinary drugs were acquired from Patterson veterinary (Bessemer, AL), except gabapentin which was compounded locally (McCracken Pharmacy, Waynesburg, PA). Animals were allowed to recover from surgery for at least 1 week before any experiments were performed.

**Drug preparation and blood sampling procedures:** Drugs for icv injection were prepared the night before injection and sterilized with UV irradiation. Animals were treated prophylactically with gentamicin before and after the blood sampling period. For studies of surge-type LH secretion, blood samples were collected at 2-4 hr intervals during the expected time of the LH surge. For studies of episodic LH secretion (Exp. 2-4), blood samples were collected at 12 min intervals from 2 hrs before until 4 hrs after drug or vehicle injection. For all experiments, jugular blood samples (3-4 mL) were collected via venipuncture and placed in heparinized tubes. Plasma was harvested and stored at -20 C until assayed for LH with RIA.

**Experiment 1: Does SST act in the hypothalamus to regulate surge-type LH secretion?**

In order to identify an effective dose of the selective SSTR2 agonist, octreotide, that would alter LH secretion, in a preliminary experiment, we administered 20 nmol of octreotide (Tocris Bioscience, Ellisville, MO) in 100 uL of artificial cerebrospinal fluid (aCSF) (25) to OVX ewes. LH concentrations were measured in blood samples collected at 12 min intervals from 3 hrs before to 7 hrs after drug administration. Octreotide was selected for these experiments because it has been demonstrated to alter LH secretion in rats (20) and humans (22). A single 20 nmol icv injection of octreotide suppressed mean LH (pre: 8.1 ± 1.5 ng/mL; post: 2.8
± 0.6 ng/mL) and pulse amplitude (pre: 8.7 ± 3.7 ng/mL; post: 2.3 ± 0.8 ng/mL) for the entire post injection sampling period (Fig. 1). However, inter-pulse interval (IPI) was not obviously altered (pre: 47.3 ± 6.4 min; post: 56.3 ± 6.7 min).

We used a well-established artificial follicular phase model in OVX ewes to test the effects of a SSTR2 agonist and antagonist on the E2-induced LH surge (26). Briefly, at the time of OVX and insertion of third ventricle cannula, a small (1 cm long) Silastic implant (inner diameter 0.34Cm, outer diameter 0.46 cm; Dow Corning Corp., Midland, MI) containing E2 was inserted subcutaneously (sc) and remained in place for the entire experiment. Two progesterone containing CIDRs (Zoetis, Parsippany, NJ) were also inserted vaginally at this time. Eight days later the CIDRs were removed and the next day 4 large (3 cm long) E2 implants were inserted sc and removed 3 days later. Blood samples were collected at 2 hr intervals for 36 hrs starting at the time of large E2 implant insertion, and then at 4 hr intervals for an additional 12 hrs. This treatment protocol reliably induces an LH surge 20-24 hrs after insertion of the large E2 implants. To test the effect of a SSTR2 agonist, six ewes were randomly assigned to receive icv injections of either the SSTR2 agonist, octreotide (20 nmol in 100uL) or aCSF every 8 hr for the first 24 hrs of blood sampling starting at the time of E2 implant insertion. This dosing regimen was chosen based on results from the preliminary experiment. Three days after removal of large E2 implants, this progesterone pretreatment, surge induction and experimental protocol (icv injections and blood sampling) was repeated with each ewe receiving the alternate treatment in a cross-over design.

To test the effects of the SSTR2 antagonist, seven adult ewes were OVX and a permanent cannula placed into the third ventricle at least 1 week before any experimental procedure. An LH surge was induced as described above. Ewes were randomly assigned to receive icv injections of
either the selective SSTR2 antagonist, CYN154806 (CYN) (27) (60 nmol in 100 uL; Tocris Bioscience, Ellisville, MO; n=4), or equal volume of saline (n=3) at 6 hr intervals for 24 hrs starting at the insertion of large E$_2$ implant treatment. This dose of antagonist altered pulsatile LH secretion in Exps. 2-5 below.

**Experiment 2: Does endogenous SST act in the hypothalamus to regulate tonic LH secretion in intact anestrous ewes?**

Eight ovary-intact anestrous ewes received a permanent cannula into the third ventricle in mid-April (non-breeding season). Ewes were randomly assigned to receive a single icv injection of CYN (60 nmol in 100uL sterile saline), or vehicle with 6 hrs of frequent blood collection. The protocol was repeated 4 days later in which each animal received the alternate treatment in a cross-over design.

**Experiment 3: Does endogenous SST act in the hypothalamus to regulate tonic LH secretion in luteal phase ewes?**

Eight ovary-intact ewes received a permanent cannula into the third ventricle in mid-October (breeding season). Estrous cycles were synchronized with 2 intramuscular injections of PGF2α (20mg, Lutalyse, Zoetis, Parsippany, NJ ) given 7 days apart (28). Eight days after the second injection, ewes were randomly assigned to receive a single icv injection of CYN (60nmol in 100uL sterile saline), or 100 μL of vehicle and frequent blood samples were collected for 2 hrs before and 4 hrs after injections as described above. The protocol was repeated 2 days later in which each animal received the alternate treatment in a cross-over design.
Experiment 4: Effects of SSTR2 blockade on GnRH and kisspeptin cell activation

To determine whether SST may act via GnRH or kisspeptin cells, we administered CYN or saline via icv injection and examined c-Fos expression in GnRH or kisspeptin cells using immunohistochemistry. Six days after the final sampling period in experiment 2, the same intact ewes were randomly assigned to receive either saline (n=4) or CYN (n=4). Animals were euthanized and hypothalamic tissue was collected 2 hrs after injection.

Ewes were treated with heparin (20,000 U) 10 min before and at the time of euthanasia, which was performed with an overdose (8-16 mL, iv) of sodium pentobarbital (Euthasol, Patterson Veterinary, Bessemer, AL). The head was rapidly removed and perfused with 6L of 4% paraformaldehyde in 0.1M phosphate buffer (PB) with 0.1% NaNO₃. A block of tissue including the hypothalamus and pre-optic area (POA) was removed and stored overnight in the same paraformaldehyde solution at 4°C. Tissue was then transferred to 20% sucrose in PB and maintained at 4°C. After sucrose infiltration, tissue was frozen and 45μm-thick-coronal sections were cut with a microtome with a freezing stage and collected in 10 series (450μm apart) and stored at -20°C in cryoprotectant solution (29).

For detection of GnRH and c-Fos, dual label immunohistochemistry was performed on one complete series of hemi-sections throughout the hypothalamus. All steps were performed at room temperature with mild agitation, unless noted differently. Tissue was rinsed in 0.1M PB 8 times (15 min each) and stored overnight in PB at 4°C. Tissue was rinsed 12 additional times in 0.1M phosphate buffered saline (PBS; 15 min each). Endogenous peroxidase activity was quenched by incubation in 1% H₂O₂ in PBS, after which tissue was rinsed 4 times for 5 mins each in PBS (typical rinsing step). Tissue was incubated in 4% normal goat serum (NGS; Jackson Immunoresearch, West Groove, PA) in PBS containing 0.04% triton X-100 (PBST) for
1 hr and then incubated in rabbit anti-c-Fos serum (dilution: 1:2000 with 4% NGS in PBST; SC-52, RRID: AB_2106783, Santa Cruz Biotechnology, Dallas, TX) for 17 hrs. Tissue was rinsed, and incubated sequentially in biotinylated goat anti-rabbit immunoglobulin (IgG; dilution: 1:500 in PBST with 4% NGS; BA-1000; Jackson Immunoresearch) for 1 hr, ABC-elite (dilution 1:500 in PBS; Vector Lab Burlingame, CA) for 1 hr and nickel enhanced 3′3′-Diaminobenzidine solution (10mg per 50mL PB with 2mL of 2% NiSO₄ and 20μL of 30% H₂O₂ to produce a black reaction product, Sigma-Aldrich, Saint Louis, MO) for 10 min with intervening rinse steps. Tissue was then rinsed in PB and placed into 1% H₂O₂ in PBS, rinsed and incubated in blocking solution containing 4% NGS in PBST for 1 hr, and was incubated in rabbit anti-GnRH serum (dilution: 1:2000 in PBST with 4% NGS, 20075, RRID:AB_572248, Immunostar, Hudson, WI) for 1 hr. Tissue was rinsed in PBS and incubated sequentially in biotinylated goat anti-rabbit IgG (dilution: 1:500 in PBST with 4% NGS), ABC-elite (dilution: 1:500 in PBS), and 3,3′-diaminobenzidine (10mg per 50mL PB with 20μL of 30% H₂O₂ to produce a brown reaction product) for 10 min. Tissue was rinsed in PB and mounted onto superfrost slides (Fisher Scientific, Pittsburgh, PA), once dried, cover glass was applied with Eukitt (Fisher Scientific, Pittsburgh, PA) mounting media.

The total number of GnRH cells and the percentage of GnRH cells that contained c-Fos was determined in the diagonal Band of Broca, POA, anterior hypothalamic area, and the mediobasal hypothalamus (MBH), (30) with a bright field microscope (Axiovert 400CFL, Zeiss) with a 20x objective. Values were averaged per animal. All cell counts were made by a single observer blinded to treatment groups.

Detection of c-Fos within kisspeptin cells was performed in one series of hemi-sections from throughout the arcuate nucleus (ARC), which contains the population of kisspeptin-
neurokinin B-dynorphin (KNDy) neurons (31,32), and in 2-4 hemi sections from the POA as described above except that 10% H₂O₂ and 20% NGS were used in blocking solutions and a kisspeptin antibody (dilution: 1:10K in PBST with 4% NGS; AB9754, RRID:AB_2296529, Millipore, Billerica, MA) was used following detection of c-Fos. The total number of kisspeptin cells and the number of kisspeptin cells that contain c-Fos were determined in 2-4 sections in the rostral, middle and caudal portions of the ARC (32) and POA. The mean number of kisspeptin cells per hemi section, and percent of kisspeptin cells that contained c-Fos were averaged per animal. All cell counts were made by a single observer blinded to treatment groups using a bright-field microscope (VS120 Olympus, Tokyo, Japan) with a 20x objective. Antibodies used to detect c-Fos (32,33), GnRH (34) and kisspeptin (35) have been validated and used extensively in sheep, additional details about these antibodies are presented in Table 1.

**Experiment 5: Does endogenous SST suppress LH in a steroid-independent manner?**

To determine whether the stimulatory effects of SSTR2 blockade observed in experiment 3 were dependent or independent of gonadal steroid inhibition of LH secretion, we administered CYN to six OVX ewes during anestrus (June). Seven to ten days after OVX, animals were randomly assigned to receive a single icv injection of CYN (60 nmol in 100uL sterile saline) or vehicle, with frequent blood samples collected for 6 hrs. They then received the alternate treatment 5 days later in a crossover design.

In light of the results of the first part of this study, we next determined if endogenous SST mediates the steroid-independent suppression of LH caused by inhibitory photoperiod by giving CYN to eight OVX ewes during the breeding season (mid-November). Animals were randomly
assigned to receive treatments (CYN or saline as described above) 6-9 days after OVX and received the alternate treatment 2 days later.

**Data Analysis**

*Radioimmunoassay*

LH concentrations were measured in duplicate as described previously (36) using a double-liquid phase RIA with reagents provided by the National Hormone and Peptide Program. The sensitivity of the assay averaged 0.077 ng/tube (NIH S24), the intra- and inter-assay coefficients of variation were 5.8% and 8.3%, respectively. Progesterone concentrations were determined using a RIA kit (MP Bioscience, Santa Anna, CA) according to the manufacturer’s directions. Progesterone was measured in duplicate in a single assay with a sensitivity of 0.012 ng/tube and an intra-assay coefficient of variation of 10.1%.

**Statistical Analysis**

For experiment 1, the time of maximal LH secretion and the amplitude of the LH surge (maximal concentration minus LH concentration of the first sample collected at time of insertion of large E2 implants) were compared between treatment groups with t-tests (paired t-tests for experiments conducted as cross-over designs). In studies of episodic LH secretion, pulses were identified with established criteria (1); briefly, a pulse had to be 2 assay standard deviations greater than the preceding nadir, the pulse had to occur within 2 samples of the preceding nadir and the amplitude had to be greater than the assay sensitivity. The total number of GnRH or
kisspeptin cells and the percentage of each that contained c-Fos within each area were compared between treatment groups with independent t-tests.

For experiments examining episodic LH secretion, mean LH concentration and LH pulse amplitude (maximal pulse value minus preceding nadir) were determined during the 2 hrs pre- and 4 hrs post-injection sampling period and analyzed by two-way ANOVA with repeated measures (time and treatment); Tukey’s multiple comparisons test was used to determine differences between individual points where appropriate. LH inter-pulse interval (IPI) was determined during the 4 hr post-injection period and was compared between treatment groups with paired t-test. Post-injection pulse latency (time from injection to the first pulse) and the amplitude of the first pulse after injection was compared between treatment groups with a paired t-test. P < 0.05 was considered statistically significant.

RESULTS

Effects of SSTR2 agonist and antagonist on surge type LH secretion

Estradiol treatment induced an LH surge in all animals. Administration of the SSTR2 agonist, octreotide, did not alter either the amplitude of the LH surge or the timing of the LH peak compared to aCSF treated animals (Fig 2, upper panels). Similarly, administration of the SSTR2 antagonist, CYN did not alter the amplitude or the peak time of the LH surge compared to saline treated animals (Fig 2, lower panels).

Effects of SSTR2 antagonist in anestrous ewes

One ewe apparently had an LH surge during one sampling period (evident by very high and sustained LH concentration patterns) and was thus removed from analysis. A single injection
of CYN into anestrous ewes induced pulse-like LH secretion in all 7 ewes within 36 min. Four of these ewes also had an LH pulse within 36 min after injection of saline (Fig 3). For mean LH, two-way ANOVA revealed a significant time by treatment interaction such that CYN significantly increased mean LH concentrations (Pre: 1.4 ± 0.2 ng/mL; Post: 4.3 ± 0.7 ng/mL), whereas saline injections did not alter mean LH (Pre: 1.3 ± 0.1 ng/mL; Post: 2.4 ± 0.4 ng/mL). Pulse amplitude during the post injection period tended (P=0.089) to be greater in animals that received CYN (4.7 ± 0.9 ng/mL) than saline (2.7 ± 0.5). Neither IPI (Fig 3) nor timing of the first pulse (data not shown) was different between the two treatments.

**Effects of SSTR2 antagonist in luteal phase ewes**

The synchronization protocol using prostaglandin resulted in luteal phase concentrations of progesterone (>1 ng/mL) in 7 of 8 ewes on both sampling days; one ewe with low progesterone concentrations was excluded from analysis. Two-way ANOVA for mean LH revealed a significant time by treatment interaction such that a single icv injection of CYN into luteal phase ewes produced a modest, but significant increase in mean LH concentrations (Fig 3, Pre: 1.0 ± 0.2 ng/mL; Post: 1.4 ± 0.2 ng/mL), whereas saline injections did not alter mean LH concentrations (Pre: 1.0 ± 0.3 ng/mL; Post: 1.1 ± 0.3 ng/mL). The first pulse after CYN injection occurred sooner (24 ± 4.5 min) than the first pulse after saline injection (58 ± 10.3 min). Neither pulse IPI (Fig 3) nor amplitude (data not shown) was significantly altered by treatment with this antagonist.

**Effects of SSTR2 antagonist on GnRH and kisspeptin cell activation**

Ewes that received CYN had a significantly greater percentage of GnRH cells that contained c-Fos within the MBH compared to ewes that received saline (Fig 4). There were no
significant differences in the percentage of GnRH cells that contained c-Fos in the diagonal band of Broca, POA or anterior hypothalamic area between treatment groups. CYN had no significant effect on the total number of GnRH cells in any of these areas (Table 2).

Ewes that received CYN had a significantly greater, though modest, percentage of kisspeptin cells that contained c-Fos within the caudal aspect of the ARC compared to ewes that received saline (Fig 4). CYN treatment did not alter the percentage of kisspeptin cells that contained c-Fos in other portions of the ARC or POA. There was also no difference in the total number of kisspeptin cells between treatment groups in any area. Although there appeared to be a large numerical difference between treatment groups in the percentage of GnRH and kisspeptin cells that contained c-Fos in the diagonal band of Broca and the POA, respectively, these differences were not statistically significant. This might be due to the relatively low number of animals (n=4/group), but more likely reflects the variability inherent in estimating percentages because there is a small number of total cells in these areas (Table 2).

Effects of SSTR2 antagonist in OVX ewes

Following CYN injection, OVX ewes during anestrus had a significantly shorter IPI (52.3 ± 6.9 min) than following saline injection (83.7 ± 9.6 min; Fig 5). Similarly, the first pulse after CYN injection tended to occur sooner (P=0.06; 32.0 ± 6.7 min) than following saline injection (64.0 ± 14 min). In contrast, during the breeding season, the IPI following CYN injection did not differ from that following saline injection, nor did the timing of the first pulse. Of note, CYN administration in anestrus reduced the IPI to a duration essentially the same as that seen in OVX during the breeding season. CYN treatment did not alter pulse amplitude (data not shown) or mean LH concentrations within either season, but mean LH levels were higher during the non-
breeding season than in the breeding season (Fig 5) because there was an increase in LH pulse amplitude during anestrus (anestrus: $11.0 \pm 2.1$ ng/mL vs breeding season $6.7 \pm 0.8$ ng/mL), which has been seen in previous studies (37,38).

**Analysis of guide cannula placement**

Cannulae placed into the third ventricle were aimed so that the tip was 2-3 mm directly above the infundibular recess (24), but there was some variation in this position. To determine if this variation affected the response to the SSTR2 antagonist, the distance from infundibular recess to the tip of the cannula was measured on radiographs captured during neurosurgery and compared to LH responses for each experiment. As illustrated in Fig 6, for experiment 2 (intact, anestrous ewes), there was a negative relationship ($R^2=0.84$) between the distance of the cannula tip to the infundibular recess and the change in mean LH concentration (mean LH concentration after CYN injection minus mean LH concentration pre-injection). Animals with the cannula tip placed further rostral and dorsal had the smallest LH response. No significant relationships between cannula placement and LH response following CYN or octreotide injection were observed in any other experiment, though there was less variability in guide cannula placement and response to CYN in the other experiments.

**DISCUSSION**

This is the first report to demonstrate a role for endogenous SST in regulation of GnRH secretion in any species. Previous work in several species has shown that SST or SST receptor agonists can alter GnRH cell activity or LH secretion, but the present study is the first to employ a SST receptor antagonist to determine whether endogenous SST suppresses LH secretion. Using this approach, we demonstrate that the SSTR2 antagonist, CYN, stimulates pulsatile LH secretion and c-Fos expression in MBH GnRH neurons, but not surge secretion.
Although several lines of correlative evidence in sheep, and pharmacological data in rodents, support a role for SST in surge type LH secretion, neither a SSTR2 agonist nor antagonist altered surge secretion in our experiments. Several methodological or theoretical considerations could account for the lack of effect of these drugs. First, as with all pharmacological studies, it is possible that an effective concentration of the drug was not achieved in the necessary locations to elicit an effect on LH surge secretion, but these drugs did effect episodic LH secretion. Second, SST could be acting through one of the other somatostatin receptors, although as in the Introduction, SSTR2 is the most likely receptor to be involved in control of GnRH secretion. Third, it is well-established that several redundant systems contribute to the generation of the LH surge (39) so that compensatory pathways could mask any effects caused by altered somatostatin signaling. Fourth, based largely on data from humans (21,40,41) it is possible that SST acts at the pituitary to suppress the response of the gonadotrope to GnRH. Despite these caveats, the simplest explanation for the lack of effect of SSTR2 agonist and antagonist is that SST does not play an essential role in generation of the LH surge in sheep.

Two groups have reported an increase in c-Fos, a marker for neuronal activation, in SST cells within the VMN around the time of the LH surge in sheep (16,19). One possible explanation for the discrepancy between these observations and our pharmacological data is that the cells that contain SST in the VMN are activated during the time of the surge but contain another signaling molecule or molecules that is/are important for surge secretion in sheep. Alternatively, it is possible that SST cells in the VMN are involved with inducing estrous behavior, since E₂ implants placed into the VMN, but not other areas, induce sexual receptivity in ewes (42). However, one report that c-Fos increased within SST cells in the VMN in animals
during the LH surge but not during the portion of estrous behavior that preceded the surge (19) argues against this possibility.

Perhaps the most dramatic effects of the SSTR2 antagonist on episodic LH secretion occurred in OVX animals, in which CYN administered to OVX ewes decreased IPI during anestrus but not during the breeding season. Interestingly, SSTR2 blockade during anestrus reduced LH IPI to levels similar to those observed for OVX ewes during the breeding season. Thus, we propose that SST acts during anestrus to mediate the steroid-independent actions of photoperiod. The only other signaling molecule that has been implicated in steroid independent suppression of LH during anestrus is serotonin. Similar to our current findings, serotonin receptor blockade increased LH pulse frequency in OVX ewes in anestrus but not during the breeding season (43–45). Serotonin depletion during anestrus also increased LH pulse frequency in OVX ewes, but had no effect in OVX ewes treated with E\textsubscript{2} (44).

Because both SST and serotonin have been implicated in the steroid-independent actions of photoperiod, these two systems may interact. The ovine hypothalamus has abundant serotonergic fiber innervation (46), and mRNA for the relevant receptor, 5-HT\textsubscript{2} (47) is concentrated in the ventral portion of the mediobasal hypothalamus (MBH), including the VMN (48). It is therefore possible that serotonin inhibits LH secretion via SST neurons that are located in the VMN. This possibility would also be consistent with the theory that serotonin suppresses LH via an inhibitory interneuron since 5-HT\textsubscript{2} receptor activation releases diacylglycerol and inositol triphosphate, resulting in increased protein kinase C activity and Ca\textsuperscript{2+} influx, which are generally considered excitatory signaling events (49).
In contrast to the seasonal difference in response to CYN seen in OVX ewes, administration of this antagonist to ovary-intact animals increased mean LH concentrations in both seasons, although the effect size was larger in anestrous ewes. Although LH pulse patterns are similar in ovary-intact luteal phase and anestrous ewes, these patterns reflect different negative feedback actions of ovarian steroids. During anestrus, the heightened sensitivity to E\textsubscript{2} negative feedback (50) produced by the inhibitory photoperiod is due, at least in part, to activation of inhibitory A15 dopaminergic neurons (51–53), which suppresses kisspeptin release from KNDy neurons in the ARC (31,32,54). During the breeding season E\textsubscript{2} and progesterone act together to suppress GnRH and LH pulse frequency and appear to act directly on KNDy neurons (36,39,55), which contain receptors for both E\textsubscript{2} and progesterone (56,57). Thus, the greater response in ovary-intact anestrous ewes could reflect the different inhibitory systems active at this time of year. Alternatively, this greater response might be due to the combination of steroid-independent and a more modest steroid-dependent suppression of LH by SST during anestrus since serotonergic inhibition of pulse frequency is sometimes evident in the presence of E\textsubscript{2} (45).

Because CYN was administered into the third ventricle the pharmacological data provide no indication of possible sites of action. However, the robust increase in the percentage of GnRH-ir cells that contained c-Fos only within the MBH, supports the possibility of an action in this region. Alternatively, this could reflect an indirect action of CYN, because an increase in episodic LH secretion by an endogenous opioid receptor antagonist or a pheromonal stimulus also selectively increased c-Fos in MBH GnRH neurons (58). Two other observations point to an action of CYN in the MBH. First, the negative correlation between the LH response and distance of the cannula site from the infundibular recess within the third ventricle is more consistent with an action in the MBH than in the POA. Second, there was a modest increase in
the percentage of kisspeptin-ir cells in the caudal aspect of the ARC that contain c-Fos following 
SSTR2 blockade. The possibility that SST acts on neural afferents to GnRH cells such as the 
KNDy neurons of the ARC (35,59), which have been implicated in the generation of GnRH 
pulses (60–64), had not been previously evaluated in any species. Thus, the stimulatory effects of 
SST2R blockade could be mediated by actions directly on a subset of GnRH neurons in the 
MBH that have been implicated in pulsatile LH secretion (58), or on KNDy neurons, or a 
combination of these substrates (Fig 7).

In summary, these experiments demonstrate that endogenous SST, acting at least partly 
through SSTR2, inhibits episodic LH secretion, but does not alter LH surge secretion. 
Furthermore, blockade of SSTR2 in ovary-intact ewes increased mean LH in both the breeding 
season and anestrus, whereas in OVX ewes, SSTR2 blockade increased LH IPI only in anestrus. 
The use of a SSTR2 antagonist allowed us to test, for the first time in any species, the role of 
endogenous SST in the regulation of LH secretion. Together, these studies provide evidence that 
SST contributes to steroid-independent suppression of LH during anestrus, and may also have a 
minor role in the inhibitory actions of ovarian steroids on LH secretion.

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FIGURE LEGENDS:

Figure 1. LH concentration patterns in 2 ewes receiving SSTR2 agonist, octreotide, injections (arrows) into the third ventricle. Black circles denote samples identified as pulses. Inset panels depict group mean (± SEM) LH concentration (MEAN), LH pulse amplitude (AMPL) and inter-pulse interval (IPI) during 3 hrs pre- (open bars) and 7 hrs post- (black bars) injection. Statistical analysis was not performed because of the low number of animals and lack of contemporary vehicle control injection.

Figure 2. Top panels, effect of SSTR2 agonist (Octreotide, black circles and bars) and vehicle (aCSF, open circles and bars) on the estrogen-induced LH surge. LH concentrations during E2-induced LH surges in one animal are shown in the left panel; right panel presents mean (± SEM) LH surge amplitude, and time (hrs after E2 implants) of maximum LH value. Bottom panels, effect of SSTR2 antagonist (CYN, black circles and bars) and vehicle (saline, open circles and bars) on the estrogen-induced LH surge. Left panel depicts LH concentrations during an E2-induced LH surge in one control and two CYN-treated animals to illustrate variability in timing of the surge; right panel presents mean (± SEM) LH surge amplitude, and time of maximum LH values.

Figure 3. Top, Representative LH concentration patterns in one anestrous intact ewe (left) and another luteal phase ewe (right) receiving either saline (top panels) or CYN (bottom panels) as icv injection (arrows). Black points represent samples identified as pulses. Note difference in y-axes between seasons. Bottom panels, mean LH (± SEM) concentration during 2 hr pre- (open bars) and 4 hr post-injection (gray bars) sampling period for saline or CYN treatments are shown.
on left and mean IPI (± SEM) during 4 hr sampling period after saline (open bars) or CYN (black bars) and shown on right. *, P<0.05 vs pre-injection period.

Figure 4. Top panels, Percentage (± SEM) of GnRH and kisspeptin immunoreactive cells that contain c-Fos in ewes that received either saline (open bars) or CYN (black bars). *, P<0.05 vs saline treated ewes. DBB: Diagonal band of Borca; AHA: anterior hypothalamic area. Lower panels, Representative photomicrographs of GnRH (mediobasal hypothalmus, middle) and kisspeptin (caudal ARC, bottom) immunoreactive soma (brown) in ewes treated with CYN (left) or saline (right). Arrow heads indicate GnRH or kisspeptin soma that contain nuclear c-Fos (black). Scale bar, 50 μm.

Figure 5. Top panels, Representative LH patterns in one OVX ewe during anestrus (left) and another ewe in the breeding season (right) receiving icv injection (arrows) of saline (top panels), or CYN (bottom panels). Black points represent samples identified as pulses. Bottom panels, mean (± SEM) IPI (left panel) and mean LH concentration (right panel) during 4 hr post-injection period in OVX ewes receiving saline (open bars) and CYN (black bars) during anestrus (left) or the breeding season (right). *, P<0.05 vs saline injection.

Figure 6. Change in mean LH concentration following CYN injection in intact anestrus ewes (Exp 3) versus chronic cannula position (distance from tip of cannula to infundibular recess on the lateral radiograph taken during neurosurgery).

Figure 7. Proposed model for SST action within the hypothalamus that mediates the steroid-independent control of LH secretion in sheep between the anestrous (left) and the breeding seasons (right). The inhibitory photoperiod in anestrous stimulates serotonergic neurons that increase the activity of SST neurons in the VMN. These SST neurons in turn inhibit GnRH
pulse frequency, which could be mediated directly on GnRH neurons, indirectly through KNDy neurons, or indirectly through some other neuron type. This system is distinct from the steroid-dependent actions of photoperiod which act via A15 dopaminergic (DA) neurons. See text for a more detailed discussion.
Table 1: Antibody table

<table>
<thead>
<tr>
<th>Peptide/protein target</th>
<th>Antigen sequence (if known)</th>
<th>Name of Antibody</th>
<th>Manufacturer, catalog #, and/or name of individual providing the antibody</th>
<th>Species raised in: monoclonal or polyclonal</th>
<th>Dilution used</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Fos</td>
<td>FOS human, mouse, rat</td>
<td>Rabbit Anti-C-Fos Polyclonal antibody, Unconjugated</td>
<td>Santa Cruz Biotechnology, SC-52</td>
<td>Rabbit, polyclonal</td>
<td>1:2000</td>
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<tr>
<td>GnRH</td>
<td>Luteinizing Hormone Releasing Hormone</td>
<td>LHRH</td>
<td>Immunostar, 20075</td>
<td>Rabbit, polyclonal</td>
<td>1:2000</td>
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<td>Kisspeptin</td>
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<td>Anti-Kisspeptin antibody</td>
<td>Millipore, AB9754</td>
<td>Rabbit, polyclonal</td>
<td>1:10,000</td>
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Table 2: Number of GnRH and kisspeptin immunoreactive cells

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<tr>
<th>GnRH</th>
<th>DBB</th>
<th>POA</th>
<th>AHA</th>
<th>MBH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>7.0 ± 2.9</td>
<td>65.5 ± 18.2</td>
<td>25.5 ± 5.2</td>
<td>51 ± 8.9</td>
</tr>
<tr>
<td>CYN</td>
<td>6.8 ± 2.9</td>
<td>74.5 ± 13.8</td>
<td>22.3 ± 6.4</td>
<td>27.8 ± 4.3</td>
</tr>
<tr>
<td>Kisspeptin</td>
<td>POA</td>
<td>Rostral ARC</td>
<td>Middle ARC</td>
<td>Caudal ARC</td>
</tr>
<tr>
<td>Saline</td>
<td>10.9 ± 5.89</td>
<td>16.7 ± 7.1</td>
<td>65.5 ± 11.2</td>
<td>46.1 ± 14.2</td>
</tr>
<tr>
<td>CYN</td>
<td>8.4 ± 3.8</td>
<td>17.2 ± 9.2</td>
<td>30.5 ± 9.6</td>
<td>39.5 ± 7.1</td>
</tr>
</tbody>
</table>
Figure 1

[Graph showing the effect of Octreotide on LH levels and IP1 duration.]

- LH levels before and after Octreotide injection.
- Showcases mean and amplitude changes.
- IP1 duration comparison before and after treatment.

**Legend:**
- Pre: Open bars
- Post: Solid bars

**Axes:**
- X-axis: Time from Injection, hrs
- Y-axis: LH, ng/mL

**Graph Details:**
- Peaks and troughs indicating LH response.
- Statistic comparisons indicated by error bars.
Figure 3

The figure shows the effect of saline and CYN injections on LH (luteinizing hormone) levels in two different stages: anestrus and breeding season. The top graph illustrates the changes in LH levels over time following injections. The bottom graphs display the mean LH levels (left) and IPl (interval between peaks) (right) for saline and CYN treatments in both stages.
Figure 4

Bar graphs showing the percentage of GnRH cells with c-Fos (left) and Kisspeptin cells with c-Fos (right) in different brain regions: DBB, POA, AHA, MBH, POA, Rostral, Middle, and Caudal ARC. The graphs compare Saline (white bars) and CYN (black bars) treatments. Significant differences are indicated by an asterisk (*). Scale bars represent 100 μm.
Figure 5

**Anestrus** vs **Breeding Season**

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>CYN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LH</strong></td>
<td>0 - 30</td>
<td>0 - 30</td>
</tr>
<tr>
<td><strong>Time from injection, hr</strong></td>
<td>-2 - 4</td>
<td>-2 - 4</td>
</tr>
</tbody>
</table>

Histograms:

- **IPI, min**
  - Anestrus: 40 ± 10
  - Breeding season: 60 ± 10
  - * indicates a significant difference

- **Mean LH, ng/mL**
  - Anestrus: 5 ± 1
  - Breeding season: 10 ± 1
  - Saline vs CYN comparison graph
Figure 6

![Graph showing the relationship between Δ mean LH after CYN injection, ng/mL and distance from infundibular recess, mm.](image-url)
Figure 7
CHAPTER 3

HYPOTHALAMIC SITES OF ACTION FOR SOMATOSTATIN IN THE REGULATION OF GONADOTROPIN SECRETION IN SHEEP


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ABSTRACT

Gonadotropin secretion is regulated by ovarian steroids and steroid independent factors. The neuropeptide kisspeptin is critical for gonadotropin secretion in many species. Several lines of evidence support the hypothesis that the neuropeptide somatostatin (SST) also contributes to the regulation of gonadotropin secretion. We recently demonstrated that a SST receptor 2 antagonist stimulates mean LH secretion in ovary intact ewes. To investigate potential sites for SST action in the ovine hypothalamus we used immunohistochemistry to determine whether cells that produce kisspeptin (KNDy cells in the arcuate nucleus) or GnRH receive direct synaptic contact from SST fibers, and whether the number of these inputs changes throughout the estrous cycle or by season. The majority of KNDy cells receive synaptic input (evident by presynaptic synaptophysin immunostaining) from SST cells, but the amount of these inputs did not differ among phases of the estrous cycle or season. A subset of GnRH cells in both the preoptic area (POA) and mediobasal hypothalamus also receive structural synaptic input from SST cells. A greater percentage of POA GnRH cells had SST synapses during the surge than in anestrus, the luteal or early follicular phase of the estrus cycle. The total number of synaptic inputs onto GnRH and KNDy cells was altered by phase of the estrous cycle and season, extending the hypothesis that changes in GnRH and KNDy cell synaptic connectivity may contribute to altered gonadotropin secretion throughout the estrous cycle and between seasons.
INTRODUCTION

The pattern of LH and GnRH secretion is regulated by ovarian steroids as well as steroid independent factors. Sheep, like most mammals, display seasonal alterations in reproductive performance, which in part reflect activation of different negative feedback systems. During the breeding season pulsatile GnRH and LH secretion are regulated by estradiol (to suppress pulse amplitude) and progesterone (to suppress pulse frequency) (1). However during the non-breeding season (anestrus) estradiol inhibits LH pulse frequency, which prevents ovulation (2). The neural substrates that mediate these feedback effects must be steroid sensitive (i.e. contain steroid receptors), since GnRH neurons do not contain estrogen receptor alpha (ERα) or progesterone receptor (3,4); they are therefore dependent on neural afferents for information on changes in steroid levels. Two populations of kisspeptin cells in the hypothalamus are important for gonadotropin secretion. The kisspeptin cells in the arcuate nucleus contain NKB and dynorphin (5) (known as KNDy neurons), as well as ERα (6) and progesterone receptors (7), and are thought to underlie GnRH pulse generation (8–12) and contribute to LH surge generation (13,14). The second population of kisspeptin neurons, located in the preoptic area (POA) also contain ERα (6) and are likely only involved in LH surge generation (13–15). However, an extensive body of research has implicated a number of other signaling molecules in the regulation of gonadotropin secretion (16).

The hypothalamic peptide somatostatin (SST) has been demonstrated to suppress pulsatile LH secretion in sheep (17) and humans (18). SST also inhibited GnRH release from rat hypothalamic explants (19), and suppressed GnRH spike firing in mice and rats (20,21). Among the five SST receptor isoforms, SSTR2 is a logical candidate to mediate these effects: the SSTR2 agonist (octreotide) blunted the LH surge in rats (22) and suppressed pulsatile LH secretion in
humans (23). Additionally, preliminary evidence indicates that GnRH cells within the POA contain SSTR2 (but not the other receptors) immunoreactivity (24). POA GnRH cells in mice contain mRNA for only SSTR2 (20) whereas rat POA GnRH cells contain only modest expression for all 5 receptors subtypes (21). Our group recently used a SSTR2 antagonist to demonstrate an endogenous role for SST acting via SSTR2 to suppress pulsatile LH secretion (Chapter 2). The SSTR2 antagonist increased mean LH secretion in intact animals during both anestrus and the breeding season, though the magnitude of the response in anestrus was larger. Additionally, we found that application of the antagonist to anestrus ewes increased c-Fos expression in a subset of GnRH neurons (only in the mediobasal hypothalamus [MBH]) and in kisspeptin neurons in the caudal aspect of the ARC. Although these neurons were considered activated, it could not be determined whether SST acts directly on them or if they are activated indirectly via interneurons. Stimulatory effects of the SSTR2 antagonist were also seen in OVX ewes in the non-breeding season. The antagonist was given into the third ventricle, therefore the specific site of action could not be determined, but indirect evidence supports the possibility that SST acts within the MBH to suppress gonadotropin secretion (Chapter 2).

To investigate potential sites for SST action in the ovine hypothalamus we used immunohistochemistry to determine whether KNDy cells and GnRH cells receive direct synaptic contact from SST fibers. The number of unidentified synaptic inputs onto GnRH cells differs with season and stage of the estrous cycle (25–27). Similarly, the number of synaptic contacts onto KNDy cells also differs throughout the estrous cycle (25). In light of the reports that synaptic inputs to GnRH and KNDy cells do change and may contribute to differences in gonadotropin secretion, we compared the number of SST synaptic contacts onto GnRH (both POA and MBH) and KNDy neurons in intact sheep during the breeding season (luteal phase,
early follicular phase and during the surge) and anestrus. The total number of synaptic contacts and the number of synaptic contacts by SST fibers was determined using triple label immunofluorescence (antibodies for SST, synaptophysin [a protein that is expressed exclusively in the presynaptic terminal], and either GnRH or NKB [a marker for KNDy cells]) and confocal microscopy. Immunohistochemistry for a marker of the presynaptic terminal (i.e. synaptophysin) and confocal microscopy has been proven to identify the same synapses as electron microscopy (28). To further characterize the neural substrates by which SSTR2 blockade stimulates LH we sought to describe SSTR2 immunoreactivity throughout the ovine hypothalamus and determine whether GnRH or KNDy cells contain SSTR2.

MATERIALS & METHODS

Animals

Mature multiparous ewes (>2yrs) were used. Animals were maintained indoors under artificial light that was adjusted every other week to match natural light periods. Ewes were fed a maintenance diet of cubed alfalfa and had free access to mineral blocks and water. Experiments were conducted in June (anestrus) and in October and November (breeding season). All procedures were approved by the West Virginia University Animal Care and Use Committee and were in accordance with the NIH guidelines for the care and use of research animals.

Experiment 1: Do SST fibers synapse onto GnRH and KNDy cells?

Animal Protocol

Adult, ovary-intact ewes were randomly assigned for tissue collection during the luteal phase (n=5), early follicular phase (n=5) or during the preovulatory LH surge (n=5). Estrus
cycles were synchronized using well established methods (29). Briefly, animals received 2 injections of prostaglandin F2α (PGF2α; 5 mg each 3 hrs apart; Lutalyse, (Zoetis, Parsippany, NJ) and 2 intravaginal progesterone containing CIDRs after the second injection. Eight days later, PGF2α was injected (5 mg each 3 hrs apart) and CIDRs were removed from all sheep; 24 hrs later, 5 sheep were euthanized after blood collection described below (“early follicular phase” group). Five days later the remaining 10 sheep received 2 intravaginal CIDRs. Five days after CIDR insertion, 5 sheep were bled and then euthanized (“luteal phase” group). CIDRs were removed from the remaining 5 ewes 7 days after CIDR insertion, and were euthanized 60 hr later (LH surge group). Jugular blood samples (3-4mL) were collected at 12 min intervals for 4 hrs before euthanasia from animals in the follicular and luteal phase groups. Jugular blood samples were collected at 4 hr intervals beginning 36 hrs after CIDR removal until the time of euthanasia in the surge group and assayed for LH. Additionally tissue was collected from 4 intact anestrous ewes during a previous experiment as described in Chapter 2.

**Blood sampling & assay**

Blood samples were collected by jugular venipuncture into heparinized tubes. Plasma was harvested and stored frozen until assayed for LH. Plasma samples (50-200μL aliquots) were assayed in duplicate for LH using a double liquid phase radioimmunoassay that has been previously validated for use in sheep (30). Assay reagents were acquired from the National Hormone and Peptide Program and LH concentrations are expressed in terms of NIH S24. The sensitivity of the LH assays averaged 0.10 ± 0.02 ng/mL and the inter- and intra-assay coefficients of variation were 6.9% and 5.1%, respectively. LH pulses were detected as described previously (1). LH concentrations at the time of euthanasia, and inter-pulse interval and pulse
amplitude during the four hours before euthanasia (for animals collected during the early follicular phase, luteal phase and anestrus) are shown in Table 1.

**Neural tissue collection & processing**

Ewes were treated with heparin (20,000 U) 10 min before and at the time of euthanasia, which was performed with an overdose (8-16mL, iv) of sodium pentobarbital (Euthasol, Patterson, Columbus, OH). The head was rapidly removed and perfused with 6L of 4% paraformaldehyde in 0.1M phosphate buffer (PB) with 0.1% NaNO₃. A block of tissue including the hypothalamus and POA was removed and stored overnight in the same paraformaldehyde solution at 4°C. Tissue was then transferred to 20% sucrose solution in PB and maintained at 4°C. After sucrose infiltration, tissue was frozen and 45μm-thick-coronal sections were cut using a microtome with a freezing stage, collected in 10 series (450μm apart), and stored at -20°C in cryoprotectant solution (31).

**Immunohistochemistry**

To determine whether the abundant SST fibers that have been reported previously (32,33) within the ovine hypothalamus synapse onto GnRH or KNDy cells we employed immunohistochemistry to detect SST, synaptophysin (presynaptic marker), and GnRH or NKB; because antibodies against SST, GnRH, and NKB were made in rabbits, TSA-amplification was used with the latter two antisera. For detection of GnRH or NKB, antisera were used at very low concentrations and detected with tyramide amplification, a procedure that has been shown to prevent cross reactivity (34). Nissl staining was used to identify GnRH or NKB cell boundaries. The number of SST synapses onto GnRH cells was determined in 1-2 hemisections from the POA and 4-8 hemisections from the MBH. Tissue was rinsed in PB 8 times (15 min each), stored
overnight in PB at 4°C, and rinsed 12 additional times in PB with 0.9% saline (PBS; 15 min each). Tissue was incubated in 1% H$_2$O$_2$ in PBS for 10 min, rinsed, then incubated in 4% normal goat serum (NGS; Jackson Immunoresearch, West Groove, PA) in PBS with 0.04% triton X-100 (PBST) for 1 hr followed by rabbit anti-GnRH serum (dilution: 1:3000 with 4% NGS in PBST; cat: 20075; Immunostar, Hudson, WI) for 17hrs. Tissue was rinsed with PBS and incubated sequentially in biotinylated goat anti-rabbit IgG (dilution: 1:500 in PBST with 4% NGS; Vector labs, Buringame, CA) for 1 hr, in ABC-elite (dilution: 1:500 in PBS; Vector Labs) for 1hr, in biotinylated tyramide (1:250 in PBS with 1uL of 3% H$_2$O$_2$ per mL; Perkin Elmer, Bridgeville, PA) for 10 min, and in Alexa 555 streptavidin (dilution: 1:200; S32355; Invitrogen, Carlsbad, CA) for 1 hr, with intervening rinsing steps. Tissue was rinsed and incubated in 4% NGS in PBST for 1 hr and then placed in rabbit anti-SST serum (dilution 1:500; T-4103; Peninsula Laboratories, San Carlos, CA) and monoclonal mouse anti-synaptophysin (dilution 1:200; S5768; Sigma-Aldrich, Saint Louis, MO) with 4% NGS in PBST for 17 h. Tissue was rinsed and sequentially incubated with Dylight 488 goat anti-mouse IgG (dilution 1:200 in 4% NGS in PBST, 35503, Invitrogen) and Alexa 647 goat anti-rabbit IgG (dilution 1:200 with 4% NGS in PBST, A-21245, Invitrogen) for 1 hr each with intervening rinsing steps. Finally, tissue was incubated in Neurotrace 435/455 (dilution 1:200 in PBS, N21479, Invitrogen). Tissue was mounted on superfrost slides (Fisher Scientific, Pittsburgh, PA) and cover glass was applied with gelvatol (35). Antibodies for GnRH (36), NKB (37), synaptophysin (38) and SST (32) have been used previously in sheep tissue.

The number of SST synapses onto KNDy cells was determined in 1-2 hemi-sections from each the middle and caudal aspects of the ARC. Immunohistochemistry was performed identically as described for SST synapses onto GnRH cells, except that rabbit anti-NKB serum
(dilution 1:10,000 with 4% NGS in PBST, G-046-26; Phoenix Pharmaceuticals, Burlingame, CA) was used in place of the rabbit anti-GnRH serum.

**Confocal analysis**

All confocal images were acquired with a Nikon A1R laser-scanning microscope (Nikon Instruments, Tokyo, Japan) with a 60x objective (aperture: 1.4). Nissl, Dylight 488, Alexa 555, and Alexa 647 were imaged with 405, 488, 561 and 640 nm lasers, respectively. For GnRH or NKB, 7-11 neurons with complete cell bodies were randomly selected from each region for analysis per animal. Confocal images were taken at 0.5 μm intervals in the z-axis. In each section the number of SST close-contacts onto either GnRH or NKB cell bodies or proximal dendrites was enumerated. The SST varicosities that contained synaptophysin were also counted and were considered SST synapses onto GnRH or NKB cells (28); single labeled synaptophysin contacts onto GnRH or NKB cells were also counted. To be considered a close contact the SST or synaptophysin staining had to be in direct contact (no intervening dark pixels) with GnRH, NKB or Nissl staining that also contained either GnRH or NKB. To minimize the risk of over-counting, markers were placed on contacts as they were counted. Analysis was performed with ImageJ software following minor adjustments to brightness and contrast. GnRH and NKB cell size was estimated by the number of optical sections required to image the entire cell; these did not differ among treatment groups (data not shown).

The percentage of GnRH and NKB cells that had SST or dual-labeled SST and synaptophysin contacts was determined in each region by an experimenter blinded to treatments. For cells that had SST or dual-labeled SST and synaptophysin contacts, the number of each type of contact was enumerated and averaged for each animal. For each GnRH and NKB cell, the
number of SST close contacts, SST synapses, total number of synapses (synaptophysin, independent of presence of SST) and percentage of cells that had SST contacts or SST synapses within each region were compared among treatment groups with one-way ANOVA. Where appropriate, Tukey’s multiple comparisons test was used to determine differences among treatment groups. P < 0.05 was considered statistically significant. Data are presented as mean ± SEM.

**Experiment 2: Do GnRH or KNDy cells contain SSTR2?**

The antiserum raised against SSTR2 that had been used in sheep neural tissue previously by other workers (24) had been discontinued by the supplier. Consequently we sought to identify an antibody suitable for detection of SSTR2 in ovine tissue; using the criteria that: 1) the antibody must produce reliable immunostaining, 2) preadsorption of the antibody with the immunogenic peptide must prevent immunostaining, and 3) western analysis must yield band(s) of the predicted size. We screened four commercially available antisera listed in Table 3, using an immunoperoxidase protocol. As most commercially available antisera are raised against human or rodent SSTR2, we selected antisera whose antigenic sequence was homologous to the ovine SSTR2 sequence for screening. All steps were performed at room temperature with mild agitation, unless noted differently. Tissue was rinsed in 0.1M PB overnight, thoroughly rinsed in PBS, incubated in H₂O₂, then blocking solution containing 4% NGS, then incubated for 17 hrs in one of the SSTR2 antisera (dilutions tested listed in Table 3) with 4% NGS in PBST. Tissue was then incubated sequentially in biotinylated goat anti-rabbit IgG (dilution: 1:500 in PBST with 4% NGS) and ABC-elite (dilution 1:500 in PBS) for one hour each with intervening PBS rinse steps and then incubated in 3,3’-diaminobenzidene (10mg per 50mL PB with 20μL of 30% H₂O₂, Sigma-Aldrich, Saint Louis, MO) for 10 min, rinsed with PB and mounted on superfrost slides.
Once dried, cover glass was applied with Eukitt mounting media (Fisher Scientific). Additionally, for each antisera, the immunohistochemistry protocol described above was repeated following antigen retrieval. Following initial rinsing steps, tissue was incubated for 20 min at 80°C in unmasking solution (Vector Laboratories) then allowed to cool for 30 min and rinsed with PBS.

To test for antibody specificity, peptide-blocking controls were performed. The antiserum was incubated with the immunogenic peptide (5-10 times more peptide than antiserum by weight; supplied by the respective vendor) for 24 hr at 4°C. Immunohistochemistry was performed as described above using either the pre-adsorbed antiserum or antiserum incubated with PBS (equal volume to blocking peptide). Slides were prepared and staining was visualized with a bright field microscope (Axiovert 400CFL, Zeiss) with a 20x objective.

RESULTS

Experiment 1: SST synaptic input to GnRH and KNDy neurons

SST synapses onto GnRH cells based on presynaptic synaptophysin immunostaining were evident in both the MBH (Fig 1) and POA (Fig 2). In the MBH, the majority of GnRH cells had SST close contacts, whereas fewer GnRH cells had contacts that colocalized SST and synaptophysin (SST+synap) (Table 4). Neither the percentage of GnRH cells that had SST or SST+synap close-contacts, nor the number of SST or SST+synap close-contacts per cell differed among groups. The total number of synaptophysin contacts on MBH GnRH cells was numerically lower in anestrus than any stage of the estrous cycle (Table 6), but this difference did not reach statistical significance (P=0.09).
In the POA, SST and SST+synap close-contacts were evident on some GnRH cells. The percentage of GnRH cells that had dual-labeled SST and synaptophysin close-contacts differed among groups such that a greater percentage of GnRH cells received SST+synap contacts during the LH surge than all other groups (Table 4). However, the number of SST+synap close-contacts per cell did not differ among groups. The total number of synaptophysin contacts on POA GnRH cells was significantly greater in animals during the LH surge than in animals during anestrus (Table 6).

Not all tissue has been analyzed for NKB-SST-Synaptophysin staining at this time; these data are from the following number of animals, anestrus: n=3, luteal: n=4, early follicular: n=5, luteal: n=4), 6-11 cells each.

SST+synap close-contacts were present on the majority of NKB cells in both the middle and caudal aspects of the ARC (Fig 3). Neither the percentage of NKB cells that had SST or SST+synap close-contacts, nor the number of these contacts per cell, differed among treatment groups (Table 5). The total number of synaptophysin contacts on NKB cells was significantly greater in surge animals than in anestrous animals in the middle ARC (Table 6). Similarly in the caudal ARC, the total number of synaptophysin contacts on NKB cells was significantly greater during the LH surge than during the early follicular phase of the estrous cycle. It should be noted that in both regions of the ARC, the total number of synaptophysin contacts on NKB cells during the LH surge was numerically greater than all other groups, though some of these comparisons did not reach statistical significance. As expected, the majority of NKB cells in both middle and caudal ARC had dual-labeled NKB and synaptophysin contacts (82-98%). The percentage of these contacts did not differ among treatment groups, however the number of these contacts per NKB cell did differ among groups such that there were more of these contacts during the LH
surge than the luteal phase in the middle ARC, and during LH surge than the early follicular phase in the caudal ARC (Figure 4).

**Experiment 2: SSTR2 immunoreactivity in the ovine hypothalamus**

None of the antibodies tested met the criteria for acceptable use in sheep neural tissue. One antiserum (ASR-002) failed to produce any immunostaining at any concentration. Another antiserum (SC25676) produced light, but detectable immunostaining in the POA resembling neurons and very faint fiber-like staining in the MBH. Peptide-blocking controls could not be performed because the immunogenic peptide was not available from the supplier and custom synthesis of the immunogenic peptide was prohibitively expensive. Because this critical test of the antibody could not be performed, no further investigation of this antiserum was performed. The third antiserum from Alomone labs (ASR-006) produced robust and vibrant immunostaining in the POA and MBH (with intense staining in the ARC). However, the pattern of immunostaining did not resemble neurons, instead appeared as a glia-like pattern (resembling staining for glial fibrillary acidic protein). Furthermore, pre-adsorption of the antiserum with the immunogenic peptide did not prevent immunostaining. The final antiserum tested (Orb11422) produced reliable immunostaining of neurons in the POA and a dense punctate staining in the ARC, however pre-adsorption of the antiserum with the immunogenic peptide did not reduce immunostaining. Because an acceptable antiserum against SSTR2 was not identified, we could not describe SSTR2 immunoreactivity throughout the ovine hypothalamus or determine whether GnRH or KNDy cells contain SSTR2.
**DISCUSSION**

This report demonstrates that both GnRH and KNDy cells have receive synaptic input from SST cells. We observed abundant SST synapses onto GnRH cells which has been observed in mice (20), but not rats (21). Though these contacts were observed, only minor neuro-plasticity of SST inputs to GnRH or KNDy cells was detected. A greater percentage of POA GnRH cells had SST synapses (evident by presynaptic synaptophysin immunostaining) during the surge than in anestrus, the luteal or early follicular phase of the estrus cycle. Despite relatively few changes in SST synaptic inputs, we did observe changes in overall synaptic inputs to GnRH and KNDy cells evident by changes in the total number of synaptophysin contacts onto these cells which is consistent with previous reports of altered synaptic contacts onto GnRH and KNDy cells (25–27,39).

Pharmacological experiments in sheep have demonstrated that SST suppresses pulsatile LH secretion (17) and that a SSTR2 antagonist stimulates pulsatile LH secretion (Chapter 2). In both of these reports, the drugs were administered into the third ventricle which does not provide information about the site of action. However two lines of evidence support the hypothesis that SST acts in the MBH. First, animals with cannulae placed more caudally in the ventricle (further from the POA) had a larger increase in mean LH concentrations in response to the SSTR2 antagonist than animals with cannulae placed more rostrally. Second, SSTR2 blockade increased c-Fos expression in a subset of GnRH neurons in the MBH and kisspeptin neurons in the caudal ARC. The presence of SST synaptic contacts onto both MBH GnRH cells and KNDy cells is consistent with the possibility that endogenous SST acts in the mediobasal hypothalamus to suppress LH secretion. In the present study we found that 23 ± 4% of GnRH cells in the MBH had SST synapses in anestrus ewes, which is quite similar to the 24 ± 5% of GnRH cells that
contained c-Fos after SSTR2 antagonist administration during anestrus. Thus it is possible that SST acts directly on a subset of GnRH cells in the MBH to regulate pulsatile LH secretion. Pulsatile LH release in sheep is likely mediated by a subset of GnRH neurons within the MBH because administration of an opioid antagonist or pheromonal stimuli, which stimulate pulsatile LH secretion, increased c-Fos expression only in GnRH cells in the MBH (40). Electrophysiological data in rodents also supports the possibly of direct action of SST on GnRH cells (20,21).

It is also possible, but not mutually exclusive, that SST acts via the KNDy cells in the ARC that are implicated in GnRH pulse generation (8–12). Though we previously observed only a modest increase in c-Fos expression in KNDy cells in the caudal aspect of the ARC, the majority of the KNDy cells in the middle and caudal ARC received synaptic inputs from SST cells. A few possibilities for this disparity exist. First, c-Fos expression may not be an effective indicator of cellular activation induced by SSTR2 blockade. Second, endogenous SST may also act via the other SST receptors since the type of these receptors in KNDy neurons has not been determined. Finally, the methods employed here (immunohistochemistry and confocal microscopy) are useful for assessing the presence of synapses, but synapse function (i.e. the ability of one cell to alter the activity of another cell) cannot be inferred from this method.

Administration of an SSTR2 antagonist to both luteal phase and anestrous ewes stimulated LH secretion, though the magnitude of response was greater during anestrus (Chapter 2). We therefore expected that during anestrus, we would observe more SST synaptic input to GnRH and KNDy reflecting a greater inhibitory tone than during the breeding season. Though not statistically significant, a greater percentage of GnRH cells in the MBH had SST synapses in anestrus (23 ± 4%) than in the luteal phase of the estrous cycle (14 ± 6%). Additionally, it is
possible that GnRH and/or KNDy cells are also more sensitive to the SST input during anestrus, possibly by increased receptor or intra-cellular signaling molecule content.

Previous studies have identified an increase in the total number of synaptic contacts onto GnRH cells during the breeding season compared to anestrus (26,27). We detected a similar pattern in which GnRH cells in the MBH had fewer total synaptic contacts during anestrus than during the breeding season (though only the comparison to the surge group was statistically significant). However, this pattern was not apparent for GnRH cells in the POA. These differences may stem from the endocrine condition of animals, Jansen et al. used (26) OVX+E2 ewes (26), whereas we used ovary intact animals in the present study. Moreover the method used to detect synapses (electron microscopy) by Xing et al.(27) differed from confocal microscopy used here. However immunohistochemistry for a presynaptic marker and confocal microscopy and electron microscopy have been shown to identify the same synapses (28). In addition to the number of synapses, differences in the type of synapses onto GnRH cells have been reported, with a net increase in stimulatory contacts during the breeding season (26). Thus changes in absolute number of synapses must be interpreted cautiously.

Surprisingly the number of NKB containing synapses onto NKB cells was not lowest during anestrus, when LH secretion is suppressed. Instead, the number of these contacts is comparable to that during the LH surge. This may reflect the importance of these connections to pulse generation. There also may be an increase in the number of inhibitory synapses onto KNDy cells during anestrus, whereas there is an increase in stimulatory inputs during the LH surge compared to the luteal phase (25). A likely source of inhibitory inputs during anestrus is the A15 dopaminergic neurons (41–45) which project to the MBH (43). In anestrus, E2 acts in the RCh (46) and the POA (47) to suppress LH via A15 dopaminergic neurons. KNDy cells in the ARC
contain the relevant dopamine receptor (D2R) and local blockade of D2R stimulates LH in a kisspeptin dependent manner (48). Thus, the increase in the total number of synaptic inputs onto KNDy cells during anestrus may reflect an increase in inhibitory dopamine inputs to suppress LH secretion.

The percentage of GnRH cells within the POA that had SST synapses was greater during the surge than in all other groups. Increased innervation by fibers containing an inhibitory molecule was not expected during the massive discharge of GnRH that occurs to elicit the LH surge. In addition, neither an SSTR2 agonist nor antagonist altered LH surge secretion (Chapter 2). A few possibilities could explain these differences. First, SST could act via a different receptor on GnRH cells, however this is unlikely because POA GnRH cells only contain SSTR2 (and not the others) (24). Second, during the LH surge the stimulatory input(s) to GnRH cells likely overpower and obviate any effects of SST. Another possibility is that these SST-containing synapses onto GnRH cells also contain additional neurotransmitters that do stimulate the GnRH cells. The origin of the SST fibers that synapse onto GnRH cells in not known, but may be the SST cells in the VMN. Reciprocal tract-tracing experiments have demonstrated that soma in the VMN project to the POA, which raises the possibility that SST cells in the VMN project onto GnRH neurons in the POA (49,50). The population of SST cells in the VMN has been implicated in the LH surge as two studies have reported an increase in c-Fos in these cells during the LH surge (51,52), which would be consistent with the increase in percentage of GnRH cells that receive SST synapses in this study.

In this study, we observed numerous SST close contacts onto GnRH or KNDy cells that did not also contain synaptophysin. The simplest explanation is that these SST fibers are indeed in close proximity, but do not actually form synapses onto GnRH or KNDy cells. Some technical
limitations may also contribute to this finding. First, the synaptophysin protein that may actually be present in the close contacts containing SST but is below the limit of detection by the antibody. Additionally, the SST antiserum which is co-incubated with the synaptophysin antiserum may further obfuscate detection of synaptophysin. Nevertheless, the dual-labeled SST and synaptophysin close contacts demonstrate the presence of synapses, though may underestimate the number of SST synapses onto GnRH or KNDy cells.

To further characterize possible sites for SST action in the hypothalamus, immunostaining for SSTR2 was planned. However, the lack of suitable antibody precluded these experiments. Detection of G protein coupled receptors with antibodies can be difficult due to the fact that the majority of the protein is situated within the plasma membrane and therefore unavailable for antibody interactions. Additionally, these receptors tend to be expressed in low copy numbers further obfuscating immuno-detection. Selection of commercial antiserum were based on published antigenic sequences that were similar to the sheep SSTR2 protein. Future work could employ in situ hybridization to detect mRNA for sstr2 in the ovine tissue.

In summary, these experiments provide evidence for somatostatinergic synapses onto GnRH and KNDy neurons within the ovine hypothalamus. The total number of synaptic inputs onto GnRH and KNDy cells was altered by phase of the estrous cycle and season, extending the hypothesis that changes in the GnRH and KNDy cell synaptic connectivity may contribute to altered gonadotropin secretion throughout the estrous cycle and between seasons.
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FIGURE LEGENDS

Figure 1: SST and Synaptophysin contacts onto a GnRH neuron in the MBH. Confocal image (0.5µm optical thickness). Upper left, GnRH; upper right, synaptophysin; lower left, SST; lower right, merge: nissl (blue), synaptophysin (green), NKB (red), SST (magenta). Bar = 10 µm.

Figure 2: SST and Synaptophysin contacts onto an NKB neuron in the POA. Confocal image (0.5µm optical thickness). Upper left, GnRH; upper right, synaptophysin; lower left, SST; lower right, merge: nissl (blue), synaptophysin (green), NKB (red), SST (magenta). Bar = 10 µm.

Figure 2: SST and Synaptophysin contacts onto an NKB neuron in the caudal ARC. Confocal image (0.5µm optical thickness). Upper left, NKB; upper right, synaptophysin; lower left, SST; lower right, merge: nissl (blue), synaptophysin (green), NKB (red), SST (magenta). Bar = 10 µm.

Figure 4: Total number of synaptophysin contacts onto NKB cells (Mean ± SEM) in the middle (top) and caudal (bottom) ARC. Bars with different letters differ (P < 0.05).
Table 1. LH concentrations at the time of euthanasia, inter-pulse interval and pulse amplitude

<table>
<thead>
<tr>
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<th>Anestrous</th>
<th>Luteal</th>
<th>Early Follicular</th>
<th>Surge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration at time of euthanasia, ng/mL</td>
<td>5.5 ± 2.4</td>
<td>1.14 ± 0.3</td>
<td>3.1 ± 1.1</td>
<td>21.9 ± 11.93</td>
</tr>
<tr>
<td>Inter-pulse interval, min</td>
<td>121.5 ± 34.5</td>
<td>115.2 ± 8.9</td>
<td>46 ± 3.2</td>
<td>n/a</td>
</tr>
<tr>
<td>Pulse amplitude, ng/mL</td>
<td>6.1 ± 1.5</td>
<td>3.2 ± 0.9</td>
<td>2.1 ± 0.4</td>
<td>n/a</td>
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<td>Peptide/protein target</td>
<td>Antigen sequence (if known)</td>
<td>Name of Antibody</td>
<td>Manufacturer, catalog #, and/or name of individual providing the antibody</td>
<td>Species raised in: monoclonal or polyclonal</td>
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<td>------------------------</td>
<td>----------------------------</td>
<td>------------------</td>
<td>-----------------------------------------------------------------</td>
<td>---------------------------------------------</td>
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<tr>
<td>SST</td>
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<td>Rbt xSST</td>
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<td>Rabbit polyclonal</td>
</tr>
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<td>GnRH</td>
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<td>LHRH</td>
<td>Immunostar; 20075</td>
<td>Rabbit, polyclonal</td>
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<tr>
<td>NKB</td>
<td>NKB</td>
<td>Anti-NKB antiserum</td>
<td>Phoenix Pharmaceuticals</td>
<td>Rabbit, polyclonal</td>
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<td>Synaptophysin</td>
<td>Rat synaptophysin</td>
<td>Mouse Anti-Rat Synaptophysin Monoclonal Antibody, Unconjugated, Clone SVP-38</td>
<td>Sigma; S5758</td>
<td>Mouse, monoclonal</td>
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Table 2: Antibody table
Table 3: SSTR2 antibodies tested

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<th>Antigen sequence (if known)</th>
<th>Name of Antibody</th>
<th>Manufacturer, catalog #, and/or name of individual providing the antibody</th>
<th>Species raised in: monoclonal or polyclonal</th>
<th>Dilutions Tested</th>
<th>RRID</th>
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<tr>
<td>SSTR2</td>
<td>Human SSTR2 aa320-369</td>
<td>H-50</td>
<td>Santa Cruz:SC-25676</td>
<td>Rabbit, polyclonal</td>
<td>1:500, 1:1000, 1:5000</td>
<td>AB_2255396</td>
</tr>
<tr>
<td>SSTR2</td>
<td>Rat SST2R aa339-59</td>
<td>ASR-002</td>
<td>Alomone</td>
<td>Rabbit, polyclonal</td>
<td>1:100, 1:500, 1:5000</td>
<td>AB_2341073</td>
</tr>
<tr>
<td>SSTR2</td>
<td>Rat SST2R 30-43</td>
<td>ASR-006</td>
<td>Alomone</td>
<td>Rabbit, polyclonal</td>
<td>1:500, 1:5000</td>
<td>AB_2040208</td>
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Table 4: Percentage of GnRH with single and dual-labeled SST contacts and number of single and dual-labeled SST contacts onto GnRH cells

<table>
<thead>
<tr>
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<th>Anestrus</th>
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<th>Early Follicular</th>
<th>Surge</th>
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</thead>
<tbody>
<tr>
<td>MBH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of cells w/SST</td>
<td>81.9 ± 11.0</td>
<td>76.6 ± 5.6</td>
<td>75.0 ± 9.6</td>
<td>84.0 ± 2.4</td>
</tr>
<tr>
<td># of SST/cell</td>
<td>3.1 ± 0.6</td>
<td>2.8 ± 0.5</td>
<td>2.3 ± 0.3</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>% w/SST+Synaptophysin</td>
<td>23.1 ± 4.5</td>
<td>13.9 ± 5.7</td>
<td>20.0 ± 4.1</td>
<td>32.0 ± 5.8</td>
</tr>
<tr>
<td># of SST+Synaptophysin</td>
<td>1.1 ± 0.1</td>
<td>2.2 ± 0.5</td>
<td>1.8 ± 0.4</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>POA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of cells w/SST</td>
<td>75 ± 6.5</td>
<td>63.5 ± 13.7</td>
<td>52.4 ± 7.6</td>
<td>70.6 ± 4.4</td>
</tr>
<tr>
<td># of SST/cell</td>
<td>2.6 ± 0.4</td>
<td>2.1 ± 0.2</td>
<td>2.0 ± 0.2</td>
<td>2.9 ± 0.5</td>
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<tr>
<td>% w/SST+Synaptophysin</td>
<td>10 ± 4.1 a</td>
<td>8 ± 3.7 a</td>
<td>14.5 ± 5.0 a</td>
<td>34.7 ± 3.0 b</td>
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<tr>
<td># of SST+Synaptophysin</td>
<td>1.7 ± 0.7</td>
<td>1.2 ± 0.2</td>
<td>1.0 ± 0</td>
<td>1.1 ± 0.1</td>
</tr>
</tbody>
</table>

Values with different subscripts differ within row (P < 0.05), mean ± SEM
Table 5: Percentage of NKB with single and dual-labeled SST contacts and number of single and dual-labeled SST contacts onto NKB cells

<table>
<thead>
<tr>
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<th>Early Follicular</th>
<th>Surge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Middle ARC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% w/SST</td>
<td>100.00%</td>
<td>82.9 ± 11.9</td>
<td>90.0 ± 4.1</td>
<td>97.2 ± 2.7</td>
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<tr>
<td># of SST</td>
<td>4.9 ± 2.7</td>
<td>7.6 ± 0.23</td>
<td>3.9 ± 0.53</td>
<td>8.2 ± 1.84</td>
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<tr>
<td>% w/SST+Synaptophysin</td>
<td>77.9 ± 5.7</td>
<td>89.3 ± 6.4</td>
<td>80.8 ± 10.8</td>
<td>61.1 ± 7.3</td>
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<tr>
<td># of SST+Synaptophysin</td>
<td>2.2 ± 0.19</td>
<td>3.6 ± 0.85</td>
<td>3.9 ± 0.59</td>
<td>2.8 ± 0.42</td>
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<tr>
<td>Caudal ARC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% w/SST</td>
<td>88.3 ± 7.3</td>
<td>86.6 ± 8.8</td>
<td>85.7 ± 3.9</td>
<td>97.5 ± 2.5</td>
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<tr>
<td># of SST</td>
<td>4.7 ± 0.7</td>
<td>4.5 ± 1.7</td>
<td>4.9 ± 0.4</td>
<td>6.1 ± 1.4</td>
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<tr>
<td>% w/SST+Synaptophysin</td>
<td>76.7 ± 14.5</td>
<td>76.7 ± 14.5</td>
<td>81.3 ± 5.3</td>
<td>80.0 ± 10.8</td>
</tr>
<tr>
<td># of SST+Synaptophysin</td>
<td>3.13 ± 0.63</td>
<td>2.6 ± 0.4</td>
<td>3.5 ± 0.5</td>
<td>3.1 ± 0.1</td>
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Values with different subscripts differ within row (P < 0.05), mean ± SEM

Table 6: Total number of synaptic contacts on to GnRH and NKB cells

<table>
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<tr>
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<th>Anestrus</th>
<th>Luteal</th>
<th>Early Follicular</th>
<th>Surge</th>
</tr>
</thead>
<tbody>
<tr>
<td>GnRH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POA</td>
<td>7.9 ± 1.3 ab</td>
<td>7.32 ± 0.3 a</td>
<td>8.6 ± 0.7 ab</td>
<td>10.32 ± 0.7 b</td>
</tr>
<tr>
<td>MBH</td>
<td>6.4 ± 0.4</td>
<td>9.8 ± 1.2</td>
<td>7.7 ± 1.3</td>
<td>9.9 ± 1.1</td>
</tr>
<tr>
<td>NKB</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle ARC</td>
<td>5.9 ± 0.3 a</td>
<td>7.9 ± 0.1 ab</td>
<td>7.4 ± 0.5 ab</td>
<td>11.01 ± 1.5 b</td>
</tr>
<tr>
<td>Caudal ARC</td>
<td>10.3 ± 1.4 ab</td>
<td>10.9 ± 1.6 ab</td>
<td>10.3 ± 0.3 a</td>
<td>14.9 ± 1.2 b</td>
</tr>
</tbody>
</table>

Values with different subscripts differ within row (P < 0.05), mean ± SEM
Figure 2
Figure 3
Figure 4

**Middle ARC**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Middle ARC</th>
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<tr>
<td>Luteal</td>
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<td>ab</td>
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<tr>
<td>E. Follicular</td>
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<td>a</td>
</tr>
<tr>
<td>Surge</td>
<td>b</td>
<td>b</td>
</tr>
</tbody>
</table>

NKB-ir contacts per NKB cell

- ANOVA was used to analyze the data.
- Significant differences are indicated by letters a and b.
CHAPTER 4

EVIDENCE THAT A SUBPOPULATION OF SOMATOSTATIN NEURONS IN THE OVINE VENTRAL MEDIAL NUCLEUS CONTAIN NEURONAL NITRIC OXIDE SYNTHASE AND ARE ACTIVATED DURING THE LH SURGE

Richard B. McCosh, Michelle N. Bedenbaugh, Katrina Porter, Justin A. Lopez, Steven L. Hardy, Stanley M. Hileman, and Robert L. Goodman.

ABSTRACT

Surge secretion of luteinizing hormone (LH) is necessary for ovulation and therefore fertility. Several lines of evidence support the hypothesis that somatostatin (SST) cells in the ventral medial nucleus (VMN) may be involved in the generation of the LH surge in sheep. However, neither a SST receptor 2 (SSTR2) agonist nor antagonist altered the timing or amplitude of the LH surge. In this study, we confirmed that SST cells in the VMN are activated during the LH surge using c-Fos as a marker for cellular activation. One explanation for the discrepancy between the observations of SST cell activation during the LH surge, but no effect of pharmacological manipulation of SSTR2, is that the cells that contain SST in the VMN also produce additional signaling molecules, such as nitric oxide. Immunohistochemistry was used to determine that a high percentage (70-80%) of these SST cells also contain neuronal nitric oxide synthase (nNOS). Furthermore, a greater percentage of the dual-labeled SST and nNOS cells were found to contain c-Fos during the LH surge compared to the early follicular phase. In contrast, the percentage of single labeled nNOS or SST cells that contained c-Fos did not differ between the LH surge and early follicular phase. Thus, we propose that this population of SST cells in the VMN also release nitric oxide and that this transmitter contributes to the generation of the LH surge in sheep.
INTRODUCTION

Surge secretion of LH causes ovulation and is critical for fertility. The high and sustained secretion of GnRH and LH secretion that characterizes the LH surge are induced by elevated estradiol (E$_2$). This stimulatory action of E$_2$ is in contrast to typical inhibitory effects of E$_2$ that suppress LH pulse amplitude throughout most of the ovarian cycle (1), and the two actions are likely mediated by alternative neural circuits within the hypothalamus (2). During the LH surge, approximately half of the GnRH neurons throughout the ovine hypothalamus express the common marker for cellular activation, c-Fos, indicating broad activation of GnRH cells (3). Because these cells do not contain estrogen receptor alpha (ER$\alpha$) (4), neural afferents must convey this signal. In sheep, E$_2$ administered into the ventral medial hypothalamus (VMN) and not into other areas, induces surge secretion of LH (5), indicating that the site of E$_2$ positive feedback is in the mediobasal hypothalamus (MBH). Neural deafferentation studies have demonstrated a critical role for neural substrates in the rostral hypothalamus in order to induce an LH surge (6). Together these studies demonstrate that multiple hypothalamic regions are necessary for the detection and transmission of the LH surge.

Several lines of evidence support a role for kisspeptin in the generation of the LH surge in sheep. First, kisspeptin cells in both the POA and ARC contain ER$\alpha$ (7). Second, kisspeptin cells in both the preoptic area (POA) and arcuate nucleus (ARC) express c-Fos during the LH surge (8–10). Third, icv kisspeptin infusion in anestrous ewes induced an LH surge (11). Additionally, the neuropeptide neurokinin B (NKB) is produced within the same neurons as kisspeptin in the ARC (12) and also contributes to the generation of the LH surge. Administration of the neurokinin 3 receptor (NK3R) agonist senktide causes surge-like secretion of LH (13,14) and administration of a NK3R antagonist (SB222200) into the retrochiasmatic
area (RCh) reduces the LH surge amplitude by ~40% (14). Similarly, icv administration of a kisspeptin receptor antagonist reduces the LH surge amplitude by ~50% (15), which indicates that other signaling molecules likely contribute to the generation of the full LH surge.

Several lines of evidence support the hypothesis that the neuropeptide somatostatin (SST) may contribute to the generation of the LH surge. First, SST cells in the VMN contain ERα (16). Second, two studies have reported an increase in c-Fos expression in SST cells in the VMN during a naturally occurring LH surge (17) or in an estradiol benzoate induced LH surge (16) compared to the early follicular phase or oil treated controls, respectively. Finally, the SST receptor 2 agonist (octreotide) blunted the LH surge and prevented c-Fos expression within GnRH cells in rats (18). However, neither a SSTR2 agonist nor antagonist altered the timing or amplitude of an E2 induced LH surge in sheep (Chapter 2). One possible explanation for the discrepancy between the observations of SST cell activation during the LH surge, but no effect of pharmacological manipulation of SSTR2, is that the cells that contain SST in the VMN also contain another signaling molecule that does alter LH surge secretion. One such signaling molecule is nitric oxide produced by neuronal nitric oxide synthase (nNOS), which has recently been demonstrated to be critical for the LH surge in mice (19).

The objective of this study was to further characterize the SST cells within the VMN throughout the estrous cycle and to specifically explore their role in the LH surge. Reports that SST cells are activated during LH surge secretion are unexpected because SST suppresses the firing of GnRH neurons in rodents (20,21) and inhibits GnRH secretion from hypothalamic explants (22), which is consistent with the generally inhibitory effects of SST reported throughout the body (23). In this study, we first sought to confirm that SST cells in the VMN are activated during the LH surge. Next we determined whether these cells also contain neuronal
nitric oxide synthase (nNOS). A high percentage of SST cells in the VMN did contain nNOS, so we determined whether the population of SST cells that contains nNOS is activated during the LH surge. Finally, because surge-like secretion of LH can also be induced by NK3R activation in sheep (13,14), we determined whether SST cells are activated following NK3R agonist administration.

MATERIALS & METHODS

Animals

Multiparous mature ewes (>2 years old) of mixed breeding were maintained in a light and temperature controlled research building. Lighting conditions were adjusted every other week to mimic local natural lighting conditions. Ewes were fed a maintenance ration of cubed alfalfa hay and had free access to water and mineral blocks. Breeding season experiments were done in October and November and anestrous studies were done in May and June. All procedures were approved by the West Virginia University Animal Care and Use Committee and were performed in accordance with the NIH guidelines for the care and use of research animals.

Tissue collection and processing

Tissue was collected as described previously (Chapter 1 and 2), briefly ewes were treated with heparin (20,000 U) 10 min before and at the time of euthanasia, which was performed with an overdose (8-16 mL, iv) of sodium pentobarbital (Euthasol, Patterson Veterinary, Bessemer, AL). The head was rapidly removed and perfused with 6L of 4% paraformaldehyde in 0.1M phosphate buffer (PB) with 0.1% NaNO₃. A block of tissue including the hypothalamus and pre-optic area (POA) was removed and stored overnight in the same paraformaldehyde solution at 4°C. Tissue was then transferred to 20% sucrose in PB and maintained at 4°C. After sucrose
infiltration, tissue was frozen and 45μm-thick-coronal sections were cut with a microtome with a freezing stage and collected in 10 series (450μm apart) and stored at -20°C in cryoprotectant solution (24).

**Immunohistochemistry**

Immunohistochemistry was performed on free-floating sections at room temperature with mild agitation unless otherwise specified. Tissue was rinsed thoroughly in PB and stored at 4°C overnight. Tissue was then rinsed 12 times for at least 15 mins each in phosphate buffered saline (PBS; 0.1M PB with 0.9% saline). We used two approaches to enable the use of two antisera raised in the same species for immunohistochemistry in the same tissue section. In experiment 1, one antiserum was visualized with nickel-enhanced 3,3’-diaminobenzidine (DAB) and the second with DAB alone, with intervening quenching steps for detection of antigens in different cellular compartments. In the other experiments, one antiserum was used at very low concentration and detected with tyramide amplification, a procedure that has been shown to prevent cross reactivity (25). Antisera used to detect NKB (26), nNOS (27) and SST (16) have been extensively used in sheep tissue and are described further in Table 1.

**Experiment 1: Are SST cells activated during the LH surge?**

Tissue collected from 15 ewes (5/group) killed during the luteal phase, early follicular phase (18 hrs after progesterone withdrawal) or during the LH surge (as described in Chapter 3) was used. For detection of SST and c-Fos, dual label immunohistochemistry was performed on one complete series of hemisections throughout the hypothalamus. Following the rinsing protocol described above, tissue was incubated in 1% H₂O₂ in PBS for 10 min, after which tissue was rinsed 4 times for 5 mins each in PBS (typical rinsing step). Tissue was incubated in 4%
normal goat serum (NGS; Jackson Immunoresearch, West Groove, PA) in PBS containing 0.04% triton X-100 (PBST) for 1 hr and then incubated in rabbit anti-c-Fos serum (dilution: 1:3000 with 4% NGS in PBST; SC-253, Santa Cruz Biotechnology, Dallas, TX) for 17 hrs. Tissue was rinsed, and incubated sequentially in biotinylated goat anti-rabbit immunoglobulin (IgG; dilution: 1:500 in PBST with 4% NGS; BA-1000; Jackson Immunoreseach) for 1 hr, ABC-elite (dilution 1:500 in PBS; Vector Lab Burlingame, CA) for 1 hr and nickel enhanced DAB solution (10mg per 50mL PB with 2mL of 2% NiSO₄ and 20μL of 30% H₂O₂ to produce a black reaction product, Sigma-Aldrich, Saint Louis, MO) for 10 min with intervening rinse steps. Tissue was rinsed in PB and placed into 1% H₂O₂ in PBS, rinsed and incubated in blocking solution containing 4% NGS in PBST for 1 hr, and was then incubated in rabbit anti-SST serum (dilution: 1:20,000 in PBST with 4% NGS, T4103, Peninsula Laboratories, San Carlos, CA) for 17 hrs. Tissue was rinsed in PBS and incubated sequentially in biotinylated goat anti-rabbit IgG (dilution: 1:500 in PBST with 4% NGS), ABC-elite (dilution: 1:500 in PBS), and DAB (10mg per 50mL PB with 20μL of 30% H₂O₂ to produce a brown reaction product) for 10 min. Tissue was rinsed in PB and mounted onto superfrost slides (Fisher Scientific, Pittsburgh, PA), once dried, cover glass was applied with Eukitt (Fisher Scientific, Pittsburgh, PA) mounting media.

The total number of SST cells and the percentage of SST cells that contained c-Fos was determined in 2-3 hemisections containing the VMN with a bright field microscope (VS-120, Olympus, Tokyo, Japan) with a 20x objective. All cell counts were made by a single observer blinded to treatment groups. Values were averaged per animal and compared among treatment groups by one-way ANOVA followed by Tukey’s Multiple Comparisons test. P < 0.05 was considered statistically significant.
Experiment 2: Do SST cells contain nNOS?

To determine whether SST cells in the VMN also contained nNOS, dual label immunohistochemistry was performed on tissue from 3 OVX ewes that were treated with \( E_2 \) (3 cm Silastic subcutaneous implant [inner diameter 0.34 cm, outer diameter 0.46 cm] Dow Corning Corp., Midland, MI containing \( E_2 \)) and progesterone (two progesterone containing intravaginal CIDRs; Zoetis, Parsippany, NJ). Tissue was collected 2 hrs after an icv injection of saline that did not alter LH secretion (data not shown). Following the rinsing and blocking protocol described above, tissue was incubated in rabbit anti-SST serum (dilution: 1:20,000 with 4% NGS in PBST) for 17hrs. Tissue was rinsed with PBS and incubated sequentially in biotinylated goat anti-rabbit IgG (dilution: 1:500 in PBST with 4% NGS) for 1 hr, in ABC-elite (dilution: 1:500 in PBS; Vector Laboratories, Burlingame, CA) for 1hr, in biotinylated tyramide (TSA; dilution: 1:250 in PBS with 1uL of 3% \( H_2O_2 \) per mL; Perkin Elmer, Bridgeville, PA) for 10 min, and in Dylight 488 streptavidin (dilution: 1:200 in PBS; S32355; Invitrogen, Carlsbad, CA) for 1 hr, with intervening rinsing steps. Tissue was then rinsed, incubated in 4% NGS in PBST for 1 hr and placed in rabbit anti-nNOS serum (dilution 1:1000; cat: 24287; Immunostar, Hudson, WI) with 4% NGS in PBST for 17 h. Tissue was rinsed and incubated with Alexa 555 goat anti-rabbit IgG (dilution 1:200 with 4% NGS in PBST, A-21245, Invitrogen) for 1 hr each with intervening rinsing steps. Preadsorption of either antibody with the alternative immunogenic peptide (i.e. SST antibody with nNOS immunogenic peptide and vice versa) did not alter patterns or density of immunostaining (Figure 1). Similarly, omission of nNOS antiserum resulted in a lack of specific staining in that color channel and did not alter patterns or density of immunostaining for SST (Fig 1).
Cells that contained SST, nNOS or both were counted in a stitched image encompassing the MBH collected with a fluorescent microscope (VS-120, Olympus, Tokyo, Japan) in 2-3 hemisections with a 20x objective following minor adjustments to brightness and contrast. Values were averaged per animal. Because this study was descriptive in nature, no statistical analyses were performed. Data are presented as mean ± SEM percent or average number of cells per hemisection.

**Experiment 3: Are nNOS cells activated during the LH surge?**

To determine whether the population of SST cells that expressed c-Fos during the LH surge also contained nNOS, triple label immunohistochemistry was performed on a subset of the tissue used in experiment 1 (mid LH surge, n=4 and early follicular phase n=4). Because all three antisera used previously were raised in rabbits, an alternative SST antiserum (rat x SST, Millipore, MAB354) (16) that required antigen retrieval was used. Two hemisections were incubated for 20 min at 80°C in unmasking solution (Vector Laboratories) then allowed to cool for 30 min and rinsed with PBS. Tissue was incubated in 1% H$_2$O$_2$ in PBS for 10 min, rinsed then incubated in 4% NGS in PBST for 1 hr followed by rabbit anti-c-Fos serum (dilution: 1:3000 with 4% NGS in PBST) for 17 hrs. Tissue was rinsed, and incubated sequentially in biotinylated goat anti-rabbit IgG (dilution: 1:500 in PBST with 4% NGS) for 1 hr, ABC-elite (dilution 1:500 in PBS) for 1 hr, TSA (1:250 in PBS with 1uL of 3% H$_2$O$_2$ per mL; Perkin Elmer) for 10 min, and in Dylight 488 streptavidin (dilution: 1:200) for 1 hr, with intervening rinsing steps. Tissue was rinsed and incubated in 4% NGS in PBST for 1 hr and then placed in rabbit anti-nNOS (dilution 1:1000 with 4% NGS in PBST) and rat anti-SST (dilution: 1:200 with 4% NGS in PBST; cat: MAB354, Millipore, San Billerica, MA) for 17 hr. Tissue was rinsed and incubated with Alexa 555 goat anti-rat IgG (dilution 1:200 with 4% NGS in PBST, A-21434,
Invitrogen) and Alexa 647 goat anti-rabbit (dilution 1:200 with 4% NGS in PBST, A21245, Invitrogen) for 1 hr each with intervening rinsing steps. Tissue was mounted on superfrost slides and coverslipped with gelvatol.

Cells that contained SST, nNOS, c-Fos and any combination of these were counted in a stitched image encompassing the MBH collected with a fluorescent microscope (VS-120, Olympus, Tokyo, Japan) with a 20x objective following minor adjustments to brightness and contrast. All cell counts were made by a single observer blinded to treatment groups and used to calculate percentage of each cell type that contained c-Fos. Cytoplasmic staining was described as: dual-labeled SST and nNOS cells, all SST cells (regardless of whether nNOS-ir was present), all nNOS cells (regardless of whether SST-ir was present), SST only (SST-ir, but not nNOS-ir) and nNOS only (nNOS-ir, but not SST-ir). Values were averaged per animal and compared between mid LH surge and early follicular phase with t-tests. P < 0.05 was considered statistically significant. Data are presented as mean ± SEM percent or average number of cells per hemisection.

**Experiment 4: Are SST cells activated during NK3R agonist induced LH surge like secretion?**

Dual-label immunohistochemistry was performed on tissue that had been collected for a previous study (14) 3 hrs after senktide-containing or blank implants was administered through chronic guide cannulae aimed at either the RCh or POA described previously (14,28). Briefly, Anestrous ewes received 18-gauge bilateral guide cannula just above the RCh at least 3 weeks before the experiment. Ewes received either a senktide- (Tocris, Minneapolis, MN) containing (n=4) or a blank implant (n=7) through both guide cannula into tissue and were euthanized 3hrs
later. During the breeding season, ewes received 18-gauge bilateral guide cannula just above the POA at least 2 weeks before the experiment. Estrous cycles were synchronized with prostaglandin F2α (2 injections 5 mg each 3 hrs apart; Lutalyse, (Zoetis, Parsippany, NJ) and each ewe received 2 intravaginal progesterone containing CIDRs after the 2nd injection, 7 days later CIDRs were removed and ewes received prostaglandin F2α again. Eighteen hours later (early follicular phase), ewes received either a senktide (Tocris, Minneapolis, MN) containing (n=4) or a blank implant (n=7) through the guide cannula as above and were euthanized 3 hrs later.

For immunohistochemistry, tissue was incubated in 1% H2O2 in PBS for 10 min, rinsed, incubated in 4% NGS in PBST for 1 hr and then incubated in rabbit anti-c-Fos serum (dilution: 1:3000 with 4% NGS in PBST; Santa Cruz Biotechnology) for 17 hrs. Tissue was rinsed, and incubated sequentially in biotinylated goat anti-rabbit IgG (dilution: 1:500 in PBST with 4% NGS; BA-1000; Jackson Immunoreseach) for 1 hr, ABC-elite (dilution 1:500 in PBS; Vector Laboratories) for 1hr and nickel enhanced DAB (10mg per 50mL PB with 2mL of 2% NiSO4 and 20μL of 30% H2O2 to produce a black reaction product, Sigma-Aldrich) for 10 min with intervening rinse steps. Tissue was then rinsed in PB and placed into 1% H2O2 in PBS, rinsed and incubated in blocking solution containing 4% NGS in PBST for 1 hr, and was incubated in rabbit anti-SST serum (dilution: 1:20,000 in PBST with 4% NGS) for 1 hr. Tissue was rinsed in PBS and incubated sequentially in biotinylated goat anti-rabbit IgG (dilution: 1:500 in PBST with 4% NGS), ABC-elite (dilution: 1:500 in PBS), and DAB (10mg per 50mL PB with 20μL of 30% H2O2 to produce a brown reaction product) for 10 min. Tissue was rinsed in PB and mounted onto superfrost slides, once dried, cover glass was applied with Eukitt mounting media.
The total number of SST cells and the percentage of SST cells that contained c-Fos was determined in 3 hemissections with a bright field microscope (Olympus AZ70, Tokyo, Japan) with a 20x objective. SST cells were counted in the ARC, VMN and ventral lateral aspect of the VMN (VL-VMN). The VL-VMN was defined as lateral to the medial edge of the fornix. All cell counts were made by a single observer blinded to treatment groups. Values were averaged per animal and analyzed with a t-test (senktide vs control) for animals treated in the POA and in the RCh separately. P < 0.05 was considered statistically significant. Data are presented as mean ± SEM percent or average number of cells per hemissection.

RESULTS

Experiment 1: Activation of SST cells throughout the estrous cycle

As previously reported (17), a greater percentage of SST cells in the ventral lateral aspect of the VMN contained c-Fos in ewes killed during the LH surge than during the early follicular or luteal phase of the estrous cycle (Fig 2). There were no significant differences in the average number of SST cells per hemissection between treatment groups (luteal, 152.4 ± 37.2; early follicular, 180.3 ± 48.9; mid LH surge, 199.3 ± 16.4).

Experiment 2: SST and nNOS colocalization within the VMN

A dense plexus of SST fiber-like staining was observed in the VMN as described previously (16,29,30). Numerous neuronal cell bodies were observed throughout the MBH with many SST cells in the ventral lateral aspect of the VMN. A large cluster of nNOS-ir neurons were also present in the ventral lateral aspect of the VMN, immediately lateral to the dense plexus of fiber-like SST staining (Fig 3, top row). Immunostaining with the nNOS antiserum produced well defined and very clear soma. Somatostatin-ir fibers and soma were evident
throughout the MBH, but few of them contained nNOS. Some of the SST-ir structures were approximately the size of soma, but based on shape, they were not unambiguous soma. Many of these structures were observed within nNOS-ir that did appear to be soma. Within the VMN (including the ventral medial aspect of the VMN) the majority of SST-ir neurons (81.7 ± 7.5%) also contained nNOS -ir, similarly 73.1 ± 2.5% of nNOS-ir neurons contained SST-ir (Fig 3, middle row).

No colocalization of nNOS-ir and SST-ir cells was observed in the ARC, though few SST cells (<10 per hemisection) were observed in the ARC. Similarly, minimal colocalization of SST-ir and nNOS-ir fibers was observed in this nucleus (Fig 3, bottom row).

**Experiment 3: Activation of the nNOS and SST cells in the VMN during the LH surge**

The total number of c-Fos-ir nuclei was greater in animals during the surge (83.6 ± 13) than the early follicular phase (45.1 ± 7.8). There was a trend (P = 0.07) for an increase in the percentage of all SST-ir cells that contained c-Fos-ir during the LH surge compared to the early follicular phase (Table 2, Fig 4). Neither the percentage of all nNOS-ir cells, nNOS only-ir cells nor SST only-ir cells that contained c-Fos differed between the stage of cycle (Table 2). However, the percentage of dual-labeled (nNOS and SST) cells that contained c-Fos was significantly greater during the LH surge than the early follicular phase (Table 2). The number of unidentified c-Fos cells (c-Fos nuclei but no cytoplasmic staining for SST or nNOS) and the total number of any type of neurotransmitter-containing cell (SST, nNOS, SST and nNOS) did not differ between groups (data not shown). The percentage of cells that colocalized SST and nNOS was lower in this experiment than in experiment 2 (47.5 ± 5.8% of SST cells had nNOS, and 18.3 ± 1.9% of nNOS-ir cells had SST-ir).
Experiment 4: Activation of SST cells during NK3R agonist-induced LH surge-like secretion

Administration of senktide into the RCh caused a surge-like increase in LH concentrations (14,28), but did not alter the mean number of SST cells per hemisection in the VMN (senktide, 35.11 ± 14.1; control 42.1 ± 22.3), VL-VMN (senktide, 30.3 ± 11.5; control, 40.0 ± 20.34) or the ARC (senktide, 6.7 ± 1.9; control, 7.8 ± 2.8). Similarly, the percentage of SST cells that contained c-Fos in either the VMN (senktide, 10.7 ± 2.6%; control, 16.8 ± 9.8%), VL-VMN (senktide, 26.9 ± 6.7; control, 14.1 ± 5.9%) or ARC (senktide, 7.8 ± 4.2%; control, 30.8 ± 16.5%) did not differ between animals that received senktide-containing or blank implants.

Administration of senktide into the POA caused a surge-like increase in LH concentrations (14,28), but did not alter the total number of SST cells in the VMN (32.4 ± 6.07; control, 58.1 ± 10.7), VL-VMN (senktide, 38.1 ± 2.62; control, 58.11 ± 10.65) or the ARC (senktide, 5.1 ± 0.8; control, 4.9 ± 0.9). However, senktide administration significantly reduced the percentage of SST-ir cells that contained c-Fos in the VL-VMN (senktide, 8.8 ± 2.6%; control, 23.3 ± 0.9%) and VMN (senktide, 10.23 ± 5.6%; control, 26.43 ± 3.1%), but not in the ARC (senktide, 21.9 ± 7.3; control, 34.4 ±7.1).

DISCUSSION

We have confirmed that SST cells in the VMN do express c-Fos during the LH surge and found that a portion of SST cells in the VMN also contain nNOS. Moreover, a greater percentage of dual-labeled SST and nNOS cells express c-Fos during the LH surge compared to the early
follicular phase, whereas neurons that contained nNOS or SST alone did not. However, when surge-like secretion of LH was induced via NK3R activation, c-Fos expression was not induced in SST cells in the MBH.

This is the first report of colocalization of nNOS and SST in the same population of neurons. This finding is supported by previous reports that SST cells in the VMN contain ERα (16,31), that progesterone receptor and ERα are colocalized throughout the hypothalamus (32) and that progesterone receptor is contained within cells stained with NADPHd (a marker for nitric oxide synthase) (33). The colocalization of SST and nNOS within neurons that are activated during the LH surge raises the intriguing possibility that the previously identified SST cells (based on Fos expression) in the VMN do contribute to the LH surge, but use NO, rather than SST, to do so. In mice, nNOS knock-out animals are infertile (34), due to an inability to ovulate (35). This ovulatory defect is mediated in large part by the lack of an LH surge, though ovary defects may also be present (19). It is possible that cells in the ovine VMN containing both SST and nNOS are the source of nitric oxide that stimulates LH surge secretion, but no data are available on the effects of nitric oxide on LH surge secretion in sheep.

The location of the fiber projections of the dual-labeled SST nNOS cells in the VMN is not known. Projections from the VMN to the POA have been demonstrated with anterograde tract-tracing agents in the sheep (36), which raises the possibility that SST cells in the VMN project to the POA. In Chapter 3, we identified structural synapses between SST fibers and GnRH cells in the POA. The number of these synapses was increased during the LH surge. GnRH neurons in the POA receive close contacts from nNOS fibers in prepubertal lambs (27). It remains to be determined whether SST and nNOS are colocalized within the same fibers within the POA. If SST fibers also contain nNOS, then the SST synapses onto POA GnRH cells
identified in Chapter 3 may also contain nNOS. Though speculative, these distinct anatomical observations provide a testable hypothesis that a population of cells in the VMN that contain SST, nNOS, and ERα project to the POA, synapse onto GnRH neurons and release the gaseous neurotransmitter nitric oxide to facilitate LH surge secretion. These neurons could also act via kisspeptin cells in the POA or ARC that have also been implicated in the LH surge in ewes (8–10). However the lack of colocalization of SST and nNOS in fibers in the ARC argues against an action via the kisspeptin neurons in this area.

The population of SST, nNOS and c-FOS triple-labeled cells observed during the LH surge were clustered in the ventral lateral aspect of the VMN, ventral to the fornix. The distribution of SST cells was similar when detected with either SST antibodies (rabbit x SST Peninsula, or rat x SST Millipore), however the total number of unambiguous cell soma staining was lower when detected with the Millipore antibody, possibly because antigen retrieval was used with this antibody. This reduced detection of SST soma likely contributes to the lower amount of colocalization between SST and nNOS cells in Experiment 3 than in Experiment 4, though there were also differences in endocrine status.

Neurokinin B and NK3R signaling are critical for fertility in humans (37). In sheep, administration of the NK3R agonist senktide into the POA or RCh caused surge-like secretion of LH (14,28). Several lines of evidence cited above support the possibility that SST may contribute to the LH surge, so we tested whether SST cells in the VMN express c-Fos during senktide induced LH surge-like secretion. A statistically significant decrease in the percentage SST cells that contained c-Fos was observed in animals that received senktide into the POA. The half-life of c-Fos protein has been estimated to be hours long (38), which makes the acute decrease in c-Fos expression within SST neurons difficult to interpret. The clear lack of an increase in c-Fos in
SST cells when senktide was administered into either area indicates that SST cells in the VMN are likely not involved in senktide-induced LH secretion. Moreover, two lines of evidence suggest that endogenous NKB does not act in the POA to induce LH surge secretion. First, a NK3R antagonist administered to the POA did not alter the timing or amplitude of an E2 induced LH surge (14). Second, there was no change in the percent of NK3R cells in the POA that expressed c-Fos during the LH surge compared to the luteal or early follicular phase of the estrous cycle (McCosh et al., unpublished observations). Thus, surge-like secretion of LH caused by senktide administration to the POA likely reflects an experimental artifact and not an important endogenous action of NKB in this area.

In conclusion, a specific population of cells in the ventral lateral aspect of the VMN contains both SST and nNOS, and is activated during the LH surge in ewes. In contrast, cells that contain nNOS or SST alone are not more active (based on Fos expression) at this time. Thus, we propose that this population of SST cells in the VMN also release NO and that this transmitter contributes to the generation of the LH surge in sheep.
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**FIGURE LEGENDS**

Figure 1: Representative immunohistochemistry controls (raw images). Top block: anti-SST (left column), nNOS antiserum preadsorbed with SST peptide (middle column), merged image (right column). Middle block: SST antiserum preadsorbed with nNOS immunogenic peptide (left column), anti-nNOS (middle column), merged image (right column). Lower block: anti-SST (left column), nNOS antiserum omitted (middle column), merged image (right column). Within each block, top row low magnification; bottom row, higher magnification. Bar = 50 µm.

Figure 2: Percent of SST immunoreactive cells in the VMN that contain c-Fos during the early follicular phase, luteal phase and mid LH surge. Bars with different letters differ, *P* <0.05.

Figure 3: SST and nNOS immunoreactivity in the ovine mediobasal hypothalamus (Top row, l0w magnification, bar = 500 µm), VMN (middle row, arrows indicate neurons that contain both nNOS and SST, triangles denote single labeled SST (top) or nNOS (bottom), bar = 50 µm. Bottom row, ARC, bar = 50 µm. Left column, SST immunoreactivity (greyscale), middle column, nNOS immunoreactivity (greyscale). Right column, merge: SST (green) and nNOS (red).

Figure 4: c-Fos, SST, and nNOS immunoreactivity in the VMN in an animal killed during the LH surge. Upper right, SST (red); upper left SST (red) and c-Fos (green); lower left, nNOS (magenta); lower right nNOS (magenta), SST (red) and c-Fos (green). Arrow indicates a dual-labeled nNOS and SST cell with c-Fos, triangles indicate dual labeled nNOS and SST cells without c-Fos. Bar = 20 µm.
Table 1: Antibody table

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<th>Peptide/protein target</th>
<th>Antigen sequence (if known)</th>
<th>Name of Antibody</th>
<th>Manufacturer, catalog #, and/or name of individual providing the antibody</th>
<th>Species raised in: monoclonal or polyclonal</th>
<th>Dilution used</th>
<th>RRID</th>
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<td>SST-14</td>
<td>Rbt x SST</td>
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<td>Rabbit polyclonal</td>
<td>1:200</td>
<td>AB_2255365</td>
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<tr>
<td>nNOS</td>
<td>C-Terminal nNOS</td>
<td>Rbt x nNOS</td>
<td>Immunostar; 24287</td>
<td>Rabbit polyclonal</td>
<td>1:1000</td>
<td>AB_572256</td>
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<tr>
<td>c-FOS</td>
<td>Fos zebrafish, human, rat, mouse</td>
<td>Rbt x c-Fos K-25</td>
<td>Santa Cruz: SC-253</td>
<td>Rabbit polyclonal</td>
<td>1:2000/1:3000</td>
<td>AB_2231996</td>
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Table 2: Percentage of immunoreactive cells that contain c-Fos within the VMN

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<th>Early follicular</th>
<th>Mid LH surge</th>
<th>Statistical comparison</th>
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<tr>
<td>dual labeled SST + nNOS cells</td>
<td>3.9 ± 1.3</td>
<td>17.3 ± 0.3</td>
<td>P = 0.0096</td>
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<tr>
<td>all SST-ir cells</td>
<td>10 ± 2.9</td>
<td>21.6 ± 4.1</td>
<td>P = 0.07</td>
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<tr>
<td>all nNOS-ir cells</td>
<td>7.2 ± 2.3</td>
<td>15.8 ± 4.7</td>
<td>not significant</td>
</tr>
<tr>
<td>SST only-ir cells</td>
<td>14.1 ± 4.0</td>
<td>24.6 ± 6.9</td>
<td>not significant</td>
</tr>
<tr>
<td>nNOS only-ir cells</td>
<td>7.1 ± 2.2</td>
<td>15.4 ± 5.4</td>
<td>not significant</td>
</tr>
</tbody>
</table>
Figure 1:
Figure 2:

![Bar chart showing the percent of SST cells with c-Fos at different stages of the menstrual cycle. The chart compares Luteal, Early Follicular, and Mid LH Surge phases.](image)
Figure 4
CHAPTER 5

GENERAL DISCUSSION
GENERAL DISCUSSION

In work published before this dissertation, several lines of evidence supported the hypothesis that somatostatin (SST) is involved in the LH surge and that SST could inhibit episodic LH secretion. However, these studies were either correlative in nature or examined the effects of exogenous SST. The preceding chapters describe pharmacological and neuroanatomical data collected to test the hypothesis that somatostatin acts in the hypothalamus to regulate LH secretion in sheep. These studies are the first to test the hypotheses that endogenous SST release contributes to the control of the LH surge and episodic LH secretion in any species.

SOMATOSTATIN AND THE LH SURGE

Several lines of correlative evidence described in previous chapters led to the hypothesis that somatostatin (SST) contributes to the LH surge, however neither a SST receptor 2 (SSTR2) agonist nor antagonist altered the timing or amplitude of the LH surge (Chapter 2). These findings led us to reject the hypothesis that SST acting via SSTR2 has a major role in surge secretion of LH. An important caveat to this interpretation is that treatment with drug may have altered the GnRH surge secretion, but not LH surge secretion. Although a surge of GnRH causes the LH surge (1,2), and the amplitudes of the GnRH and LH surge are correlated, GnRH is often secreted in excess of that necessary to induce an LH surge (3). Additionally, the duration of the GnRH surge is much longer than that of the LH surge (4). Therefore, treatments administered in Chapter 1 may have had minor effects on GnRH surge secretion but did not manifest in a change in LH surge secretion.
We used SSTR2-selective agonist, octreotide (5), and competitive antagonist, CYN154806 (6,7), that have been demonstrated to be selective for human and rodent SSTR2, but have not previously been tested in sheep. SSTR2 was selected as a target based on evidence that this receptor mediates the actions of SST in rodents (8,9) and that GnRH cells in the preoptic area (POA) of sheep contain SSTR2, but not the other SST receptor subtypes, as determined by immunohistochemistry in a preliminary report (10). These findings do not preclude the possibility of action at other receptors. In mice, an SSTR2 agonist was shown to be equally as effective at inhibiting GnRH cell firing as SST itself. This finding is good evidence that SSTR2 is important but does not preclude the possibility that other receptors may be involved. In mice, SSTR2mRNA only (8) and in sheep only SSTR2 protein (10) was detected in POA GnRH cells. These anatomical findings support a role for SSTR2, however the lack of detection of a molecule does not prove its absence. Furthermore, data presented in this dissertation support the hypothesis that SST acts via GnRH cells in the MBH and kisspeptin cells in the ARC although there is no information on which receptors they contain.

The observation that SST cells within the ventral medial nucleus (VMN) also contain neuronal nitric oxide synthase (nNOS) raises the possibility that a different signaling molecule produced by the same cells may contribute to the LH surge (Chapter 4). Projections from the VMN to the POA have been demonstrated with anterograde tract-tracing agents in the sheep (11), which supports the possibility that SST cells in the VMN project to the POA. In Chapter 3, we identified structural synapses between SST fibers and GnRH cells in the POA. The number of these synapses was increased during the LH surge, possibly indicating increased transmission of neuromodulators. GnRH neurons in the POA also receive close contacts from nNOS fibers in prepubertal lambs (12). Based on these two observations, we hypothesize that the SST synapses
onto GnRH neurons also contain nNOS. Though speculative, these distinct anatomical observations provide a testable hypothesis that a population of cells in the VMN that contain SST, nNOS, and estrogen receptor α project to the POA and synapse onto GnRH neurons to release the gaseous neurotransmitter nitric oxide (NO) to facilitate LH surge secretion. It is also possible that SST-nNOS fibers project onto POA kisspeptin cells that have also been implicated in surge secretion of LH in sheep (13,14).

Several experiments are needed to test the hypothesis that neurons in the VMN that express both SST and nNOS contribute to the generation of the LH surge. First, to determine if NO stimulates LH surge secretion in sheep, a nNOS inhibitor could be administered during the induction of the LH surge. Second, immunofluorescence could be used to determine whether fibers that contain SST and nNOS are in close apposition to GnRH neurons in the POA and increase during the LH surge. If these dual labeled fibers are present, retrograde tract tracing experiments could demonstrate the origin of these fibers (VMN if the present hypothesis is correct). If this SST/nNOS neural circuit exists, it may serve as a redundant mechanism for the generation of the LH surge that acts in concert with the NKB/kisspeptin system described in Chapters 1 and 4.

**SOMATOSTATIN AND PULSATILE LH SECRETION**

Experiments in Chapter 2 demonstrate that endogenous SST, acting at least partly through SSTR2, inhibits episodic LH secretion. Furthermore, blockade of SSTR2 in ovary-intact ewes increased mean LH in both the breeding season and anestrus, whereas in ovariectomized (OVX) ewes, SSTR2 blockade increased LH pulse frequency only in anestrus. Together, these studies provide evidence that SST contributes to steroid-independent suppression of LH during
anestrus, and may also have a minor role in the inhibitory actions of ovarian steroids on LH secretion.

Despite the observation of steroid-independent suppression of gonadotropin secretion in response to photoperiod over 30 years ago, we still know very little about possible mechanisms for this effect. The only other signaling molecule that has been implicated in steroid-independent suppression of LH during anestrus is serotonin. Serotonin receptor antagonists increase LH pulse frequency in OVX ewes in anestrus but not during the breeding season (15,16). Serotonin depletion during anestrus increased LH pulse frequency in OVX ewes but not in OVX ewes treated with estradiol (E\textsubscript{2}) (16). Similarly, a 5-HT\textsubscript{2} receptor antagonist (cyprohetadine) did not alter LH pulse frequency in intact anestrous ewes (17). But in another study, cyprohetadine increased LH pulse frequency and mean LH in E\textsubscript{2}-treated OVX ewes during anestrus (18). Thus, inhibitory effects of serotonin have been consistently demonstrated in OVX ewes, but are only sometimes apparent in steroid-treated or intact ewes. Selective (ketanserin and methysergide) and non-selective (cyprohetadine) 5-HT receptor antagonists, as well as serotonin depletion, all increased LH pulse frequency, results that are consistent with action at the 5-HT\textsubscript{2} receptors (19).

Similar to the effects of serotonin receptor blockade, blockade of SSTR2 increased mean LH in intact ewes during both the breeding season and anestrus, though there are some differences between studies. First, cyprohetadine increased LH pulse frequency in E\textsubscript{2}-treated OVX ewes acutely exposed to a stimulatory photoperiod, but before LH secretion had risen (18), whereas in the present study (Chapter 2, experiment 2), CYN did not alter LH pulse frequency in luteal phase ewes. Whether this is due to differential action of SST and serotonin neural systems or if this is due to the presence of progesterone, a potent inhibitor LH secretion (20,21), in our study (Chapter 2) is not known. Second, cyprohetadine increased LH pulse frequency in E\textsubscript{2}
treated OVX ewes in anestrus (18) whereas in the present study, CYN did not alter LH pulse frequency in intact anestrus ewes. One possible difference between these two studies is the endocrine status (OVX +E vs ovary-intact animals). Notably, disruption of serotonin signaling failed to increase LH secretion in OVX+E anestrous ewes in other studies (16) and a stimulatory effect on pulse frequency in our study (chapter 2, experiment 2) could have been masked by an unexpected high pulse frequency when intact anestrous ewes were treated with vehicle.

Despite subtle differences in response to antagonists, SST and serotonin may interact in the ovine hypothalamus. The ovine MBH contains abundant serotonergic fiber innervation (22) and mRNA for 5-HT2 receptor (23), therefore serotonin may suppress LH secretion by activating SST neurons. This possibility would also be consistent with the theory that serotonin suppresses LH via an inhibitory interneuron since 5-HT2 receptor activation releases diacylglycerol and inositol triphosphate, resulting in increased protein kinase C activity and Ca++ influx, which are generally considered excitatory signaling events (24). Functional tests of this hypothetical neural system with pharmacological approaches will be difficult because it involves inhibiting an inhibitor. If the SST cells in the VMN contain 5-HT2 receptor, a 5-HT-saporin conjugate could be used to ablate SST cells to determine whether the stimulatory effects of 5HT2 receptor antagonists act via SST. However, this technique will ablate all cells with 5-HT2, which may include cells other than just SST cells, complicating the interpretation of results. Therefore the first step in determining the feasibility of this approach is a detailed description of 5-HT2 content within the VMN. Correlative evidence could be provided if serotonin receptor antagonist induces c-Fos in SST neurons. Anatomical evidence for this neural circuit could be demonstrated by showing serotonergic fibers in close apposition to SST neurons and the presence of 5-HT2 receptor in SST neurons.
SITES OF ACTION FOR ENDOGENOUS SST

Although inhibitory actions of exogenous SST have now been demonstrated in humans, rodents and sheep, the specific sites of action of this neurotransmitter remain to be determined. In humans, iv SST infusion (25) and subcutaneous injections of octreotide (26) suppressed LH secretion. However, these peripherally administered drugs likely act at the pituitary gland because SST (27), a SST analogue (28), or a SSTR2 agonist (26) blunted the increase in LH caused by GnRH or GnRH agonists. These results do not preclude the possibility that SST also acts in the human hypothalamus to regulate GnRH secretion as it likely does in sheep and rodents; however, this possibility has not been specifically tested.

In rodents, SST has been demonstrated to act in the hypothalamus. Electrophysiological experiments have shown that SST can potently inhibit GnRH cell firing in the POA (8,29). Furthermore, icv SSTR2 agonist administration blunted the LH surge and also prevented an increase in c-Fos within these GnRH cells (9). Thus, there is both in vitro and in vivo data for action of SST within the hypothalamus, and more specifically in the POA, of rodents.

In sheep, icv administration of SST inhibited episodic LH secretion (30) and GnRH neurons within the POA contain SSTR2 (10), however little else was known about potential sites of SST action. Therefore, we administered the SSTR2 agonist and antagonist into the third ventricle because this approach has the advantage of testing a functional role for SST signaling without a priori information of sites of action.

Several lines of evidence reported here support the hypothesis that SST acts in the MBH to suppress pulsatile LH secretion. First, analysis of the radiographs of cannula placement in ewes for experiment 3 (intact, anestrum, Chapter 2) indicate that cannula placed closer to the
infundibular recess had a greater response to CYN injections. Second, CYN induced an increase in percentage of GnRH and kisspeptin cells that contained c-Fos only in the MBH. Moreover, we found that 23 ± 4% of GnRH cells in the MBH had SST synapses in anestrous ewes, which is strikingly similar to the 24 ± 5% of GnRH cells that contained c-Fos after SSTR2 antagonist administration during anestrus. Thus, SST may act directly on a subset of GnRH cells in the MBH to regulate pulsatile LH secretion.

Another possibility is that SST acts on KNDy cells in the ARC. KNDy cells are so named because they contain three neuropeptides kisspeptin, NKB, and dynorphin and have been implicated in pulsatile LH secretion. In these experiments we used two different markers for this population of neurons: kisspeptin (Chapter 2) and NKB (Chapter 3). The switch in markers was necessitated by the unavailability kisspeptin antiserum for later experiments. However the high degree of colocalization of these peptides in sheep (31) indicates that both peptides are adequate markers of the same population of neurons. The majority of KNDy cells received SST synapses (Chapter 3), but there was a relatively modest increase in the percentage of kisspeptin cells (and only in the caudal ARC) that contained c-Fos after SSTR2 antagonist administration.

There are several possible experimental approaches to clarify the sites of SST action in the regulation of episodic LH secretion. A detailed description of SST receptor expression in the ovine hypothalamus could indicate possible sites of action. Specifically assessing SSTR2 colocalization within KNDy cells would determine whether KNDy cells are a possible site of SST action. If SST does act via KNDy cells to suppress LH secretion, then administration of a Kiss1R antagonist should block the stimulatory effects of a SSTR2 antagonist.
CONCLUSIONS

In this work I have demonstrated that endogenous SST plays an important role in the steroid-independent actions of photoperiod to suppress LH pulse frequency, but does not contribute to the control of the LH surge. Under many physiological conditions, female fertility is dependent on adequate episodic LH secretion, which is regulated by steroid-dependent and steroid-independent mechanisms. Although steroid-dependent suppression of gonadotropin secretion is more dramatic than steroid-independent suppression of LH in sheep, steroid-independent suppression of LH is evident and critical for prepubertal suppression of LH in agonal monkey s (32,33) and children (34,35). The finding that SST contributes to steroid-independent suppression of LH pulse frequency during anestrus raises the exciting possibility that SST may contribute to steroid-independent suppression of LH in other contexts, such as prior to puberty.
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