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Casey C. Nestor
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Evidence of a Role for Three Neuropeptides that Mediate Steroid Negative Feedback on Gonadotropin Releasing Hormone/Luteinizing Hormone Secretion in the Ewe: Kisspeptin, Neurokinin B and Orphanin FQ

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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

Cellular and Integrative Physiology

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Key Words: gonadotropin releasing hormone, luteinizing hormone, kisspeptin, neurokinin B, orphanin FQ, sheep

ABSTRACT

Evidence of a Role for Three Neuropeptides that Mediate Steroid Negative Feedback on Gonadotropin Releasing Hormone/Luteinizing Hormone in the Ewe: Kisspeptin, Neurokinin B and Orphanin FQ

Casey C Nestor

Gonadotropin-releasing hormone (GnRH) is the final common output from the central nervous system for controlling luteinizing hormone (LH) secretion from the anterior pituitary. GnRH, and subsequently LH, secretion for most of the menstrual/estrous cycle occurs in an episodic pattern that is controlled by the inhibitory actions of estradiol and progesterone. However, GnRH neurons are devoid of the appropriate steroid receptors, therefore, estradiol and progesterone must mediate their effects on GnRH/LH secretion through interneurons. Three likely candidates are neurons containing kisspeptin, neurokinin B (NKB) and orphanin FQ (OFQ).

Puberty in the ewe is marked by an increase in GnRH/LH secretion which in part results from a lessening of estradiol negative feedback. Kisspeptin and NKB are coexpressed in the same subsets of neurons in the arcuate nucleus (ARC) of the hypothalamus and are proposed to be critical for normal timing of puberty. Given that greater than 95% of kisspeptin/NKB neurons colocalize with estrogen receptor-α (ERα) and progesterone receptor in the ewe, we examined changes in kisspeptin- and NKB-positive neurons in the ARC of pre- and postpubertal ewes in the presence and absence of sex steroids. We observed a greater number of kisspeptin, but not NKB, positive neurons in the ARC of postpubertal ewes compared to prepubertal ewes, which corresponded with changes in LH secretion. Also, we showed that an intravenous injection of senktide, an NKB receptor agonist, stimulates LH secretion in prepubertal ewes. These results support a role for kisspeptin and NKB in ovine puberty.

OFQ when given centrally has been shown to inhibit LH secretion in rats and ewes and OFQ localizes to the external zone of the median eminence. Given its effect on LH secretion and its location, we investigated whether OFQ acts at the pituitary to inhibit LH release and/or at the hypothalamus to inhibit GnRH secretion. OFQ had no direct influence on ovine anterior pituitary cells in cell culture.

However, we did observe a decrease in GnRH secretion in hypophyseal portal blood samples following infusion of an OFQ agonist into the third cerebroventricle of ovariectomized ewes. From this we conclude that OFQ acts centrally at the hypothalamus to inhibit GnRH secretion.

Given its central inhibitory action on GnRH secretion in the ewe, we examined if OFQ neurons in the preoptic area (POA) and hypothalamus contain steroid receptors. Furthremore, we investigated a role for endogenous OFQ by infusing an OFQ receptor antagonist into steroid-treated ewes. POA OFQ neurons did not contain steroid receptors, while a majority of OFQ neurons in the ARC contained both ERα and progesterone receptor. Furthermore, ovariectomized (OVX) ewes with luteal phase concentrations of progesterone and estradiol, but not OVX ewes implanted with only estradiol or ovary-intact anestrous ewes, showed a significant increase in LH pulse frequency during infusion of an OFQ receptor antagonist. Therefore, we conclude that OFQ mediates, at least in part, the negative feedback action of progesterone on GnRH/LH secretion in the ewe.

DEDICATION

I dedicate this collection to the men and women who donated their bodily remains to further the education and wellbeing of generations to come.

Respectfully yours, Casey C Nestor

ACKNOWLEDGEMENTS

Let me take a moment to recognize several individuals that have made this work possible. I'll do my best to include all those that participated in my doctoral education.

I walked into Bob Goodman's office almost eight years ago hoping to gain insight into what research had to offer. I never knew that he would have the patience to put up with me for this long. To say the least he deserves many thanks for all the hours of surgical expertise, repeated explanations, continuous funding and the congratulatory drinks following many successful, and some not so successful, experiments. Thanks Bob for being an excellent mentor and a wonderful friend. With help from Stan Hileman I gained better editorial and surgical skills and learned how to laugh at myself while pulling a second golf ball from my pocket. He helped bring me home safely from Slovakia and for that my mother thanks him. I thank John Connors who gave me my first opportunity to teach and provided excellent surgical advice. He is full of great humor and from May to July each year has one of the best looking lawns in Morgantown. Ida Holaskova was sentenced to share an office with me for two years. I thank her for listening to my personal and profession ideas. Stan, John and Ida joined forces in an attempt to teach me statistics. I still use the 'eye squared' test, but can follow it with legitimate statistical analysis. Jorge Flores brought his *in vitro* expertise to the table reminding me that specificity should be one of my main priorities. Dave Smith contributed with pharmacological advice and kept me thinking, as did all of my committee members, in terms of the big picture. I would like to thank Miro Valent for escorting Stan and I through Slovakia. He taught me the ins and outs of the farm and the laboratory from day one and treated me as if I was his own son. I thank him for all the radioimmunoassays and for the pictures throughout the years. Also, I'm thankful for his wife's wonderful cooking. Those apple things are delicious. Gail Nesselrod had to endure hours of bad language and even worse jokes. I thank her for all her help working with the sheep. Sushma Singh and Adrienne Bogusz warned me about what I was getting myself into and I didn't listen. I thank them for their help early in my degree with experimental design and friendly encouragement. Karen Martin was a great help in navigating the imaging facility. I

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ABBREVIATIONS

GnRH	Gonadotropin-Releasing Hormone
LH	Luteinizing Hormone
OFQ	Orphanin FQ
NKB	Neurokinin B
ARC	Arcuate nucleus
OVX	Ovariectomized
POA	Preoptic area
FSH	Follicle Stimulating Hormone
AHA	Anterior Hypothalamic Area
ERα	Estrogen Receptor-alpha
PR	Progesterone Receptor
OVX + E_2	Ovariectomy plus estradiol treatment
EOP	Endogenous opioid peptides
OVX + P	Ovariectomy plus progesterone treatment
OVX + P + E_2	Ovariectomy plus progesterone plus estradiol treatment
MBH	Medial Basal Hypothalamus
ME	Median Eminence
ORL-1	Opioid-Receptor Like-1
NK3R	Neurokinin 3 Receptor
GPR54	G-coupled protein receptor 54
OVLT	Organum Vasculosum of the Lamina Terminalis
AVPV	Anteroventral Periventricular Nucleus
VMH	Ventromedial Hypothalamus
EF	Early Follicular
mPOA	medial Preoptic Area

RIA	Radioimmunoassay
GNDX	Gonadectomized
DAB	3,3'-diaminobenzidine tetrahydrochloride
POMC	Proopiomelanocortin
TH	Tyrosine Hydroxylase
AgRP	Agouti-related Peptide
GABA	gamma-aminobutyric acid
icv	intracerebroventricular

TABLE OF CONTENTS

Evidence for a Role of Three Neuropeptides that Mediate Steroid Negative Feedback on Gonadotropin Releasing Hormone/Luteinizing Hormone in the Ewe: Kisspeptin, Neurokinin B and Orphanin FQ…………………………………..……………………..…………….i

ABSTRACT……………………………..……………………………….……..……...…….ii
DEDICATION…………………………..…….…………………………….………….…….iv
ACKNOWLEDGEMENTS………………..…….………………………….…………..…….v
ABREVIATIONS………………………..………….…………………………….…………vii
TABLE OF CONTENTS. ………………..……………………………………….….………....ix

CHAPTER 1: GnRH/LH SECRETION……………………………………………..……………….1

GONADOTROPIN-RELEASING HORMONE: ANATOMY AND FUNCTION………………2
FEEDBACK CONTROL OF GnRH AND LH DURING THE OVARIAN CYCLE…………….3
ROLE OF CHANGES IN STEROID NEGATIVE FEEDBACK IN PUBERTY AND SEASONAL BREEDING…………………………………………………………...5
ENDOGENOUS OPIOID PEPTIDES (EOP)……………………………………………….....10
NEUROKININ B (NKB)…………………………………………………………………………13
KISSPEPTIN…………………………………………………………………………………..14
POSSIBLE ROLES FOR KISSPEPTIN AND NKB IN PUBERTY ONSET…………………...17
SPECIFIC AIMS………………………………………………………………………………19
REFERENCE LIST……………………………………………………………………………21

CHAPTER 2: EVIDENCE OF A ROLE FOR KISSPEPTIN AND NEUROKININ B IN PUBERTY OF FEMALE SHEEP……………………………………………………43

ABSTRACT……………………………………………………………………………………44
INTRODUCTION………………………………………………………………………….45
MATERIALS AND METHODS……………………………………………………………46
RESULTS…………………………………………………………………………………...51
DISCUSSION……………………………………………………………………………….54
REFERENCE LIST…………………………………………………………………………59
FIGURE LEGENDS………………………………………………………………………...66
FIGURES……………………………………………………………………………………68

CHAPTER 3: ORPHANIN FQ ACTS AT THE HYPOTHALAMUS TO SUPPRESS GONADOTROPIN RELEASING HORMONE SECRETION IN THE EWE…….73

ABSTRACT……………………………………………………………………………………74
INTRODUCTION………………………………………………………………………….75
MATERIALS AND METHODS…………………………………………………………...76
RESULTS…………………………………………………………………………………...80
DISCUSSION……………………………………………………………………………….82
REFERENCE LIST…………………………………………………………………………85
FIGURE LEGENDS………………………………………………………………………...89
FIGURES…………………………………………………………………………………....91

CHAPTER 4: EVIDENCE THAT ORPHANIN FQ MEDIATES PROGESTERONE NEGATIVE FEEDBACK IN THE EWE……………………………………..........98

ABSTRACT………………………………………………………………………………99
INTRODUCTION……………………………………………………………………...101
MATERIALS AND METHODS…………………………………………………………..102
RESULTS………………………………………………………………………………107
DISCUSSION…………………………………………………………………………...109
REFERENCE LIST……………………………………………………………………..113
FIGURE LEGENDS……………………………………………………………………119
FIGURES………………………………………………………………………………..121

CHAPTER 5: GENERAL DISCUSSION……………………………………………………128

KISSPEPTIN AND NKB IN PUBERTY…………………………………………………129
SITE OF ACTION OF OFQ……………………………………………………………132
ROLE FOR ENDOGENOUS OFQ……………………………………………………..132
REFERENCE LIST……………………………………………………………………..135

PERMISSION TO REPRODUCE COPYRIGHT MATERIALS…………………………………..137

APPENDIX………………………………………………………………………………...138

SUPPLEMENTAL FIGURE LEGENDS.......……………………………………………..138
SUPPLEMENTAL FIGURES……………………………………………………………..139

CURRICULUM VITAE……………………………………………………………………..143

CHAPTER 1

GnRH/LH SECRETION

GONADOTROPIN-RELEASING HORMONE: ANATOMY AND FUNCTION

Gonadotropin-releasing hormone (GnRH) is the final common output from the central nervous system that governs reproduction. As its name implies, GnRH stimulates the gonadotropes of the anterior pituitary to release luteinizing hormone (LH) and follicle stimulating hormone (FSH). The GnRH gene is found on chromosome 8 in humans with exon 2 encoding the GnRH decapeptide (1-3). During midgestation, GnRH neurons differentiate in the olfactory placode, then migrate through the nasal septum and enter the ventral forebrain establishing an adult distribution prior to birth. In rodents, GnRH cell bodies are found almost exclusively in the preoptic area (POA) and anterior hypothalamic area (AHA), while in sheep and primates, GnRH perikarya are found in the POA, AHA and medial basal hypothalamus (MBH;(4)). In rats and sheep, an equal percentage of GnRH neurons from the before mentioned areas send projections to the external zone of the median eminence (ME), while in primates the ventral hypothalamic tract of the MBH has the greatest percentage of neuroendocrine GnRH projections (5-8).

Once secreted, GnRH enters a network of fenestrated capillaries in the external zone of the ME and travels to the anterior pituitary via the hypophyseal portal veins where it acts via a G-coupled protein receptor to stimulate the synthesis and secretion of LH and FSH. In the female, gonadotropin action depends upon the targeted structure in the ovary (follicle and corpus luteum). During the follicular phase, LH and FSH stimulate follicles in the ovary to develop and produce estradiol. During the luteal phase, LH stimulates the corpus luteum to produce progesterone and, in some species, estradiol. These steroids, estradiol and progesterone, complete a negative feedback loop by inhibiting GnRH secretion at the hypothalamus as well as by inhibiting LH secretion at the pituitary. Since GnRH and LH secretion closely coincide throughout most of the estrous cycle, these two hormones are commonly referred to in tandem (*i.e.,* GnRH/LH)

The estrous cycle in ewes (9) and the menstrual cycle in primates (10), averages 16 to 17 and 28 days, respectively, and consist of two phases, follicular and luteal, each named for its dominant ovarian structure. The difference in cycle length between the ewe and primate is due to a shortened follicular

phase in the ewe. During the early to mid-follicular phase, follicular growth occurs with gradually increasing GnRH/LH and estradiol concentrations, while in the late follicular phase, a large increase in estradiol leads to a large increase in GnRH/LH secretion (the GnRH/LH surge) which causes ovulation. Following ovum release, the remaining follicular structure becomes luteinized, forms a corpus luteum and survives for approximately 14 days producing progesterone, which keeps GnRH/LH concentrations relatively low. If fertilization does not occur, the corpus luteum regresses and the cycle begins again. The length of the estrous cycle in rodents (11) is 4 or 5 days because rodents have eliminated a normal length luteal phase. The rodent cycle can be separated into four distinct parts: metestrus, diestrus, proestrus and estrus. During metestrus and diestrus, follicular development occurs within the ovary. On the afternoon of proestrus, a large increase in LH secretion occurs and results in follicular rupture and release of an ovum; this is followed by an estrous period when the female is sexually receptive to the male. If fertilization does not occur, the reproductive cycle can begin again.

FEEDBACK CONTROL OF GnRH AND LH DURING THE OVARIAN CYCLE

During the estrous/menstrual cycle, GnRH/LH secretion occurs in two patterns: surge and tonic. LH concentrations on the afternoon of proestrous in rats can reach 200 times greater than basal levels (12, 13). The stimulus for this GnRH/LH surge in rodents appears to be estradiol. Several groups have shown that ovariectomized (OVX) rats given exogenous estradiol on the morning of proestrus respond with a surge-like release of LH similar to intact proestrous rats on the afternoon of proestrus (14-20). Also, the LH surge can be blocked by giving an estrogen antagonist (21), estradiol antiserum (22) or by OVX on diestrus (23). Also in rodents, progesterone plays a role in the timing and magnitude of the surge given that administration of progesterone can advance the LH surge a few hours and dramatically increase its amplitude in estrogen primed rats on the day of proestrous, but not on other days of the estrous cycle (24). Therefore, in rodents both estradiol and progesterone act together to elicit GnRH/LH surge secretion.

In sheep and primates, estadiol alone appears to be the driving stimulus for the GnRH/LH surge. The GnRH/LH surge is preceded by elevated estradiol concentrations in ewes (25) and female monkeys (26). Furthermore, estradiol alone is enough to induce a GnRH/LH surge in OVX sheep (27, 28) and

monkeys (29-33). Also, immunoneutralization of estradiol can block the surge in intact, cycling monkeys (34) and ewes (35-39). In contrast to rodents, progesterone does not appear to play an analogous role in these species. In the ewe, increasing the circulating concentrations of progesterone during the follicular phase does not accelerate the timing of the surge (40). Furthermore, a progesterone receptor antagonist, RU486, fails to block surge induction in sheep (41). Although having no stimulatory effects on the LH surge in these species, progesterone, if present, can block the GnRH/LH surge in sheep (42) and primates (34, 43-45). However, the fall in progesterone following regression of the corpus luteum appears to contribute to the timing of the GnRH/LH surge in ewes given that prolonging the elevation in progesterone concentrations by progesterone administration can delay the timing of the surge in ewes (46). Nonetheless, as intriguing as GnRH/LH surge secretion may be, my experimental focus herein will be placed solely on the regulation of GnRH/LH secretion by steroid negative feedback in the ewe.

For most of the estrous cycle in rodents and sheep as well as most of the menstrual cycle in primates, GnRH/LH secretion occurs in a pulsatile pattern, consisting of brief episodes of secretion separated by relatively long periods of no secretion (*i.e.,* tonic secretion). Tonic secretion during the follicular phase consists of GnRH/LH pulses of high frequency and low amplitude, while in the luteal phase GnRH/LH pulses have a low frequency and high amplitude (47). This tonic mode of secretion in rodents appears to be primarily controlled by estradiol since estradiol alone in OVX rats or progesterone in estrogen-primed rats can suppress LH secretion, but progesterone by itself cannot (24). In sheep and monkeys, tonic GnRH/LH secretion during the early follicular phase is controlled by the negative feedback of estradiol given that estradiol can inhibit GnRH/LH pulse amplitude, while increasing pulse frequency in OVX ewes (48, 49) and monkeys (50). In sheep and primates, progesterone is the primary steroid responsible for controlling tonic GnRH/LH secretion during the luteal phase. Progesterone administration in OVX ewes can decrease LH pulse frequency (48), while progesterone in combination with estradiol can suppress LH secretion below detectable levels in the OVX monkey (26). Therefore, during the estrous/menstrual cycle, estradiol negative feedback inhibits GnRH/LH pulse amplitude, while progesterone negative feedback inhibits GnRH/LH pulse frequency (Figure 1).

ROLE OF CHANGES IN STEROID NEGATIVE FEEDBACK IN PUBERTY AND SEASONAL BREEDING

Changes in steroid negative feedback can cause alterations in GnRH/LH pulse patterns that have been shown to control important hallmarks of fertility, such as pubertal progression and seasonality. Puberty is defined as a time when the individual first gains the ability to reproduce. Regardless of species, prepubertal GnRH/LH concentrations are low with minimal GnRH/LH pulse frequency. What does differ between species is the reason for low gonadotropin levels. In the rat, OVX as early as postnatal day 6 yields maximal LH concentrations, while estradiol alone is not sufficient to suppress gonadotropin levels to prepubertal values (51). Changes in the shape of GnRH neurons (52) and other hypothalamic neurons in the ARC (53) occurs during this pubertal transition. Furthermore, the lack of elevated LH secretion prior to puberty cannot be solely accounted for by the absence of GnRH activity and estrogen receptors (54, 55) or at the pituitary by its inadequate response to GnRH (56). Therefore, the absence of LH prepubertally in rodents most likely is a combination of hypothalamic inhibition and pituitary insufficiency. In primates, the ovary appears to play little part in the prepubertal brake on GnRH/LH secretion as removal of the ovaries in monkeys does not cause any increase in GnRH/LH secretion (57). The reason for the lack of LH secretion appears to be central in nature because exogenous pulsatile administration of GnRH stimulates LH secretion (58), thus verifying that the pituitary is not a limiting factor in puberty onset for this species.

The current working model for puberty in the ewe can be found in what has been called the gonadostat hypothesis, in which it is postulated that decreased hypothalamic release of GnRH/LH secretion is due to steroid negative feedback from the ovary (Figure 2). Indeed, puberty in the ewe is well-defined by the increase in GnRH/LH secretion that is followed by the first ovulation and often includes a short luteal phase followed by a complete estrous cycle (59). Prior to puberty, estradiol negative feedback is the primary contributor to the inhibition of GnRH/LH pulse frequency. Thus, OVX of prepubertal ewes as early as 6 weeks of age increases LH pulse frequency and this can be readily reversed by estradiol replacement up until 27 weeks of age (60). In further support for the concept of

lessening estradiol negative feedback at puberty, it has been reported that OVX ewes replaced with estradiol (OVX+E_2) have LH concentrations below detectability until 28 weeks of age, after which LH secretion increases, at a time similar to when intact ewes begin to display estrous cycles (61). This age-dependent increase of LH in OVX+E_2 ewes reflects a loss in the ability of estradiol to inhibit GnRH/LH pulse frequency (62); as noted above estradiol inhibits pulse amplitude in adult breeding season ewes. Therefore, the gonadostat hypothesis holds particularly true for the ewe with the pubertal increase in GnRH/LH pulse frequency being due to a lessening of steroid negative feedback. However, the neural systems mediating this reduction in estradiol negative feedback remains to be determined.

Another instance when GnRH/LH secretion is altered by steroid negative feedback is during photoperiod-induced (or seasonal) changes in fertility. Mice and rats are not seasonal breeders, but sheep have a distinct window of reproductive activity during the fall and winter months (63). For half of the year, non-pregnant ewes become reproductively quiescent wherein estrous cycles come to a stop (a period of time known as anestrus or the non-breeding season). The lengthening days of spring and summer bring about a reduction in GnRH/LH secretion in the ewe caused by increased sensitivity to estradiol negative feedback as evidenced by lower LH secretion during long-day, but not short-day, photoperiods in OVX+E_2 ewes (64). Melatonin secretion from the pineal gland, which is only secreted at night and thus inversely proportional to the amount of daylight, appears to be driving this seasonal effect because pinealectomized OVX + E_2 ewes given a long-day pattern of melatonin while on a short-day photoperiod regimen have low LH secretion (65). As with puberty, the seasonal changes in response to estradiol negative feedback are reflected by a change in GnRH/LH pulse frequency (Figure 1): the effect of estradiol on GnRH/LH secretion during anestrus is a reduction in pulse frequency, while during the breeding season it inhibits pulse amplitude (66-68). This seasonal change in the ability of estradiol to inhibit LH pulse frequency is suggestive of a primary change in the hypothalamus. Furthermore, since administration of pentobarbital, which decreases neural activity, can increase LH secretion in anestrous ewes, but not breeding season ewes (69), it has been theorized that inhibitory afferent neurons mediate the negative feedback of estradiol on GnRH/LH secretion during seasonal anestrous in the ewe. Considerable

work has lead to idea that dopaminergic and kisspeptinergic neural systems participate in this inhibition (70).

Two figures that quite succinctly summarize the effects of steroids on LH secretion in the adult ewe during breeding and non-breeding seasons (Figure 1) and during the pubertal transition (Figure 2) can be seen on the next two pages.

Figure 1

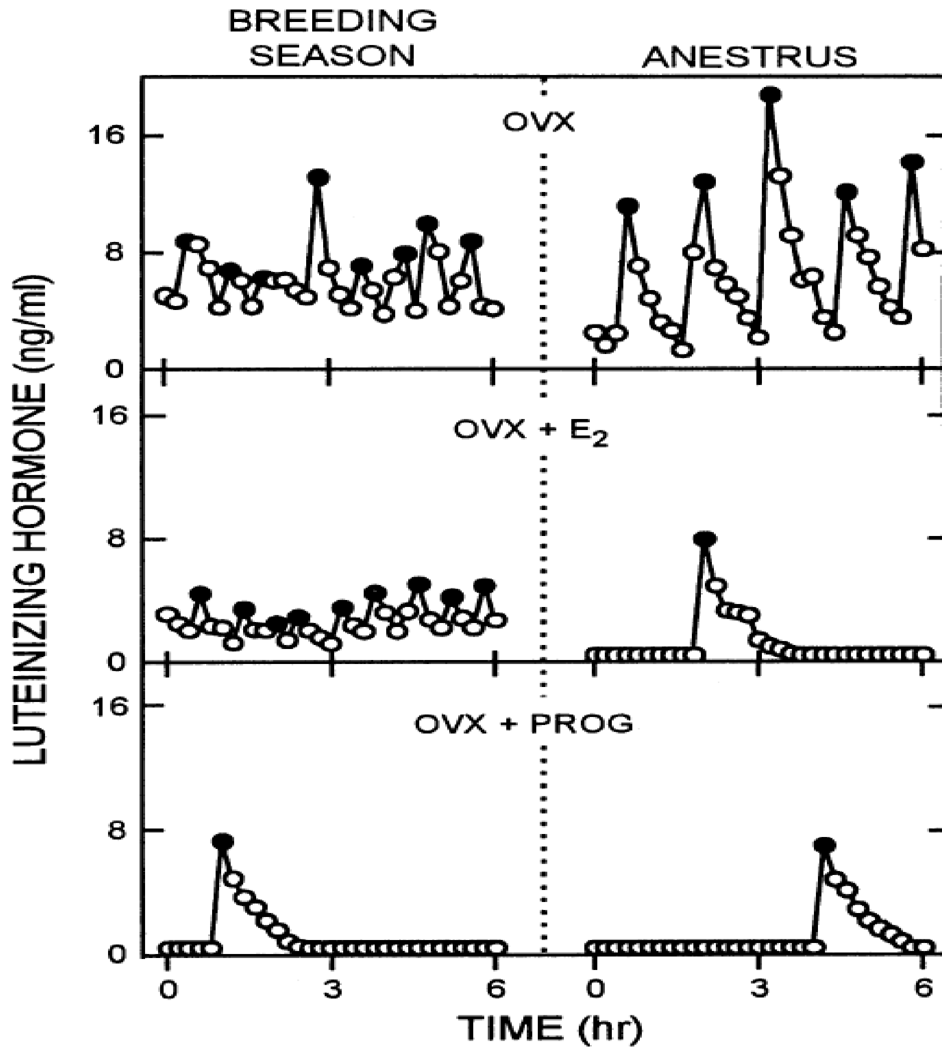

Figure 1: Effects of steroid negative feedback on LH secretion in the ewe. During the breeding season estradiol and progesterone act to inhibit GnRH/LH pulse amplitude and frequency, respectively. During anestrus, estradiol inhibits GnRH/LH pulse frequency (from (71)).

Figure 2

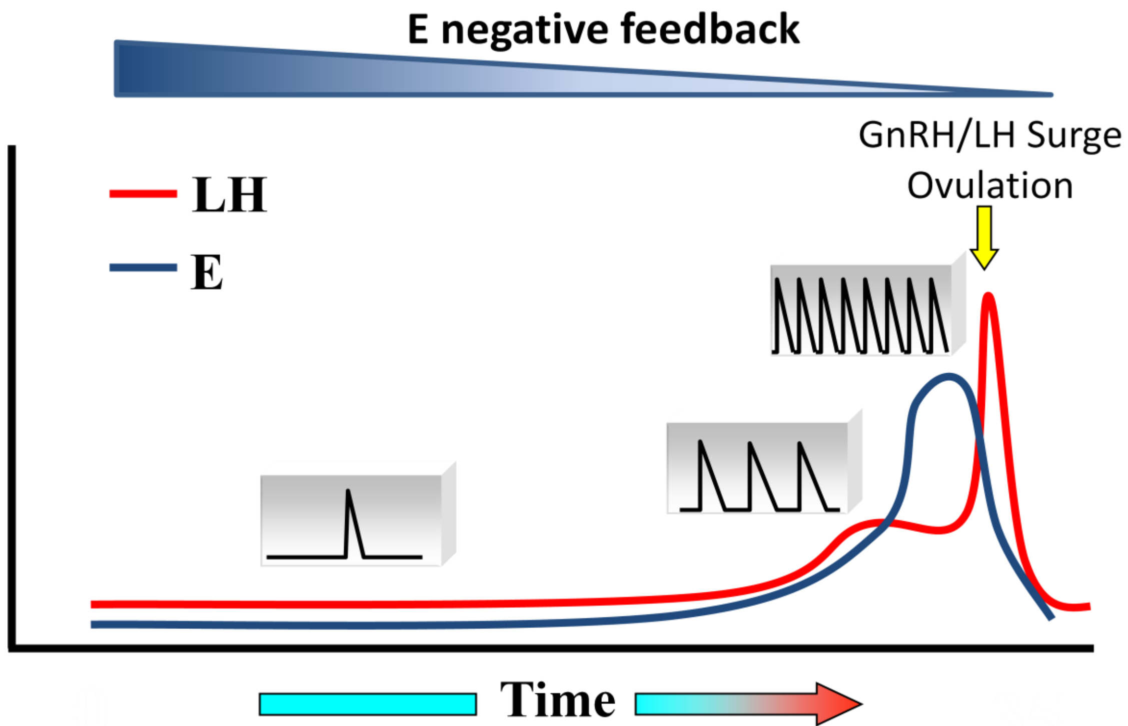

Figure 2: The gonadostat hypothesis for pubertal development in the ewe. In the prepubertal ewe, estradiol negative feedback potently inhibits GnRH/LH pulse frequency as seen in the leftmost inset with the single LH pulse. As the animal grows and matures, estradiol negative feedback lessens allowing for an increase in GnRH/LH pulse frequency (rightmost inset) which leads to the first ovulation.

The simplest explanation for a central inhibitory effect of estradiol or progesterone on LH secretion would be via a direct action on GnRH neurons. However, GnRH neurons are devoid of the appropriate steroid receptors, estrogen receptor-α [ERα; (72, 73)] and progesterone receptor [PR; (74)]; therefore, steroid feedback must occur through afferent neurons that have yet to be identified. Upcoming sections will consider the possible roles of three such afferent inputs, orphanin FQ (OFQ), neurokinin B (NKB) and kisspeptin, in the control of GnRH/LH secretion. Each neuropeptide will appear in the chronological order in which they were discovered, with consideration given primarily to female rodents, sheep and primates.

ENDOGENOUS OPIOID PEPTIDES (EOP)

Endogenous opioid peptides consist primarily of three major families: enkephalins, endorphins and dynorphins. The enkephalins and endorphins preferentially bind the delta (δ) and mu (μ) receptors, while the dynorphins preferentially bind kappa (κ) receptors (75). Shortly after their discovery, evidence began to accumulate indicating that EOP were involved in steroid negative feedback.

Through the use of opioid antagonists, it is now well established that EOP contribute to control of GnRH/LH secretion. An early study in normal women reported that an infusion of naloxone, a nonspecific opioid receptor antagonist, increased LH concentrations in the late follicular and luteal phases (76). Subsequent studies in humans revealed that an infusion of naloxone increased LH concentrations in steroid-replaced women, but not in women lacking the inhibitory effect of sex steroids (77, 78). Such studies led to the conclusion that in humans EOP mediate steroid negative feedback. In partial agreement with the human work, in other primates naloxone increased LH secretion over two fold in luteal phase monkeys, but was without effect in follicular phase monkeys (79). Thus, in the monkey, EOP appear to mediate progesterone, but not estradiol, negative feedback. In rats, LH increases in response to naloxone treatment 2 days post OVX, which is likely due to the negative feedback that remains until steroids are cleared following OVX since these effects were absent when tested 7 and 21 days post OVX (80). Furthermore, during early pregnancy in rats when estradiol and progesterone are elevated, naloxone

stimulates an increase in LH secretion (81), supporting the idea that EOP can mediate steroid negative feedback on GnRH/LH secretion.

In the ewe, results from experiments with naloxone vary somewhat with increases in LH seen in ewes with high serum progesterone (82), follicular and luteal phase ewes (83, 84) and OVX plus progesterone (OVX + P) ewes (85). With the use of a long acting opioid antagonist, WIN 44,443-1, Whisnant and Goodman (86) reported an increase in LH pulse amplitude in follicular phase and OVX + E_2 ewes as well as an increase in LH pulse frequency in luteal phase and OVX + P or OVX ewes with progesterone and estradiol (OVX + P + E_2). Yang et al. (87) showed similar results in the breeding and non-breeding season as OVX ewes exibited increased LH release following WIN 44,443-1 in the presence of progesterone alone. Since naloxone can increase GnRH pulse frequency and amplitude in luteal phase ewes (88), it was concluded that EOP could mediate progesterone and estradiol negative feedback in the ewe. However, naloxone increased GnRH pulse amplitude to the same extent in both OVX and OVX+E_2 ewes (89). Therefore, EOP do not appear to be involved in estradiol negative feedback in the ewe.

It is thus now generally accepted in the ewe, and several other species including women, that progesterone negative feedback occurs, at least in part, through EOP, however the specific EOP involved remains controversial. Knife cuts in the ewe disconnecting the POA from the MBH do not disrupt estrous cyclicity (90) nor does it affect EOP mediation of progesterone negative feedback (91). These results led researchers to investigate the role of EOP within the MBH, with specific attention to the arcuate nucleus (ARC). Greater than 90% of dynorphin neurons in the ARC of the ewe contain PR (92), while 33% and 21-25% of ARC β-endorphin neurons coexpress PR in guinea pigs (93) and monkeys (94), respectively. Furthermore, in multiple species dynorphin and β-endorphin neurons synapse on GnRH neurons (95-98). Moreover, progesterone can increase preprodynorphin expression and proopiomelanocortin (POMC) mRNA (the precursor of β-endorphin) in the ewe (99, 100), further supporting the idea that progesterone could regulate GnRH through EOP.

Weesner and Malven (101) demonstrated with the use of antiserum that β-endorphin inhibits LH secretion via actions in the POA and met-enkephalin acts in the MBH, while Conover et al., (102) showed, with infusion of β-endorphin, that this opioid exerts its inhibition at the median eminence. However, with the use of specific opioid antagonists for the μ-, δ- and κ-receptors, Goodman et al. (95) reported an increase in LH pulse frequency only in luteal phase ewes treated with the κ-receptor antagonist. A more recent report by the same group (103) concluded that dynorphin neurons in the ARC alone may not be sufficient to account for the inhibitory effect of progesterone on LH secretion. In that study, progesterone microimplants placed into the caudal ARC to target the area where dynorphin neurons reside failed to inhibit LH secretion in the ewe. However, suppression of LH secretion with systemic administration of progesterone by constant intravaginal drug release was reversed by microimplants of a progesterone receptor antagonist, RU486, in the caudal ARC. The simplest explanation for these data is that other neurons outside the caudal ARC must also be involved in mediating progesterone negative feedback and likely form a network with dynorphin neurons. One such neuropeptide that may serve such a role is orphanin FQ (OFQ).

Overshadowed, for good reason, by other neuropeptides (kisspeptin, NKB, etc.) that will be a later focus of this literature review, OFQ has received little attention in reproduction since it was discovered by two independent groups (104, 105) to bind an orphan G protein-coupled receptor, opioid receptor like 1 (ORL-1). OFQ has been shown to participate in various physiological processes including pain perception, learning and memory, as well as renal and cardiovascular function (106). Although OFQ shares structural similarities with dynorphin A, classic opioids do not activate ORL-1 nor does OFQ activate classic opioid receptors (107, 108).

In rodents, OFQ (109, 110) and ORL-1 (110-112) are found in many areas of the central nervous system including the POA and throughout the hypothalamus. Likewise in the ewe, neurons expressing OFQ are also found in the POA and hypothalamus (113). In this same study, greater than 90% of GnRH neurons in the POA contained OFQ with each neuropeptide in separate secretory vesicles within the same neuron. OFQ-containing terminals were also present in the external zone of the ME, but separate from

GnRH terminals. Therefore, it is possible that OFQ reaches the anterior pituitary, as does GnRH, via the hypophyseal portal veins and could have actions at the anterior pituitary.

Given the hypothalamic location of OFQ neurons and ORL-1, a number of groups have examined a role for OFQ in controlling GnRH and LH secretion. Work done with hypothalamic slice preparations in rodents has revealed that OFQ can inhibit GnRH release (114, 115) by increasing conductance of an inwardly-rectifying potassium channel (116). *In vivo* reports in the rat have also shown that intracerebroventricular (icv) administration of OFQ reduces GnRH concentrations in push-pull perfusates (115, 117), while administration of an OFQ receptor antagonist results in an increase in LH secretion (115). In the ewe, icv infusion of OFQ elicited a robust suppression in LH secretion (113). Although OFQ appears to affect LH through an inhibition of GnRH secretion, at least in rats, previous limitations in experimental design have made this less than a certainty. Administration via the ventricular system is a useful means of drug delivery to establish a central effect, but does not eliminate the possibility of an action at the pituitary because this gland is bathed in cerebrospinal fluid. In addition, it remains controversial as to whether or not ORL-1 is present in the anterior pituitary (112, 115, 118). Thus, without direct pituitary assessment one cannot exclude an action of OFQ at the level of the pituitary to inhibit LH secretion.

NEUROKININ B (NKB)

Although NKB was discovered shortly after the EOP, it only recently gained increased attention following the 2009 discovery that in humans mutations in the genes that encode for NKB or its receptor, neurokinin 3 receptor (NK3R), lead to hypogonadotropic hypogonadism (119). As a member of the tachykinin family, NKB is the only peptide synthesized from the preprotachykinin-B gene (120, 121) and preferentially binds NK3R (122).

In rodents, NKB mRNA (123-126) and protein (123-125, 127, 128) are seen in various areas of the hypothalamus with the majority of cell bodies residing in the ARC. In sheep, NKB mRNA (129, 130) and protein (129, 131, 132) are found almost exclusively within the ARC. In primates, NKB mRNA (133, 134) is predominantly in the infundibular nucleus, the primate homolog of the ARC, which is

consistent with reports in women for NKB mRNA (135) and protein (136). Furthermore, NKB cell numbers within the ARC of female sheep are higher than males, which clearly results from the organizational effects of testosterone during prenatal development (129, 137). NKB afferent neurons project to GnRH neurons in multiple species (138). NK3R is found in several areas of the hypothalamus in rodents (128, 139) and sheep (140) and in GnRH terminals in the ME in rats (193), but not in sheep (140).

In multiple species, ARC NKB mRNA levels are inhibited by estradiol (130, 133, 141, 142). Thus it was proposed that NKB may have a stimulatory action on GnRH/LH secretion. However, in rodents the first reported effect of senktide, an NK3R agonist, on LH secretion was inhibitory (134). With the renewed interest in the role of NKB, several new studies have reported stimulatory actions of this agonist. It now appears that the actions of senktide are largely dependent on steroid milieu. In OVX mice (126), OVX rats (142, 143) and OVX rats treated with low levels of estradiol (134) senktide inhibits LH secretion. In contrast, senktide stimulates LH secretion in diestrous rats (142, 143). Work done in non-rodent species has reported a stimulatory role for NKB on GnRH/LH secretion. In sheep, NKB and senktide can stimulate LH secretion in follicular phase and ovary-intact anestrous ewes (144). Therefore, inhibition of NKB may contribute to steroid negative feedback on GnRH/LH secretion.

KISSPEPTIN

Kisspeptin, which was discovered in 1996 (145), has created quite a stir within the field of reproductive biology. Kisspeptin was named in honor of the popular treat made in Hershey, Pennsylvania, the “Hershey’s Kiss”. The KiSS-1 gene encodes for a 145 amino acid peptide that is cleaved to bioactive lengths of 10, 11, 13 and 54 amino acid lengths (146), collectively known as kisspeptins. Four separate groups discovered the receptor for this family to be the orphan G-coupled protein receptor 54 (GPR54; (146-149), which is now also referred to as Kiss1r. In 2003, when two independent groups (150, 151) reported that mutations in the human GPR54 lead to hypogonadotropic hypogonadism, kisspeptin became, to say the least, a popular topic in neuroendocrinology.

Several groups have shown that kisspeptin can potently stimulate LH secretion in such species as rodents (152, 153), sheep (154), monkeys (155) and humans (156, 157). That this stimulation of LH secretion is due to a direct stimulation of GnRH release is suggested by several observations. One, colocalization of GPR54 mRNA with GnRH neurons is greater than 90% in female mice (158) and 78-90% in the ewe (159, 160). Second, GPR54 knockout mice show no increase in LH in response to exogenous kisspeptin, but are still responsive to exogenous GnRH (151, 161). Third, in mice kisspeptin can depolarize GnRH cell bodies *in vitro* (158) and stimulate GnRH release from mouse (162) and sheep (160) median eminence explants *in vitro*. Fourth, administration of a GnRH receptor antagonist, acyline, blocked kisspeptin effects on LH secretion (163). Lastly, following subcutaneous administration of kisspeptin, 88% of GnRH neurons in and around the organum vasculosum of the lamina terminalis (OVLT) contained c-fos, an immediate early gene product commonly used as a marker of neural activation (164). Taken together, this evidence indicates that kisspeptin stimulates GnRH neurons directly and is important for normal GnRH/LH secretion.

In all mammalian species to date, kisspeptin neurons exist in two distinct areas of the midbrain, the anteroventral periventricular nucleus (AVPV)/POA and ARC. In female rodents, kisspeptin neurons, identified by mRNA abundance (152, 165, 166) and protein content (166, 167), are localized with greater number in the ARC vs the AVPV. Although there is no distinct AVPV in the ewe, kisspeptin immunoreactive cells are present in the medial POA (168, 169) and ARC (168-170). In women (171), kisspeptin mRNA abundance was observed in a dense population of cells within the ARC with some scattered distribution in the medial POA, which is consistent with kisspeptin protein distribution in women (136). In female monkeys, kisspeptin mRNA appears to be restricted to the ARC alone, with no detectable change in kisspeptin mRNA abundance outside the MBH (163). As with rodents, numbers of kisspeptin cells in these species are greater in the ARC than the AVPV.

The effects of steroids on kisspeptin in these two areas, ARC and AVPV/POA, has led to the hypothesis of differing roles for each kisspeptin population in control of GnRH/LH secretion. In the ARC, ovariectomy of adults results in an increase in kisspeptin mRNA levels in rodents (165), sheep

(169) and monkeys (171), which is consistent with data in women where kisspeptin mRNA levels are greater following menopause (171). Estradiol replacement in rodents, sheep and monkeys readily reduced ARC kisspeptin mRNA levels to those of intact animals. Although progesterone alone does inhibit kisspeptin mRNA levels in the ARC, it is not sufficient to suppress kisspeptin levels to that of an intact, luteal phase ewe (169). Since greater than 90% of kisspeptin neurons within the ARC of sheep contain ERα (129), it is possible that estradiol controls tonic GnRH/LH secretion through kisspeptin in the ARC of multiple species.

Although, the negative feedback of estradiol generally appears to involve the ARC, the region wherein it acts to elicit the GnRH surge appears to differ between species. In rodents the positive feedback action of estradiol appears to reside in the AVPV since microimplants of estradiol in the AVPV of rats can elicit a preovulatory GnRH/LH surge (172). Multiple reports support the idea for a role of AVPV kisspeptin in estradiol positive feedback in rodents. First, kisspeptin mRNA levels in the AVPV peak on the afternoon of proestrus in rats (173) at a time when the GnRH/LH surge occurs in rodents. Second, kisspeptin mRNA levels increase in the AVPV following estradiol replacement in OVX rodents (165, 174). Third, colocalization of c-fos and kisspeptin in the AVPV is greatest at the time of the GnRH/LH surge (173), while kisspeptin receptor knockout mice display no increase in neuronal activity within this neural population (175). Finally, kisspeptin antiserum in the POA of rodents can block the LH surge (166, 176). Since nearly all kisspeptin cells in the AVPV of rodents contain ERα (165), estradiol may act directly on kisspeptin neurons in the AVPV to promote GnRH surge induction in rodents.

In contrast to rodents, estradiol positive feedback in sheep (177) and primates (178) occurs in the MBH since microimplants of estradiol in the ventromedial hypothalamus (VMH) and ARC can elicit a GnRH/LH surge and microimplants in the POA have no effect on LH secretion. Together with the before mentioned high degree of colocalization of ERα and kisspeptin in the ARC and the site of estradiol positive feedback in these species, it is highly probable that kisspeptin neurons within the ARC mediates the effects of estradiol on tonic and surge GnRH/LH secretion in sheep and primates. Interestingly, kisspeptin neurons of the ARC form a unique population because almost all kisspeptin cells express NKB

and dynorphin, a finding that was first reported in sheep (132). Since that initial observation, a similar population of arcuate kisspeptin/NKB/dynorphin neurons has been described in female mice (126), female rats (139), goats (179) and women (136). Whether the same kisspeptin neurons participate in tonic and surge GnRH/LH secretion or whether there are specific subpopulations for each remains to be determined.

The role of POA kisspeptin neurons in non-rodent species remains somewhat controversial. Increases in kisspeptin mRNA in both the POA and ARC during the late follicular phase of sheep have been reported (159), but others report changes only in ARC kisspeptin expression (170). Moreover, earlier studies reported that steroid replacement in OVX ewes (169) and primates (171) did not alter POA kisspeptin mRNA levels, but more recent work in ewes found that estradiol increases kisspeptin in the POA (180). Furthermore, elevated colocalization of c-fos and kisspeptin in the POA during the preovulatory surge in the ewe (181) fits with the idea that POA kisspeptin contributes to estradiol positive feedback. Nevertheless, the role of POA kisspeptin cells in sheep and primates remains to be determined.

In addition to steroid effects, sexual dimorphism adds another level of complexity to kisspeptin expression. Within the AVPV of rodents, females have many more kisspeptin cells than their male counterparts (167, 173), a difference that is still present in gonadectomized animals (166, 182). These results led to the idea that sex steroids during development cause organization of this neuronal population. Within the ARC of rodents, multiple reports show no difference in kisspeptin expression between males and females (167, 182-186), with one recent exception in the rat ARC where kisspeptin cells numbers are greater in females than males (187). Sheep (137) and humans (136) display a sexual difference in both the POA and ARC kisspeptin populations with females having higher numbers of neurons than males. Whether the sexual dimorphism in kisspeptin content is due to organizational or activational effects of gonadal steroids remains to be determined in these species.

POSSIBLE ROLES FOR KISSPEPTIN AND NKB IN PUBERTY ONSET

A few lines of evidence make kisspeptin a prime candidate for involvement in the pubertal increase in GnRH/LH secretion. First, as mentioned before, mutations in GPR54 of humans and mice

blocks normal pubertal development and ARC kisspeptin expression in multiple species is controlled by steroid negative feedback. Second, kisspeptin can stimulate LH secretion in prepubertal rats (153), sheep (188) and primates (155, 189), demonstrating that the kisspeptin/GnRH network is intact prior to puberty. Third, kisspeptin expression is greater in the AVPV with no consistent changes in ARC kisspeptin in postpubertal compared to prepubertal female mice (158, 190), which leads to the idea that in mice AVPV kisspeptin neurons are responsible for the pubertal increase in GnRH/LH secretion. However, recent evidence shows that ablation of kisspeptin neurons in the ARC prior to puberty at a time when AVPV kisspeptin neurons have yet to be detected can disrupt estrous cyclicity in mice (191). In the rat, kisspeptin expression in both the AVPV and ARC is greater in postpubertal compared to prepubertal females (192, 193). Similarly, in the monkey, kisspeptin mRNA in the MBH is greater in pubertal females compared to juvenile females (163). Together the results of these studies are strongly suggestive that kisspeptin is a gatekeeper to the pubertal increase in GnRH/LH secretion.

NKB may have a role in puberty as well. Although data for changes in NKB in relation to puberty are limited, in the mouse NKB mRNA abundance is under steroid negative feedback both prepubertally and postpubertally, but does not change across puberty (183). Furthermore, the response to NKB appears to be intact prior to puberty in monkeys since NKB and senktide stimulate LH secretion in prepubertal male monkeys (194). However, it remains to be determined if NKB can stimulate LH secretion in the prepubertal female. Although a direct action of NKB on GnRH might exist given that GnRH terminals in the ME contain NK3R in rats (195), several groups have investigated the possibility that NKB may act through kisspeptin to stimulate GnRH secretion. In rodents and sheep, a majority of ARC kisspeptin neurons contain NK3R (126, 139, 140), a finding consistent with the depolarizing effect of NKB on kisspeptin neurons in whole-cell patch recordings mice (142). Also, an icv administration of senktide produces an increase in c-fos containing kisspeptin cells in the ARC of rats with no change in the AVPV (142). Furthermore, mice lacking GPR54 do not exhibit an increase in LH secretion after icv injection of senktide (196). These data support the notion that NKB acts via kisspeptin to stimulate GnRH/LH secretion.

SPECIFIC AIMS

Given the effects of steroids on GnRH/LH secretion and that GnRH neurons are devoid of ERα and PR, it seems clear that other, intermediate neurons must serve to link the two. We propose three specific aims to test the roles for kisspeptin, NKB and OFQ in mediating steroid negative feedback on GnRH/LH secretion in the ewe.

Specific Aim 1: To determine if kisspeptin and/or NKB are involved in puberty onset in the ewe.

We hypothesize that the kisspeptin network is intact, but merely inhibited by heightened estradiol negative feedback prior to puberty. To test this hypothesis we examined numbers of kisspeptin positive cells in the ARC of prepubertal and postpubertal ewes in the presence and absence of estradiol and used age-matched males as a control for any effect of age that might be independent of reproductive function. Furthermore, we analyzed kisspeptin close contacts onto GnRH neurons in pre- and postpubertal ewes in the presence and absence of estradiol.

Similar to kisspeptin, we hypothesize that NKB expression is inhibited by estradiol in the prepubertal ewe, but not in the adult. We tested this by examining numbers of NKB positive cells in the ARC of pre- and postpubertal ewes in the presence and absence of estradiol. Also, we investigated if NKB can stimulate LH secretion prior to puberty by administering senktide, an NKB receptor agonist, to prepubertal ewes.

Specific Aim 2: To determine the site where OFQ acts to inhibit LH secretion in the ewe.

We hypothesize that OFQ inhibits GnRH release from the hypothalamus. To test this we administered an OFQ receptor agonist into the third ventricle of OVX ewes while simultaneously collecting hypophyseal portal blood and jugular blood for the measurement of GnRH and LH, respectively. Also, to test a direct effect at the anterior pituitary, we challenged anterior pituitary cells collected from ewes in various steroid milieus with increasing doses of GnRH in the presence or absence of OFQ.

Specific Aim 3: To determine a role for endogenous OFQ in steroid negative feedback in the ewe.

We hypothesize that OFQ mediates steroid negative feedback in the ewe. To test this hypothesis, we evaluated the effect of a centrally infused OFQ receptor antagonist, UFP-101, on LH secretion in OVX +

E_2 or OVX + P + E_2 ewes in the breeding season and ovary-intact ewes in the non-breeding season. Furthermore, in collaboration with our colleagues at The University of Michigan, we assessed colocalization of OFQ and ERα or PR in the POA and hypothalamus of luteal phase ewes.

As mentioned above, I am using the sheep to address my specific aims as it has several advantages for the performance of this work. First, the sheep is the only model to date that can be used to measure GnRH from hypophyseal portal blood in an unanesthetized animal. This technique allows for simultaneous collection of portal and jugular blood to determine if effects are central via alterations in GnRH secretion or at the level of the pituitary and will be used in Chapter 3 to evaluate the third ventricle infusion of an OFQ agonist. Second, with a relatively large blood volume compared to rodents, sheep can be bled multiple times per hour for several hours to analyze GnRH and/or LH pulse amplitude and pulse frequency. We will analyze LH pulse dynamics in Chapters 2-4 and GnRH pulse dynamics in Chapter 3. Third, if neural tissue is not needed immediately following treatment, each animal can be used for multiple treatments (with some limitation) allowing each ewe to serve as its own control, which will be used in Chapter 4 with the infusion of an OFQ receptor antagonist. Finally, sheep have a larger hypothalamus and pituitary relative to rodents. The former allows for more detailed anatomical analysis, which we use to our advantage in Chapters 2 and 4 to examine multiple POA and hypothalamic sections for immunocytochemical labeling of various neuropeptides, and the latter produces a greater number of anterior pituitary cells for cell dispersion in the *in vitro* examination of OFQ effects on LH release as seen in Chapter 3.

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CHAPTER 2

EVIDENCE OF A ROLE FOR KISSPEPTIN AND NEUROKININ B IN PUBERTY OF FEMALE SHEEP

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ABSTRACT

Puberty onset in female sheep is marked by a decrease in estradiol-negative feedback, allowing for the increase in GnRH and LH pulses that heralds the first ovulation. Based on recent genetic studies in humans, two possible neuropeptides that could promote puberty onset are kisspeptin and neurokinin B (NKB). Our first experiment determined whether the NKB agonist, senktide, could stimulate LH secretion in prepubertal ewes. A second study used prepubertal and postpubertal ewes that were intact or ovariectomized (OVX) to test the hypothesis that expression of kisspeptin and NKB in the arcuate nucleus increased postpubertally. For comparison, kisspeptin and NKB expression in age-matched intact, and castrated males were also examined. In experiment 1, the percentage of ewes showing an LH pulse immediately after injection of senktide (100 µg, 60%; 500 µg, 100%) was greater than that for water-injected controls (experiment 1a, 25%; experiment 1b, 20%). In experiment 2, kisspeptin-positive cell numbers in the arcuate nucleus increased after puberty in intact females and were increased by OVX in prepubertal but not postpubertal ewes. Changes in kisspeptin cell numbers were paralleled by changes in kisspeptin-close contacts onto GnRH neurons in the medial preoptic area. NKB cell numbers did not differ significantly between intact prepubertal and postpubertal ewes but increased with OVX in both age groups. NKB fiber immunoreactivity was greater in postpubertal than in prepubertal intact ewes. In age-matched males, kisspeptin and NKB cell numbers increased with castration, but decreased with age. These results support the hypothesis that kisspeptin is a gatekeeper to female ovine puberty and raise the possibility that NKB may also play a role, albeit through different means.

INTRODUCTION

Puberty is defined as the time when an individual gains the ability to reproduce. In sheep, achievement of puberty is caused by a decrease in response to estradiol-negative feedback, resulting in an increased frequency of GnRH, and subsequently LH, pulses (1). In ewes, this increase in LH pulse frequency leads to increased estradiol production that in turn induces the subsequent GnRH/LH surge and first ovulation (2). An escape from estradiol-negative feedback and the resultant increase in LH secretion occurs around 25–32 wk of age in ewes (2– 4), whereas in rams, this occurs earlier at 10–15wk of age (5). Because GnRH neurons are devoid of estrogen receptor-α (6, 7), this neuroendocrine change in sensitivity to estradiol most likely occurs through interneurons that have yet to be identified. Kisspeptin and neurokinin B (NKB) are two likely neuropeptide candidates that could mediate such interneuronal communication.

Kisspeptin has received considerable attention due to reports that mutations in the kisspeptin receptor, G-protein coupled receptor 54, block pubertal development, and lead to hypogonadotropic hypogonadism in humans (8, 9) and mice (9). In subsequent studies across several species, it has been demonstrated that kisspeptin potently stimulates the GnRH/LH axis both before (10–13) and after puberty (14). Moreover, kisspeptin may drive the awakening of reproductive function at puberty because an increase in hypothalamic kisspeptin mRNA abundance is observed after puberty onset in rats (10) and monkeys (15). It has also been shown that kisspeptin mRNA in the hypothalamus increases after ovariectomy (OVX) and is inhibited by steroid replacement (10, 16–18). These data are consistent with the possibility that the kisspeptin/GnRH network is intact before puberty but merely inhibited by heightened sensitivity to estradiol negative feedback. One recent study has challenged the hypothesis that kisspeptin is essential for puberty and normal reproduction, because congenital ablation of kisspeptin neurons did not alter puberty or prevent ovulatory cycles in mice (19). However, they also reported that ablation of these neurons at 20 d of age in prepubertal mice completely abolished ovulatory cycles, suggesting that kisspeptin neurons are essential for reproduction in mice that have developed normally.

More recently, NKB has also been implicated in puberty, because mutations in the gene that encodes for NKB or its receptor, neurokinin 3 receptor (NK3R), block pubertal development in humans

(20). Interestingly, within the arcuate nucleus (ARC) of the hypothalamus, NKB is found in the same neurons as kisspeptin in mice (21), sheep (22), goats (23), monkeys (24), and humans (25). Much like kisspeptin, ARC NKB mRNA abundance is inhibited by estradiol in adult female mice (26), rats (27), sheep (28), and monkeys (29). However, little information is available on changes in NKB expression across puberty. Although NKB action on the GnRH/LH axis in adult females varies depending on endocrine status (26, 30), recent reports in intact adult female rats (26) and sheep (31) show that an NKB receptor agonist, senktide, stimulates LH release. Furthermore, although NKB and senktide increase GnRH/LH secretion before puberty in the male monkey (24), the question still remains as to whether NKB can stimulate LH secretion in prepubertal females. Thus, we hypothesized that kisspeptin and NKB play critical roles in puberty onset in female sheep. This hypothesis leads to three testable predictions. First, we predict that the NKB network is intact before puberty so that senktide will stimulate LH release in prepubertal ewes. Second, because both appear to have stimulatory effects on GnRH/LH, we predict that kisspeptin and NKB expression will be greater in postpubertal than in prepubertal ovary-intact ewes. Third, because puberty is driven by a decreased response to estradiol-negative feedback, we predict that OVX will produce a larger increase in kisspeptin and NKB expression in pre- than postpubertal ewes. Because male sheep show sperm production (3) and a decreased sensitivity to estradiol-negative feedback (5) at 10–15 wk of age, we used age-matched males that were presumed to be postpubertal as a control for any effect of age that was independent of reproductive function.

MATERIALS AND METHODS

Animals

For experiments 1a and 1b, prepubertal ewes (5–6 months old) of mixed breeding were housed and studied in an open barn. They received a daily diet of hay and water *ad libitum*. For experiment 2, a different group of mixed-breed prepubertal female sheep (5–6 months old), postpubertal female sheep (>9 months old), and age-matched males was used during the breeding season (October to February). As mentioned above, all males were presumed to be postpubertal. Sheep were housed in an open barn until 3–14 d before the study, when they were moved indoors. While indoors, they received an alfalfa pellet

food ration and had open access to water and mineral supplement. Indoor lighting simulated the natural changes in day length. OVX were performed by midlateral laparotomy under gas anesthesia (oxygen + nitric oxide + 3% halothane) 2 wk before tissue collection. Blood samples were collected via jugular venipuncture into heparinized tubes, and plasma was stored at -20 C. All procedures were approved by the West Virginia University Animal Care and Use Committee and followed National Institutes of Health guidelines for use of animals in research.

Experimental design

Experiment 1a

Fourteen ovary-intact prepubertal ewes were weighed and placed into one of three treatment groups: sterile water (n=4), senktide (n=5; Tocris Bioscience, Ellisville, MO), or senktide+acyline (n=5) (National Institute of Child Health and Human Development, Rockville, MD). Acyline (60 µg/kg), a GnRH receptor antagonist, was administered im immediately before blood collection. Blood samples were collected at 12-min intervals from ewes for 6 h with senktide (100 µg) or water (2 ml) administered iv at 3 h of sampling. Mean body weight of ewes injected with water (36.6±5.2 kg), senktide (35.3±3.4 kg), or senktide + acyline (36.6 ±3.6 kg) did not differ among treatment groups.

Experiment 1b

Based on the results from experiment 1a, a second experiment was performed to examine a higher dose of senktide (500 µg). Ten ovary-intact prepubertal ewes from experiment 1a were randomly selected and received either water (n=5) or senktide (n=5). Blood samples were collected every 12 min for 6 h with injection of water (3 ml) or senktide (500 µg) administered iv at 3 h of sampling.

Experiment 2

Four groups (five per group) of females (prepubertal intact, prepubertal OVX, postpubertal intact-early follicular, and postpubertal OVX were used for experiment 2. Early follicular (EF) phase ewes were used, because at this time in the ovarian cycle, the dominant steroid is estradiol and as such are similar in steroid environment to prepubertal intact ewes. The ovarian cycles of EF ewes were synchronized by two

im injections of prostaglandin-F2α (6 mg/injection; Lutalyse; Henry Schein, Melville,NY) given 3 h apart and followed 7 d later by another two im injections given 3 h apart (32). Killing of ewes in this group occurred 24 h after the fourth injection of prostaglandin-F2α. Early follicular endocrine status was confirmed by absence of corpora lutea and low plasma progesterone (0.32 ± 0.1 ng/ml) on the day of killing. Four groups of age-matched males were either intact (rams) or castrated (wether) (young ram, young wether, older ram, and older wether). Blood samples (3 ml) were taken every 12 min for 4 h from all sheep immediately before killing. Tissue was collected as described previously (33). Briefly, all sheep were heparinized (20,000 U) and killed using an iv overdose of sodium pentobarbital (Euthasol; Webster Veterinary, Devens, MA). Heads were removed and perfused with four liters of 4% paraformaldehyde in 0.1M phosphate buffer (PB)(pH=7.4) containing 0.1% sodium nitrite via the carotid arteries. Blocks of tissue containing the preoptic area (POA) and the hypothalamus were then removed and stored in4%paraformaldehyde for 24 h at 4 C and transferred to 20% sucrose until sectioned. Frozen coronal sections (50 µm) were cut with a freezing microtome and stored in cryopreservative until the time of immunocytochemical staining.

Immunocytochemistry for kisspeptin or NKB

On d 1, sections were washed 4x5 min in 0.1 M PBS and stored overnight at 4 C. On d 2, sections were washed 4x5 min in PBS then placed in 10% H_2O_2 for 10 min followed by 4x5 min washes in PBS. Tissue was then incubated for 1 h with 0.4% Triton X (Sigma-Aldrich, St. Louis, MO) in 20% normal goat serum for kisspeptin or 4% normal goat serum for NKB, both made in PBS. Kisspeptin and NKB neurons were identified using primary antibodies for kisspeptin (gift from A. Caraty) and NKB (Peninsula Laboratories, San Carlos, CA) that have been validated for use in sheep (22, 33). Five to six sections of the middle to caudal ARC from each animal were incubated with 1:50,000 kisspeptin antiserum raised in rabbit or 1:100,000 NKB antiserum raised in rabbit for 18 h at room temperature; one to two sections from the POA were also analyzed immunocytochemically for kisspeptin (1:50,000). On d 3, biotinylated goat antirabbit antibody (Vector Laboratories, Burlingame, CA) at 1:500 and Vectastain ABC-elite (Vector Laboratories) at 1:500 were applied sequentially for 1 h each with 4x5 min washes of PBS

between incubations. Sections were then placed in a 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution (10mg of DAB; Sigma-Aldrich) in 50 ml of PB with 20 µl of 30% H_2O_2 added just before incubation for 10 min. After 3x5 min washes in PB, sections were mounted on Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA), dehydrated using a series of increasing alcohol baths, and coverslipped using DPX Mounting Medium (Electron Microscopy Sciences, Hatfield, PA).

Dual immunocytochemistry for kisspeptin and GnRH

One section from the medial POA, which included the organum vasculosum of the lamina terminalis for each female ewe used in experiment 2 was chosen to analyze kisspeptin-positive close contacts on GnRH neurons. Kisspeptin staining was the same as described above through the incubation with ABC solution, except that the kisspeptin antibody was used at a 1:75,000 dilution. After the 1-h incubation with ABC solution, tissue was incubated for 30 min with Alexa Fluor 555 streptavidin (Invitrogen, Carlsbad, CA) at 1:300 in PBS followed by 4x5 min in PBS; sections and reagents with fluorescent substrates were covered to prevent light exposure from here on. Sections were incubated in 0.4% Triton X in PBS containing 4% normal goat serum for 1 h followed by overnight incubation in PBS with 4% normal goat serum containing mouse anti-GnRH antibody (1:3000, lot no. 3; Sternberger Monoclonal, Inc., Lutherville, MD) at room temperature. On d 4, sections were washed 4x5 min in PBS and then incubated in Alexa Fluor 488 conjugated goat antimouse antibody (Invitrogen) at 1:200 in PBS for 30 min. Tissue was washed 4x5 min in PB, then coverslipped using gelvatol and stored in the dark at 4 C.

Data analysis

Immunocytochemistry

For single antigen staining, cell bodies, identified by cells containing a brown cytoplasmic staining, were counted manually using an Olympus AZ70 transmitted light microscope (Center Valley, PA) from four (NKB) or five (kisspeptin) sections of middle to caudal ARC, and mean cell numbers/section for each group were averaged, because there was no difference in cell numbers between the middle and caudal

ARC. NKB fiber immunoreactivity was assessed for one representative caudal ARC section from each intact and OVX ewe by three individuals blinded to treatment group. Each individual ranked fiber staining from zero to four, with zero being minimal fiber immunoreactivity and four being the most dense. For kisspeptin/GnRH staining, kisspeptin-positive close contacts (red) on GnRH neurons (green; 9–11 neurons per animal) were acquired from a three-dimensional (3D) reconstruction using a Zeiss LSM 510 laser scanning confocal (Hornwood, NY) on a Zeiss Axio Image Z1 upright microscope with a Plan Apochromat 63x/1.4 oil objective. 3D images presented here were taken at 1-_m increments through each GnRH cell, the image stack was deconvoluted using Auto Quant X2.2 (Media Cybernetics, Bethesda, MD), and 3D rendering was performed using NIS Elements AR 3.2 (Melville, NY).

Assays

LH concentrations were measured in duplicate with a radioimmunoassay (RIA) using 100–200 µl of plasma and reagents provided by the National Hormone and Peptide Program (Torrance, CA) as previously described (34). LH assay sensitivity averaged 0.07 ng/ml (NIH S24) with intra- and interassay coefficients of variation being 12.7 and 18.2%, respectively. Progesterone concentrations were measured in duplicate 150 µl aliquots of two plasma samples from each EF ewe using a RIA that has been validated for use in sheep (35); assay sensitivity was 0.01 ng/ml.

Statistics

Pulses were identified using previously described criteria (36). For experiment 1, the comparison of percentages of animals that displayed an LH pulse within 24 min (two samples) after iv administration of water or senktide was analyzed using the χ^2 test. Also for experiment 1, mean LH for two samples immediately before iv injection was averaged and compared with the mean of two samples taken immediately after iv injection using a paired *t*-test. For experiment 2, mean LH, LH pulse amplitude, mean number of cell bodies between groups within sex, and number of kisspeptin-positive close contacts per GnRH neuron were analyzed using two-way ANOVA. The percentage of GnRH neurons contacted by kisspeptin was transformed using natural log and then analyzed using a two-way ANOVA. The three rankings of NKB fiber immunoreactivity were averaged, and differences between prepubertal

and postpubertal intact ewes or prepubertal and postpubertal OVX ewes were analyzed using the nonparametric permutation test for two independent samples. LH pulse frequency was analyzed using the Mann-Whitney *U* test. Differences were considered to be significant at $P < 0.05$.

RESULTS

Experiment 1a. Can senktide, an NK3R agonist, stimulate LH secretion in prepubertal lambs?

Representative LH pulse profiles from prepubertal ewes receiving an iv injection of water (open circles) or 100 µg of senktide (closed circles) are illustrated in Fig. 1A. Senktide had no significant effect on pulse frequency (preinjection, 1.4±0.3 pulses/3 h *vs.* postinjection, 1.8±0.4 pulses/3 h). Because it was possible that the effects of the senktide were immediate but transitory, we analyzed the percentage of animals that responded with an LH pulse within 24 min of injection and found a significant difference between senktide and water-injected animals. Senktide stimulated an LH pulse in three of five (60%) prepubertal ewes within 24 min, whereas only one of four (25%) prepubertal ewes receiving water displayed an LH pulse. Mean LH 24 min after injection of water (5.14 ± 2.58 ng/ml) did not significantly differ from mean values 24 min before injection (2.63±1.45 ng/ml) but there was a strong tendency ($P = 0.052$) (Fig. 1B) for mean LH to be greater 24 min after 100 µg of senktide (6.51 ± 1.93 ng/ml) than 24 min before senktide (1.18 ± 0.21 ng/ml). None of the five prepubertal ewes that received the GnRH receptor antagonist, acyline, just before blood collection showed an LH pulse in response to 100 µg of senktide (Appendix: Supplemental Fig. 1).

Experiment 1b

Given the partial response in experiment 1a, we next tested the response to a higher dose (500 µg) of senktide. Representative LH pulse profiles from prepubertal ewes receiving an iv injection of either water (open circles) or 500 µg of senktide (closed circles) is illustrated in Fig. 1C. This dose of senktide stimulated an LH pulse in all five (100%) prepubertal ewes, whereas only one of five (20%) prepubertal ewes receiving an injection of water displayed an LH pulse within 24 min of injection, a difference in response that was significant. Furthermore, mean LH for 24 min after injection of 500 µg of senktide (6.22 ±0.68 ng/ml) was significantly greater than mean values for 24 min before injection (3.05±1.08

ng/ml), whereas vehicle had no significant effect (preinjection, 3.56±0.44 ng/ml; postinjection, 4.36 ± 1.27 ng/ml) on mean LH concentrations (Fig. 1D).

Experiment 2. Does expression of kisspeptin or NKB protein increase with puberty and/or gonadectomy (GNDX)?

Mean LH (Fig. 2A) and LH pulse amplitude (Fig. 2C) were significantly increased after GNDX in both ewes and rams at all ages, but there was no effect of age and no age by GNDX interaction. LH pulse frequency (Fig. 2B) was also significantly increased after GNDX in prepubertal females and in both young and older males, but not in postpubertal females. Analysis of kisspeptin cell numbers in the ARC indicated that the number of kisspeptin-positive cells was higher after puberty (358 ± 77 per ewe) than before puberty (204 ± 81 per ewe) in ovary-intact females (Fig. 3, A, B, and E). Furthermore, kisspeptin-positive cell numbers significantly increased after OVX (Fig. 3E) in prepubertal ewes (447 ± 88 per ewe), but not in postpu- bertal ewes (473 ± 151 per ewe). In males, there was a significant effect of GNDX, because kisspeptin cell numbers were higher in castrated males compared with intact rams (Fig. 3E and Appendix: Supplemental Fig. 2). There also was a significant effect of age, indicating that cell numbers were lower in both intact and castrated older males compared with younger males (Fig. 3E) but no interaction of age and GNDX. Kisspeptin cell numbers strongly tended ($P = 0.068$) to be greater in postpubertal OVX ewes (473±96 per ewe) than age-matched GNDX males (138 ± 105 per wether) but were not different between prepubertal OVX ewes (447 ± 89 per ewe) and age-matched GNDX males (486 ± 96 per wether).

To determine whether puberty-related changes in kisspeptin cell numbers altered kisspeptin synaptic input onto GnRH neurons, we examined kisspeptin-positive close contacts on GnRH neurons in the medial POA in females (Fig. 4). The change in the percentage of GnRH neurons displaying kisspeptin-positive close contacts (Fig. 4C) paralleled the changes that we observed in kisspeptin cell numbers. Specifically, a significantly higher percentage of GnRH neurons received close contacts after puberty in ovary-intact females (prepubertal, 34.9 ± 6.5%; postpubertal, 71.7 ± 1.8%). This percentage significantly increased after OVX in prepubertal ewes (64.9±6.6%), but not in postpubertal ewes (61.8 ±

2.7%). However, the number of kisspeptin-positive close contacts per GnRH neuron (Fig. 4D) did not differ significantly among groups. POA sections containing GnRH neurons were also examined at lower magnification to quantify kisspeptin-containing cell bodies in this region with immunofluorescence. In contrast to previous analysis of tissue from adults (22), only one kisspeptin-positive cell body was observed in all sections from these young females. Therefore, we examined kisspeptin in a wider range of POA sections from these same females using single-label immunocytochemistry. Even using DAB as a chromogen, kisspeptin immunoreactivity in the POA was light and inconsistent across ewes (Appendix: Supplemental Fig. 3), making accurate identification and counting of cells impossible. Therefore, we did not quantify kisspeptin cells in the POA for this study. NKB cell numbers were examined only in the ARC, because NKB-containing neurons are largely limited to this nucleus in the ovine hypothalamus (37). The number of NKB cells (Fig. 5) significantly increased after OVX in both prepubertal and postpubertal females (from 188 ± 75 to 347 ± 37 per ewe and from 158± 46 to 357 ± 56 per ewe, respectively) (Fig. 5E). However, in contrast to kisspeptin, there was no change in the number of NKB containing cells between prepubertal (188 ± 75 per ewe) and postpubertal (158 ±46 per ewe) ovary-intact ewes (Fig. 5E). While counting cell numbers, we noted obvious variation among animals in NKB fiber density. After further assessment, we found that NKB fiber immunoreactivitywas significantly greater in EF ewes (rank of 2.7 ± 0.8) compared with prepubertal intact ewes (rank of 1.2± 0.4) with no significant difference in fiber density between prepubertal (rank of 1.3 ± 0.8) and postpubertal OVX ewes (rank of 2.8 ± 0.6). In males, GNDX significantly increased NKB cell numbers at both ages (Fig. 5E). There was an interaction of age and GNDX on NKB-positive cell numbers, likely due to the larger absolute decrease in NKB cell numbers observed in GNDX males (δ,-143 per wether) with age compared with intact males (δ,-73 per ram) (Fig. 5E and Appendix: Supplemental Fig. 4). However, the percent decrease with age appeared to be greater for the intact (89% decrease) than GNDX (60% decrease) males. NKB cell numbers were significantly greater in postpubertal OVX ewes (357 ± 37 per ewe) than age-matched GNDX males (108 ± 45 per wether), but

were not different between prepubertal OVX ewes (347 $\pm$ 37 per ewe) and age-matched GNDX males (272 $\pm$ 37 per wether). In addition, NKB cell numbers decreased with age in GNDX males.

DISCUSSION

The data herein provide evidence of a role for kisspeptin and NKB in puberty of female sheep. Our observation that kisspeptin cell numbers within the ARC increase after puberty in females, but not in age-matched postpubertal males, is consistent with an important role for this peptide in the onset of puberty. Furthermore, we show an increase in kisspeptin- positive close contacts on GnRH neurons in the medial POA of ewes that closely mirrored the increase in ARC kisspeptin cell numbers after puberty. The stimulatory effect of senktide on LH secretion and the OVX induced increase in NKB cell number in prepubertal ewes indicate a possible role for NKB in estradiol-negative feedback before puberty. Although there was not an increase in NKB-positive cell bodies after puberty, the increased density of NKB immunoreactive fibers leads us to suggest that NKB expression may also be increased.

It is generally thought that kisspeptin input to GnRH neurons is a limiting factor for induction of the onset of puberty, but whether it is the kisspeptin neurons in the ARC or more rostral areas [anteroventral periventricular nucleus (AVPV) in rodents, POA in other species, (38)] that are involved remains controversial. In female rats, postpubertal increases in kisspeptin mRNA (39, 40) and protein (39) occur in both the AVPV and ARC. In female mice, kisspeptinmRNAand protein increase in the AVPV (41–44), but changes in ARC kisspeptin mRNA expression are inconsistent (41, 42), leading to the hypothesis that AVPV kisspeptin drives puberty onset in mice (43). However, it was recently reported (19) that ablation of ARC kisspeptin neurons at postnatal d 20 disrupted ovarian function in mice, a result consistent with ARC kisspeptin neurons playing a critical role in puberty. The authors argued that these lesions were ARC specific because the time of kisspeptin neuronal ablation occurred before appearance of kisspeptin in the AVPV, but provided no immunocytochemical staining of the AVPV or ARC to support the specificity of the lesion. In estradiol-treated OVX ewes, kisspeptin mRNA increased with age in the POA, but not in the ARC, during pubertal development (45). In the present study, we observed that kisspeptin cell numbers in the ARC are greater in postpubertal than prepubertal ewes, whereas kisspeptin

immunoreactivity in the POA was insufficient to accurately quantify cell numbers. Comparison of these two studies in ewes suggests a possible disconnect between mRNA and protein, as has been observed in pubertal mice (41). Notably, both studies observed a positive association between ARC kisspeptin expression and LH pulse frequency during pubertal development, a finding consistent with the hypothesis that an increase in ARC kisspeptin is required for pubertal progression in the ewe.

Interestingly, we also observed that kisspeptin-positive close contacts on GnRH neurons increase after OVX in prepubertal ewes and are significantly greater in postpubertal compared with prepubertal ovary-intact lambs. A similar increase in kisspeptin inputs onto GnRH neurons after puberty has been observed in mice (43), but the origin of this input remains unclear. The coinciding changes in kisspeptin content within the ARC with the changes in kisspeptin-containing close contacts on GnRH neurons strongly suggests that kisspeptin cells in the ARC, and not the POA, are the source of this input in ewes. A similar correlation of kisspeptin in the ARC and kisspeptin input onto GnRH neurons was seen between breeding and non-breeding seasons in the adult ewe (46). Although it has been reported that only 1–2% of kisspeptin contacts onto GnRH neurons originate from the ARC (47), this tract tracing study used a small injection volume of Fluoro-Gold, which may have covered only a relatively small area of the POA, in which GnRH neurons reside. A different anatomical approach was used to show that approximately 50–70% of GnRH neurons receive close contacts containing both kisspeptin and dynorphin (48), a percentage consistent with that observed in OVX or intact postpubertal ewes from our study. Because the only set of neurons known to contain both kisspeptin and dynorphin in the hypothalamus are the subset of neurons in the ARC that also contain NKB (22, 38), we conclude that kisspeptin input from within the ARC is primarily responsible for the increased input to GnRH neurons during puberty in the ewe.

Because puberty onset in the ewe is marked by an increase in GnRH/LH secretion due to a decrease in estradiol-negative feedback (1), we predicted that a pubertal increase in kisspeptin production within the ARC would occur in response to a lessening of steroid-negative feedback. The increase in kisspeptin expression after OVX of prepubertal, but not postpubertal, ewes is consistent with this

hypothesis. It should also be noted that the proposed negative feedback inhibition of kisspeptin expression in the ARC before puberty is consistent with findings that estradiol exerts negative feedback effects on kisspeptin mRNA and protein expression in the adult ovine ARC during anestrus (46). The lack of an increase after OVX in our postpubertal ewes contrasts with previous data in ewes using *in situ* hybridization (17). One explanation for these differences is that by using immunocytochemistry, we are unable to detect any increase in kisspeptin production within cells that were already producing immunoreactive peptide. However, this apparent contradiction most likely reflects differences in cell numbers between ovary intact groups in the luteal (17) and follicular (this study) phases, because the number of kisspeptin cells in the ARC increases after luteolysis in the ewe (49).

As previously mentioned, kisspeptin cells in the ovine ARC of the hypothalamus also contain NKB (22). In humans, NKB and NK3R are crucial for puberty onset (20). We report here that a peripheral injection of senktide, an NK3R agonist, stimulated LH release in the prepubertal ewe. Because acyline abolished LH secretion and blocked any effect of senktide, we suggest that the effect of senktide occurs at the level of the hypothalamus to cause GnRH release. These data fit well with recent reports that intact adult ewes (31) and prepubertal male primates (24) both respond to senktide administration with an increase in LH release. In OVX goats, NKB stimulates multiunit activity within the ARC but decreases LH secretion; no effects of senktide were seen in OVX ewes (31). In rodents, both stimulatory (26, 50, 51) and inhibitory (30, 50) effects of senktide on LH have been observed, a response that seems largely dependent upon steroid milieu.

Unlike kisspeptin, NKB cell numbers did not increase postpubertally in intact ewes, which is similar to a previous report in mice using *in situ* hybridization to identify these cells (52). It is possible that NKB cell numbers increased in prepubertal ewes before the time analyzed in this study. If so, this increase did not lead to stimulation of LH release or induction of puberty, perhaps due to insufficient kisspeptin. This would be consistent with the hypothesis that NKB acts via kisspeptin to stimulate GnRH/LH release (21, 23, 53, 54) and with the idea that although NKB may be necessary, kisspeptin is the gatekeeper to puberty onset. Our data also indicate that NKB and kisspeptin may be differentially

regulated in the same cells, as has been observed previously within kisspeptin/NKB/dynorphin neurons (55) as well as other neurons in the ARC that express more than one neuropeptide (56). Although cell numbers were unaltered, NKB fiber density did increase in EF ewes compared with prepubertal intact ewes. Increases in kisspeptin immunoreactive fibers within the ARC during the pubertal transition have been reported in rodents (43, 44), but this is the first evidence for similar increases in NKB immunoreactive fibers. One simple explanation for this observation is that parallel increases in synthesis and transport of NKB occur in these neurons. Interestingly, NKB cell numbers did increase after GNDX in both prepubertal and postpubertal ewes, suggesting that estradiol was limiting NKB expression at both ages. This is consistent with the report where estradiol failed to suppress NKB gene expression in estrogen receptor-_ knockout mice (57), because NKB neurons contain ERα (53).

Sex differences in kisspeptin expression in sheep (55) and humans (25) and NKB expression in sheep (35, 55) have been reported to exist within the ARC. Sex differences in NKB expression are clearly due to organizational effects of testosterone during prenatal development (37,55), but whether the sexual dimorphism in kisspeptin content is due to organizational or activational effects of gonadal steroids remains unclear (55). The inclusion of OVX ewes and castrated males of a similar age in our study allows us to address this question. Our data show that in the ovine ARC, kisspeptin cell numbers strongly tended to be, and NKB cell numbers were, significantly greater in postpubertal OVX ewes compared with castrated males of a similar age. Because these differences cannot be due to activational actions of gonadal steroids, we conclude that sex differences in both kisspeptin and NKB are caused by organizational actions of steroids. Interestingly, prepubertal OVX ewes did not differ from castrated males of a similar age in kisspeptin and NKB cell numbers. Thus, the organizational effects of steroids on these neurons in sheep may only become evident as they reach adulthood and appear largely to be due to a loss of expression in older males (Figs. 3 and 5).

In conclusion, the data here support the hypothesis that kisspeptin is a gatekeeper to puberty onset in the ewe and raise the possibility that increases in NKB may also contribute to this process.

Furthermore, we suggest the increase in kisspeptin input is reflected in the medial POA at the GnRH cell bodies. These data also clearly demonstrate that NKB expression is under the control of steroid negative feedback in both prepubertal and postpubertal ewes and support the proposed stimulatory role for this neuropeptide in the ewe.

ACKNOLEDGEMENTS

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FIGURE LEGENDS

Figure 1. A, LH profiles from individual prepubertal ewes that were treated with water (*open circles*) or 100 μg of senktide (*closed circles*). B, Mean LH 24 min before (Pre) and 24 min after (Post) iv injection. C, LH profiles from individual prepubertal ewes that were treated with water (*open circles*) or 500 μg of senktide (*closed circles*). D, Mean LH 24 min before (Pre) and 24 min after (Post) iv injection. *Arrows* indicate time of iv injection. *, LH pulses. Significance ($P < 0.05$) is indicated by [stroke]T.

Figure 2. Group means (±SEM) for mean LH (A), LH pulse frequency (B) and LH pulse amplitude (C) in prepubertal and postpubertal females (*left*) and age-matched males (*right*). *Open bars* represent gonad-intact animals; postpubertal ewes are in the early follicular (EF) phase. *Closed bars* represent GNDX animals. Significance ($P < 0.05$) within sex is indicated by *differing superscripts*.

Figure 3. A–D, Photomicrographs of kisspeptin-positive neurons in the caudal portion of the ARC. Sections are from representative prepubertal and postpubertal females that were intact (A and B) or OVX (C and D). E, Mean (±SEM) number of kisspeptin-positive neurons in the ARC of prepubertal and postpubertal females (*left*) or age-matched males (*right*). Significance ($P < 0.05$) within sex is indicated by *differing superscripts*. *, Significant ($P < 0.05$) difference between intact female groups. *Scale bar*, 200 μm.

Figure 4. *Top panels*, 3D reconstruction of confocal microscopic images of GnRH neurons (*green*) lacking (A) and with (B) kisspeptin (*red*) close contacts from a prepubertal and postpubertal ewe, respectively. B, Kisspeptin-positive close contacts (*arrowheads*) with incoming kisspeptin fiber (*arrows*) adjacent to the GnRH neuron. *Bottom panels*, Mean (±SEM) percentage of GnRH neurons with at least one kisspeptin close contact (C) and the mean (±SEM) number of close contacts per GnRH neuron that received at least one close apposition (D). *Open bars* represent intact ewes, and *closed bars* represent OVX ewes. Significance ($P < 0.05$) is indicated by *differing superscripts*. Images were visualized using a 63x/1.4 oil immersion objective.

Figure 5. A–D, Representative photomicrographs of NKB-positive neurons in the caudal portion of the ARC from ovary-intact (A and B) and OVX (C and D) prepubertal or postpubertal females. E, Mean (±SEM) number of NKB-positive neurons in the ARC from prepubertal and postpubertal females (*left*) and age-matched males (*right*). *Open bars* represent gonad-intact animals, and *closed bars* represent GNDX animals. Significance ($P < 0.05$) within sex is indicated by difference in superscripts. *, Significant ($P < 0.05$) age by GNDX interaction. *Scale bar*, 200 µm.

FIGURES

Figure 1

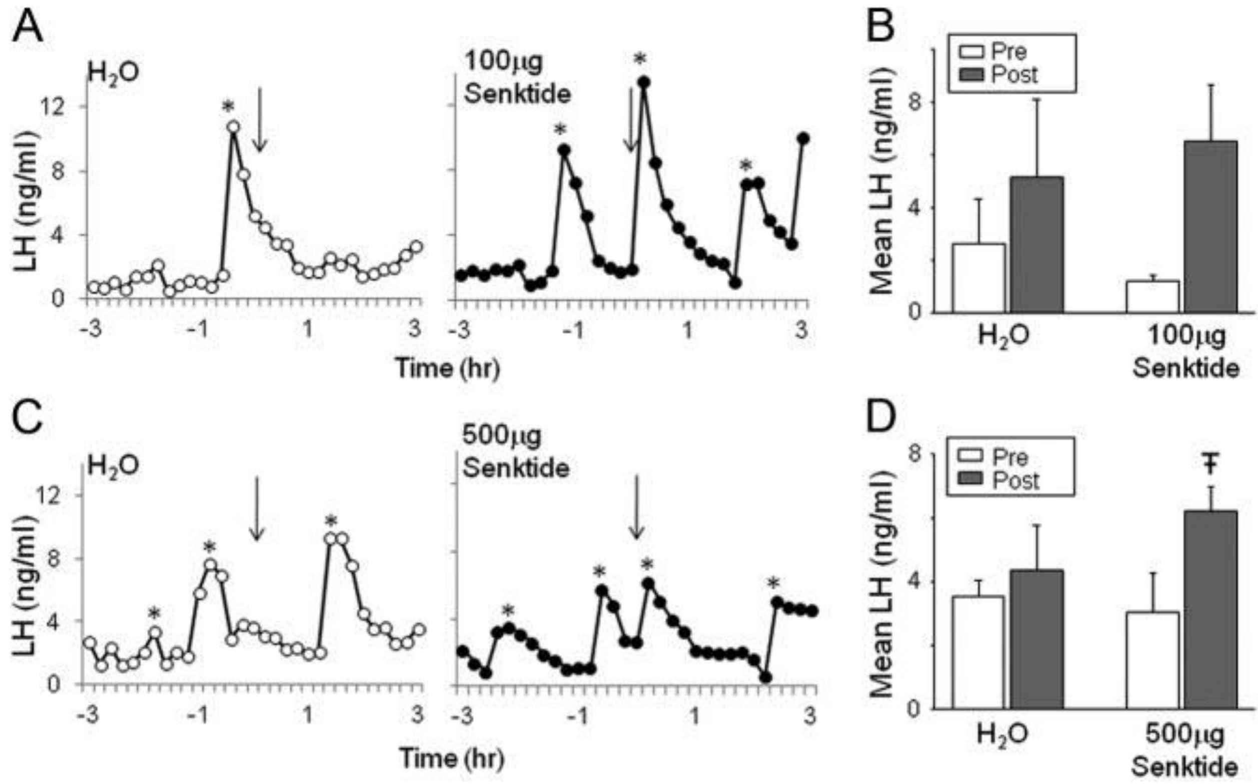


Figure 2

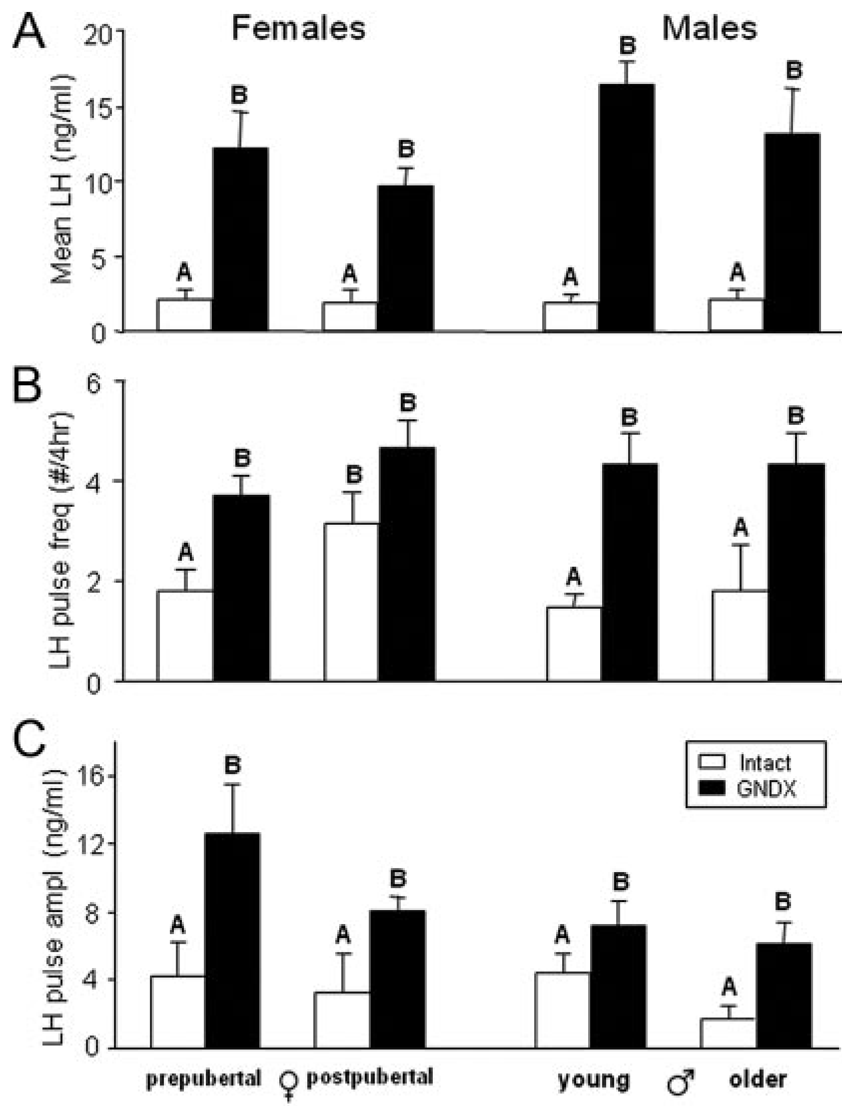


Figure 3

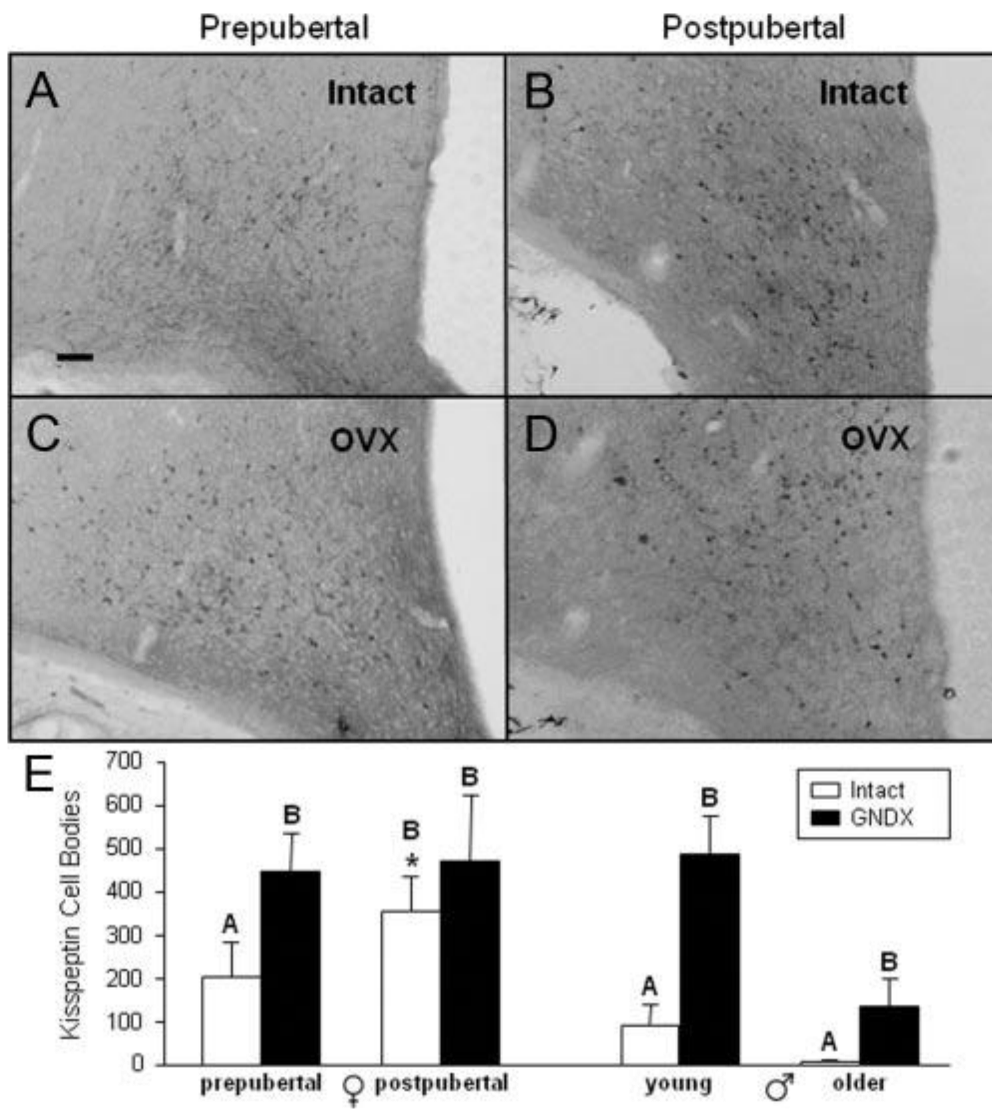


Figure 4

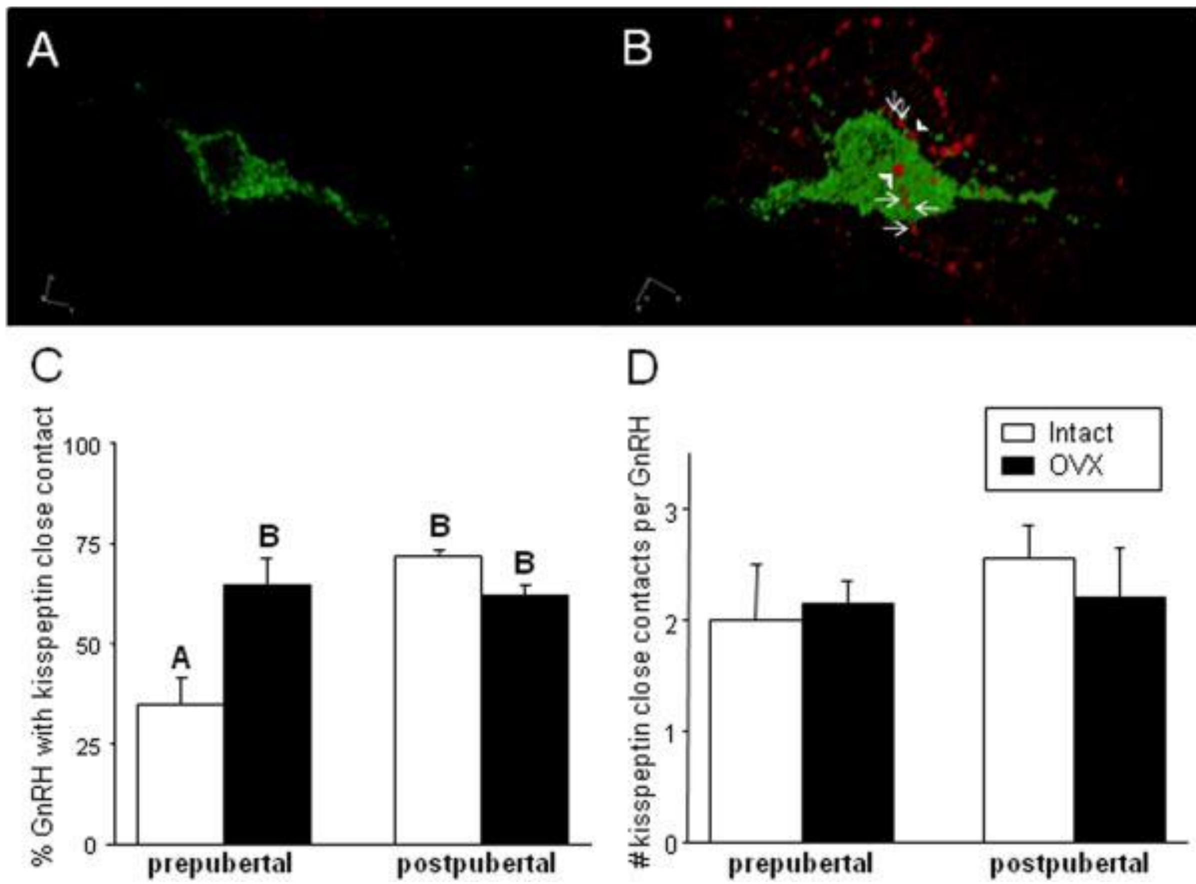
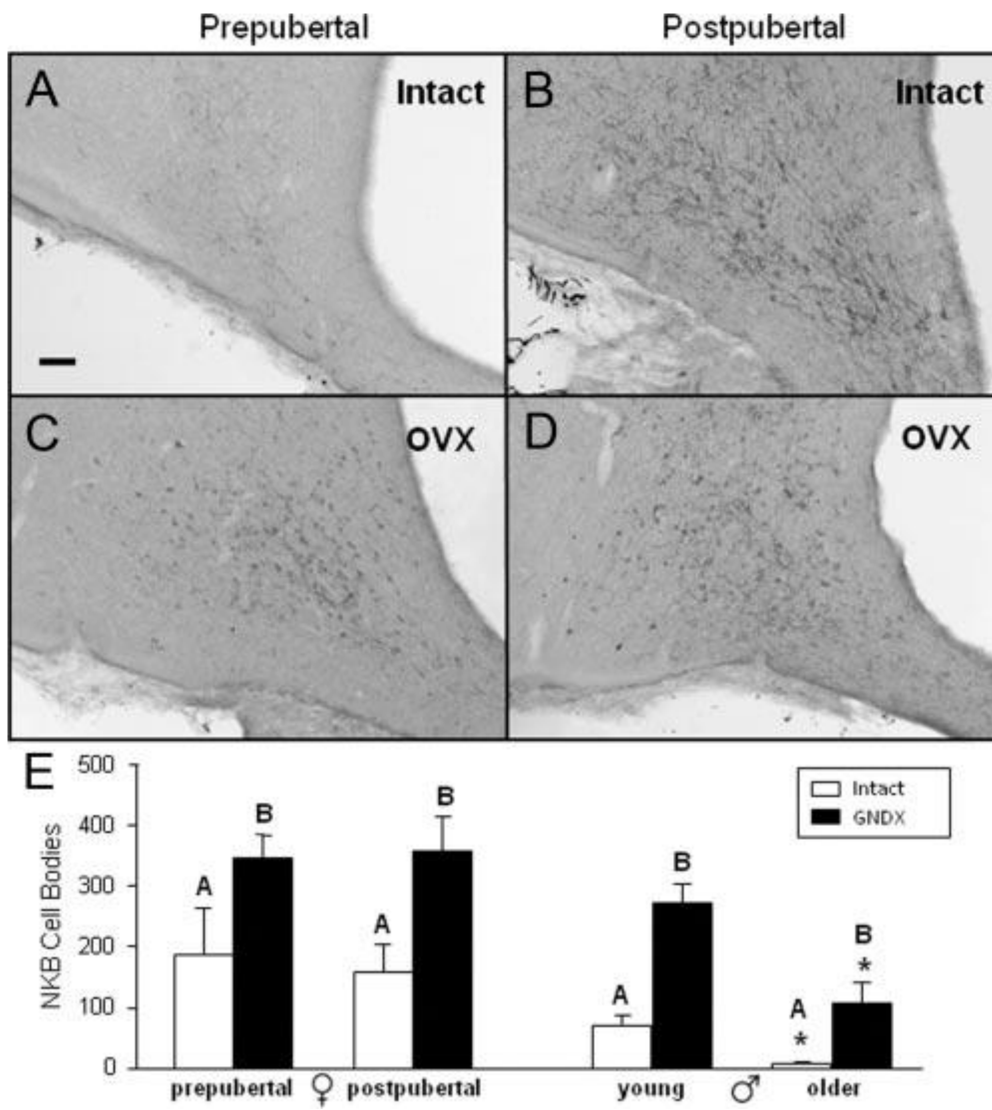


Figure 5



CHAPTER 3

ORPHANIN FQ ACTS AT THE HYPOTHALAMUS TO SUPPRESS GONADOTROPIN RELEASING HORMONE SECRETION IN THE EWE

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Running Heading: OFQ inhibits GnRH in sheep

ABSTRACT

Recently, orphanin FQ (OFQ), an opioid-like peptide that acts via its receptor, ORL-1, has been shown to be expressed throughout the ovine POA and hypothalamus including the external zone of the median eminence. Previously it has been shown that infusion of an OFQ agonist into the third ventricle suppressed luteinizing hormone (LH) secretion in ovariectomized (OVX) ewes. In this study, we determined if OFQ acts at the pituitary and/or the hypothalamus to inhibit LH secretion. To test the hypothesis that OFQ acts at the pituitary, an *in vitro* cell culture approach was used. Pituitaries were removed from eight sheep, dispersed and incubated with or without OFQ (550 pmole or 5500 pmole) and treated with increasing doses of GnRH ($4x10^{-12}$ M - $4x10^{-9}$ M). Following a three hour incubation, media was harvested and LH was measured using radioimmunoassay. The two highest doses of GnRH added elicited a significant increase in LH when compared to anterior pituitary cells incubated with no GnRH. Neither dose of OFQ inhibited GnRH-induced LH secretion. To test the hypothesis that OFQ acts at the hypothalamus, we administered an OFQ agonist ([Arg^{14}, Lys^{15}]OFQ) into the third ventricle of OVX ewes and measured GnRH secretion in the hypophyseal portal circulation. A single chronic guide tube was placed into the third ventricle of OVX ewes. Portal and jugular blood were simultaneously collected every ten minutes for four hours before and for four hours during infusion of either the OFQ agonist (n=5; 5 nmole/hr) or saline (n=5; 60 µl/hr) into the third ventricle. Infusion of OFQ agonist into the third ventricle inhibited GnRH pulse frequency during the last two hours of infusion, while infusion of saline has no effect on GnRH or LH secretion. In summary, OFQ appears to act primarily at the hypothalamus to inhibit GnRH, and subsequently LH, secretion.

INTRODUCTION

For most species, subpopulations of GnRH cell bodies exist in the diagonal band of the broca, preoptic area, the anterior hypothalamic area and the medial basal hypothalamus with axonal projections that terminate in the external zone of the median eminence (1-4). GnRH is transported via hypophyseal portal vessels to the anterior pituitary and controls the release of the pituitary gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). In the ewe, GnRH and LH secretion occur in episodic patterns; a pulse of GnRH secretion stimulates a subsequent pulse of LH secretion. Late in the follicular phase of the estrous cycle, rising levels of plasma estradiol (E_2) act as a stimulus for the preovulatory GnRH/LH surge. In contrast, during the rest of the estrous cycle relatively low levels of E_2 serves to inhibit GnRH/LH pulse amplitude while progesterone acts to primarily inhibit GnRH/LH pulse frequency (5). However, GnRH neurons are devoid of the receptors, estrogen receptor-α (ERα; (6, 7)) and progesterone receptor (PR; (8)), that mediate steroid positive and negative feedback. Therefore, other interneurons must mediate the effects of these steroids on GnRH release. Consequently, neurons that release OFQ are possible candidates to mediate steroid negative feedback.

Orphanin FQ (OFQ), also named nociceptin, was discovered by two independent groups (9, 10). OFQ was named for its binding to an orphan receptor, opioid receptor-like 1 (ORL-1), and the first and last amino acids in the peptide sequence, phenylalanine (F) and glutamine (Q) as well as called nociceptin for its pro-nociceptive properties. The peptide and its receptor are found throughout the central nervous system and, since identified, OFQ has been shown to participate in various physiological processes including pain perception, learning, and memory as well as renal and cardiovascular function (11). OFQ is found in the preoptic area, hypothalamus and median eminence in rats (12, 13) and sheep (14). Furthermore, OFQ given icv has been shown to inhibit GnRH and LH secretion in the rat (15, 16) and LH secretion in the ewe (14). Although most evidence suggests that the effect of OFQ on LH in the rat is at the hypothalamus, the presence of OFQ in the external zone of the median eminence of sheep raises the possibility of a direct action of OFQ at the pituitary. The sheep model is ideally suited to differentiate

hypothalamic vs anterior pituitary action of this opioid because both GnRH in hypophyseal portal blood and LH in peripheral blood can be assessed simultaneously in an unanesthetized animal (17).

Herein we hypothesize that OFQ acts directly at the anterior pituitary to inhibit LH secretion and at the hypothalamus to inhibit GnRH release. To examine a potential direct effect of OFQ at the anterior pituitary, we measured LH release from cultured adult ewe anterior pituitary cells with increasing concentrations of OFQ alone and with increasing concentrations of GnRH in the absence or presence of OFQ. To examine a potential effect of OFQ at the hypothalamus, we infused an OFQ receptor agonist into the third ventricle of ewes and collected hypophyseal portal and jugular blood simultaneously for the measurement of GnRH and LH, respectively.

MATERIALS AND METHODS

Animals

Adult ewes of predominantly the Suffolk breed (>3yrs) were used in this study. For Experiment 1, anterior pituitaries (n=8) were collected from breeding season ewes housed outdoors on natural photoperiod who had received a daily diet of hay and water *ad libitum*. For Experiment 2, non-breeding season ewes (n=10) were housed indoors for 14-17 days on a photoperiod that simulated natural daylength and received a daily ration of alfalfa pellets with free access to water. All procedures were approved by the West Virginia University Animal Care and Use Committee and followed NIH guidelines for use of animals in research.

Pituitary dispersal

For experiment 1, ewes (n=8) were euthanized with an overdose of sodium pentobarbital and pituitaries were removed and dispersed as previously described (18). Briefly, each anterior pituitary was cut into 1mm cubes using a sterile scalpel and forceps in Hanks balanced salt solution (Sigma), then centrifudged at 900 rpm for 2 min. Tissue was then incubated in a flask containing 10 ml Hanks solution, 10 mg collagenase type XI (Sigma), 125 µg DNase 1 (Sigma) and 100 mg bovine serum albumin (BSA; Sigma) which was placed in a 37°C shaking water bath at 150 rpm for 30 min. Contents of the flask were next moved in and out of a plastic large bore pipette approximately 20 times and then the flask was returned to

the 37°C shaking water bath for 30 min. The contents were then centrifuged for 5 min at 900 rpm and the supernatant was discarded. Cells were placed in 10 ml Hanks solution, 25 mg pancreatin, 125 μg DNase1 and 100 mg BSA and incubated for 1 hr in a 37°C shaking water bath. This suspension was then moved in and out of a sterile Pasteur pipette approximately 20 times and returned to the water bath for another 30 min. After centrifugation for 5 min, the supernatant was discarded and cells added to 1 ml DMEM (Dulbecco's Modified Eagle Medium) with 10 % FBS (fetal bovine seurm), 1mg DNase1 and 1mg trypsin inhibitor. 10 ml of the DMEM-FBS solution was added to cells, they were lightly vortexed and centrifuged for 5 min, after which the supernatant was discarded. After two more repetitions of the prior step, cells were diluted to 1 ml with DMEM-FBS, counted using a hemocytometer and placed in 24 well plates (100,000 cells in 1 ml DMEM-FBS/well; Fisher). Plates were incubated in a humidified chamber at 37°C with 5% CO_2 for 3 days prior to treatment. Cell viability was assessed using the trypan blue exclusion method (19) and was greater than 95%.

Surgeries

For Experiment 2, ewes were first ovariectomized (OVX) by midventral laparotomy under gas anesthesia (oxygen + 3% isoflurane), followed immediately by permanent insertion of a 16 gauge guide tube into the third ventricle as previously described (20). Briefly, a 2 cm hole was drilled in the skull at bregma, the sagittal sinus ligated, and 1.5 ml of a radioopaque dye (Omnipaque 350; Webster Veterinary Supply; Devens, MA) injected into one lateral ventricle. Lateral and frontal x-rays were taken to visualize placement of the guide tube to the middle area of the third ventricle and 3 to 4 mm posterior to its rostral edge. The needle hub was plugged to prevent loss of cerebrospinal fluid and, together with a plastic protective cap, was cemented in place using dental acrylic. All ewes were treated pre- and post-operatively with Penicillin Procaine G (Webster Veterinary Supply), Dexamethosone (Webster Veterinary Supply), and FluMeglumine (Webster Veterinary Supply) as previously described (20) and allowed at least 1 week for recovery. Following recovery, a hypophyseal portal cannula was surgically inserted under anesthesia as previously described (17). Briefly, a skin incision was made to expose the nasal bone with caution taken to avoid facial veins. A pentagonal piece of nasal bone was removed using

an osteotome and mallet, allowing access to the nasal turbinates and septum, which were removed using bone rongeurs to expose the face of the sphenoid bone. Using a bone drill, a tunnel was created that was large enough to accommodate the portal cannula and extended to the dura mater adjacent to the face of the anterior pituitary (approximately 10-10.4 cm). The dura mater was removed and the portal cannula placed 1-2 mm from the surface of the anterior pituitary. The apparatus was cemented into place using dental acrylic, filled with saline, and plugged. Ewes were allowed to recover for 7 to 10 days before portal blood collection.

Experimental Design

Experiment 1a: Does OFQ alone inhibit LH secretion?

Each pituitary (n=4) was dispersed and 100,000 cells were placed in each well of a 24-well plate/ewe. Each plate was divided into six groups for OFQ treatment (n=4 wells/treatment): no OFQ, 0.55 pmoles, 5.5 pmoles, 55 pmoles, 550 pmoles and 5500 pmoles of OFQ (Tocris). OFQ was applied for 3 hrs and media was harvested, after which cells were challenged with 50 mM KCl, which stimulates maximal LH release from anterior pituitary cells (21), for 3 hrs and media was again harvested. All media was stored at -20°C until time of assay.

Experiment 1b: Does OFQ inhibit GnRH-induced LH secretion *in vitro*?

Each pituitary (n=8) was dispersed and 100,000 cells were placed in each well of a 24-well plate. Enough cells were recovered from each ewe for three 24-well plates/ewe. These plates were divided into three groups for OFQ treatment: one plate received no OFQ, the second plate received 550 pmoles, and the third plate received 5500 pmoles of OFQ. These doses of OFQ were chosen based on administration in previous *in vitro* (22) and *in vivo* (14-16, 23) studies. Each plate was divided into six groups for GnRH treatment (n=4 wells per dose): no GnRH, 4 pM, 40 pM, 220 pM, 400 pM and 4000 pM of GnRH. These doses of GnRH were chosen based on a previous study using ovine anterior pituitary cells (24). OFQ treatment was applied 30 min prior to and during a 3 hr incubation with each dose of GnRH followed by harvesting of the media. At the end of incubation, all cells were challenged with 50 mM KCl

in DMEM for 3 hrs and again media was harvested. All harvested media was stored at -20°C until time of assay.

Experiment 2: Does an OFQ agonist, [Arg14,Lys15] OFQ, inhibit GnRH secretion *in vivo*? Hypophyseal portal and jugular blood samples were collected from ten ewes at 7-10 days after portal cannula insertion. Twenty-four hours prior to the collection, each ewe had a 16 gauge indwelling catheter inserted into each jugular vein using local anesthetic. On the day of the collection, each ewe was heparinized and portal veins lesioned as previously described (17). Portal and jugular blood samples were collected using peristaltic pumps every 10 min for 4hrs before and 4 hrs during infusion of either saline (n=5; 60 µl/hr) or OFQ agonist (n=5; 5 nmoles/hr; Tocris). Portal samples were collected into tubes containing 0.5 ml bacitracin (Sigma; 423 mg/100 ml saline) submersed in an ice bath to prevent the loss of GnRH. Portal plasma (750 µl) for GnRH measurements was extracted within 1-2 hrs of collection using 2 ml methanol, then dried and stored at -20 °C. Jugular plasma for LH measurements was stored at -20 °C.

Data analysis

Assays: LH concentrations were measured in duplicate with a radioimmunoassay using 100–200 µl of media and plasma with reagents provided by the National Hormone and Peptide Program (Torrance, CA) as previously described (25). LH assay sensitivity averaged 0.12 ng/tube (NIH S24) and intra- and interassay coefficients of variation were 17.1% and 22.5%, respectively. GnRH concentrations were measured in extracts of portal plasma after reconstitution with 250 µl of phosphate buffered saline with 0.1% Gelatin (Sigma) or phosphate buffer with 0.1% triton-x (Sigma) by radioimmunoassay (26, 27) using two different antibodies (BDS 037; R 1245). Samples were assayed in duplicates of 100 µl (equivalent to 300 µl of plasma) with all samples for each ewe being analyzed in the same assay. Intra- and interassay variability were 15.9% and 23.4%, respectively. GnRH assay sensitivity was 0.14 pg/tube.

Statistics: In experiment 1a, pituitaries were collected from follicular phase (n=1), luteal phase (n=1) and OVX ewes (n=2). In experiment 1b, pituitaries were collected from follicular phase (n=3), luteal phase (n=2) and OVX ewes (n=3). Since there was no difference in LH response among these groups, data

from all three groups were combined for analysis and presentation. For experiment 1a, mean LH was calculated by averaging wells in each treatment group (4 wells/group) to determine mean concentration for each ewe after each dose of OFQ. These data were then transformed to the reciprocal to establish normality and analyzed using one-way ANOVA. For experiment 1b, mean LH was calculated by averaging wells in each treatment group (4 wells/group), transformed to the reciprocal to establish normality and analyzed using two-way ANOVA with a Holm-Sidak post-hoc test.

For experiment 2, GnRH values are reported in pg/min to compensate for changes in blood flow during time of collection. GnRH pulses were identified using Cluster 8 (M. Johnson; Keswick, VA) and LH pulses were identified using previously described criteria (Goodman and Karsch, 1980). For analysis of mean GnRH and LH, pulse frequencies and amplitudes, all variables were divided into 2 hour time bins (*i.e.,* 0-2, 2-4, 4-6 and 6-8 hours). Within each treatment group, GnRH and LH pulse frequency were analyzed using Friedman Repeated Measures Analysis of Variance on Ranks with a Tukey post-hoc test. Mean LH concentrations, mean GnRH concentrations, mean LH pulse amplitude, mean GnRH pulse amplitude and mean GnRH baseline were analyzed using a repeated measure one-way ANOVA with a Holm-Sidak post-hoc test. Mean GnRH baseline (identified by Cluster 8 software) was calculated by averaging concentrations for each time bin with the exclusion of values during a pulse episode. GnRH pulse amplitude was calculated by summation of the GnRH concentrations during a pulsatile event as determined by Cluster 8. Differences were considered to be significant at $p < 0.05$.

RESULTS:

Experiment 1: Can OFQ inhibit LH release from anterior pituitary cells *in vitro*?

In experiment 1a, OFQ alone had no significant effect on LH secretion (Fig 1A) and cells responded to a 50 mM KCl with an increase in mean LH concentration slightly higher than that seen during the OFQ challenge (Fig 1B), which confirmed cell viability. In experiment 1b, mean LH release from anterior pituitary cells was significantly greater than control values following application of the two highest GnRH concentrations, 400 and 4000 pM (Fig 2A). The greatest increase in LH release, greater than 2 fold compared to control, occurred at the highest dose of GnRH. Incubation with 550 pmole or 5500 pmole of

OFQ had no significant effect on the GnRH-induced LH release (Fig 2A). Following a 50 mM KCl challenge, cells in culture responded with an increase in mean LH concentration comparable to that previously observed following administration of the highest dose of GnRH (Fig 2B).

Experiment 2: Can an OFQ receptor agonist, [Arg14,Lys15] OFQ, inhibit GnRH secretion *in vivo*?

As can be seen in the representative GnRH and LH profiles in Figure 3, infusion of an OFQ receptor agonist in the third ventricle, in contrast to saline, produced a dramatic reduction of LH secretion as well as decrease in GnRH pulse frequency and an increase in GnRH baseline in OVX ewes (Fig 3). Saline infusion had no significant effect on mean LH concentration (Fig 4A) or mean GnRH concentration (Fig 4B). Infusion of the OFQ agonist significantly reduced mean LH within the final two hours of treatment (Fig 4 C), while having no significant effect on mean GnRH concentration (Fig 4D). Mean LH pulse amplitude (Fig 5A) and mean GnRH pulse amplitude (Fig 5B) was unaffected by saline infusion. Infusion of the OFQ agonist significantly reduced LH pulse amplitude in the final two hours of infusion (Fig 5C), while having no significant effect on mean GnRH pulse amplitude (Fig 5D). Mean LH and GnRH pulse frequency (Fig 6A and 6B, respectively) were unaltered by saline infusion, while in OFQ agonist-infused OVX ewes, mean LH and GnRH pulse frequency (Fig 6C and 6D, respectively) were significantly reduced during the final two hours of infusion. Interestingly, we did observe an increase in baseline GnRH concentration for 4 out of 5 OFQ agonist-infused ewes. Mean GnRH baseline concentration significantly increased during the final two hours of OFQ agonist infusion, but not during saline infusion (Fig 7). Finally, given that each GnRH pulse should result in a subsequent LH pulse, we analyzed whether this association was altered in OFQ agonist-infused ewes and as expected almost all GnRH pulses were followed by LH pulses. Although rare, one OFQ agonist infused ewe had one GnRH pulse unaccompanied by a detectable LH pulse, while another OFQ agonist infused ewe had an LH pulse in the absence of a detectable GnRH pulse. This did not occur in saline infused ewes. In addition to the studies reported here, we also tested the effect of OFQ agonist infusion into the lateral ventricle on LH secretion in OVX ewes. Given the distance from the third ventricle and the relative increase in cerebrospinal fluid volume experienced with this approach, we infused a dose that was four-times the

dose (20 nmole/hr) used for the third ventricle infusions reported here and collected jugular blood samples every 12 minutes. After one hour, the infusion of OFQ agonist was discontinued because both ewes became ataxic and after another 30 min could not stand and appeared to be sedated for a total of four to five hours. Moreover, a similar alteration in locomotor activity was observed in rodents (9, 28, 29). LH secretion in both ewes was clearly reduced (data not shown) and since no behavioral effect is seen with infusion of the OFQ agonist in the third ventricle, we suggest that this inhibition of GnRH is independent of sedation.

DISCUSSION

Herein we investigated the site of action of OFQ in controlling LH secretion in the ewe. OFQ alone had no effect on LH release from anterior pituitary cells *in vitro*. Furthermore, OFQ did not block GnRH-induced LH release from isolated anterior pituitary cells. Our *in vivo* work demonstrated that OFQ acts at the level of the hypothalamus to inhibit GnRH pulse frequency and LH release as well as increase GnRH baseline concentration. Thus, we suggest that the inhibitory action of OFQ on LH secretion appears to reside primarily at the level of the hypothalamus.

Evidence of a central role for OFQ in control of LH has accumulated over the past fifteen years. However, one cannot rule out the possibility that OFQ inhibits LH secretion directly at the anterior pituitary given that the pituitary is bathed in the same cerebrospinal fluid in which the drug is administered. Furthermore, in rodents (12, 13) and sheep (14) OFQ exists in the median eminence and thus may be released into the hypophyseal portal vessels. However, to date, an adequate assay for measurement of OFQ in hypophyseal portal blood does not exist. And, while presence of the OFQ receptor, ORL-1, in the rat pituitary remains controversial (15, 30, 31), the question of whether or not OFQ acts to inhibit LH directly at the anterior pituitary remains open. Herein we report that, as expected, GnRH induced an increase in LH release from cultured anterior pituitary cells, but that OFQ alone had no effect on LH release nor did this opioid have an effect on GnRH-induced LH release. Although our two largest doses of OFQ were similar to doses used during icv administration that have been shown to inhibit

LH secretion in the rat (15, 16) and the ewe (14), and thus should have been adequate, we cannot eliminate the possibility that higher doses of OFQ might have been effective.

In vivo experiments in rats demonstrate that icv administration of OFQ inhibits GnRH concentrations in push-pull perfusates at the median eminence (15, 16). However, the push-pull technique is an indirect measure of the amount of GnRH that reaches the anterior pituitary. Herein we simultaneously collected hypophyseal portal blood and jugular blood in sheep, making this animal an excellent *in vivo* model to study the effects of OFQ agonist administration on GnRH secretion. Consistent with these earlier reports, we showed a similar inhibitory effect of an OFQ agonist on LH secretion. We did not observe a decrease in mean GnRH concentration or GnRH pulse amplitude following OFQ agonist infusion, which is not surprising given the low concentrations of GnRH even during a pulsatile event. We did observe a decrease in GnRH pulse frequency during the OFQ agonist infusion, which leads us to conclude that OFQ acts at the hypothalamus to inhibit LH secretion.

Although rare, an LH pulse did appear in the absence of a GnRH pulse during the last two hours of OFQ agonist infusion in one ewe. One plausible explanation for this would be the difficulty in detecting low amplitude GnRH pulses since the amplitude of GnRH pulses in our study were somewhat lower than would be expected based on a previous report (32). Also, during the latter part of the OFQ infusion period, we observed an increase in GnRH baseline in four of five OFQ-infused animals. This increase in baseline might be due to low amplitude, but high pulse freqendcy of GnRH secretion, but given our 10 min sampling we could not discern such an alteration in GnRH pulse patterns. We are currently investigating this possibility with a shorter collection interval between samples (2 minutes) which will allow us to analyze GnRH pulse dynamics. Conversely, yet equally rare, was a GnRH pulse in the absence of a subsequent LH pulse. Therefore, it is possible that OFQ could cause the release of an inhibitory neuropeptide, which could act directly at the anterior pituitary to inhibit LH secretion. One such inhibitory neuropeptide is gonadotropin inhibiting hormone which can inhibit GnRH-induced LH secretion in the ewe (33) and might explain the decrease in mean LH and LH pulse amplitude while mean GnRH concentrations and GnRH pulse amplitude did not change.

In vitro cellular recordings from GnRH neurons from guinea pig hypothalamic slices have shown that OFQ inhibits these cells by increasing inwardly-rectifying potassium channel conductance (34). Further work with hypothalamic slice preparations reveals that OFQ can inhibit forskolin-induced GnRH secretion (23) and basal GnRH release (15). The simplest explanation for OFQ inhibition of GnRH/LH secretion would be direct inhibition on GnRH perikarya. A recent report in the rat showed OFQ cell bodies exist throughout the hypothalamus (13) , which is consistent with data in the ewe that OFQ cell bodies are found in the POA and hypothalamus (14, 35). Furthermore, in the ewe, OFQ neurons in the hypothalamus, but not the POA, contain steroid receptors (35), raising the possibility that these neurons could mediate steroid negative feedback in sheep, a possibility that is tested in the next chapter. Although there is a general description of where ORL-1 is located (30), the individual neurons within the POA and hypothalamus that OFQ could influence directly have yet to be identified. Based on OFQ cell body distribution, work has been done to identify if OFQ neurons contain other neuropeptides. Within the POA of sheep, OFQ highly colocalizes (>90%) with GnRH neurons (14), which is consistent with the recent evidence that OFQ neurons in the POA of sheep do not contain steroid receptors (35). Furthermore, OFQ colocalizes with approximately 14 % and 50% of proopiomelanocortin (POMC) neurons in the arcuate nucleus in the rat (13) and the ewe (35), respectively. Together with colocalization and its hyperpolarizing effects on GnRH and POMC neurons in the ARC (34), OFQ might possess an autocrine function through an ultrashort negative feedback loop on GnRH neurons. In summary, we demonstrate that the inhibitory action of OFQ on LH secretion occurs at the hypothalamus and not at the level of the anterior pituitary.

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FIGURE LEGENDS

Figure 1. Mean LH concentration (± SEM) from adult ewe (n = 4) anterior pituitary cells with increasing doses of OFQ (Panel A) followed by KCl treatment (Panel B).

Figure 2. Mean LH concentration (± SEM) from adult ewe (n = 8) anterior pituitary cells treated with increasing doses of GnRH with or without OFQ (Panel A) followed by KCl treatment (Panel B). No OFQ (white bars), 550 pmol OFQ (grey bars), and 5500 pmol OFQ (black bars). Significance ($p<0.05$) between GnRH treatment groups is indicated by differing superscripts.

Figure 3. Secretory profiles of LH (top) and GnRH (bottom) from OVX ewes for 4 hrs before and during 4 hrs of either saline (Panel A) or OFQ agonist ([Arg14,Lys15] OFQ; Panel B) infusion. LH and GnRH pulses are identified by closed symbols. Infusion time is indicated by solid grey (saline) or solid black (OFQ agonist) bar.

Figure 4. Mean LH concentration (± SEM; Panel A) and mean GnRH concentration (± SEM; Panel B) for saline infused OVX ewes (n = 5). Mean LH (± SEM; Panel C) and mean GnRH (± SEM; Panel D) concentration for OFQ agonist, [Arg14,Lys15] OFQ, infused OVX ewes (n = 5). Measurements prior to infusion are represented by open bars. Significance ($p<0.05$) within treatment group is indicated by differing superscripts.

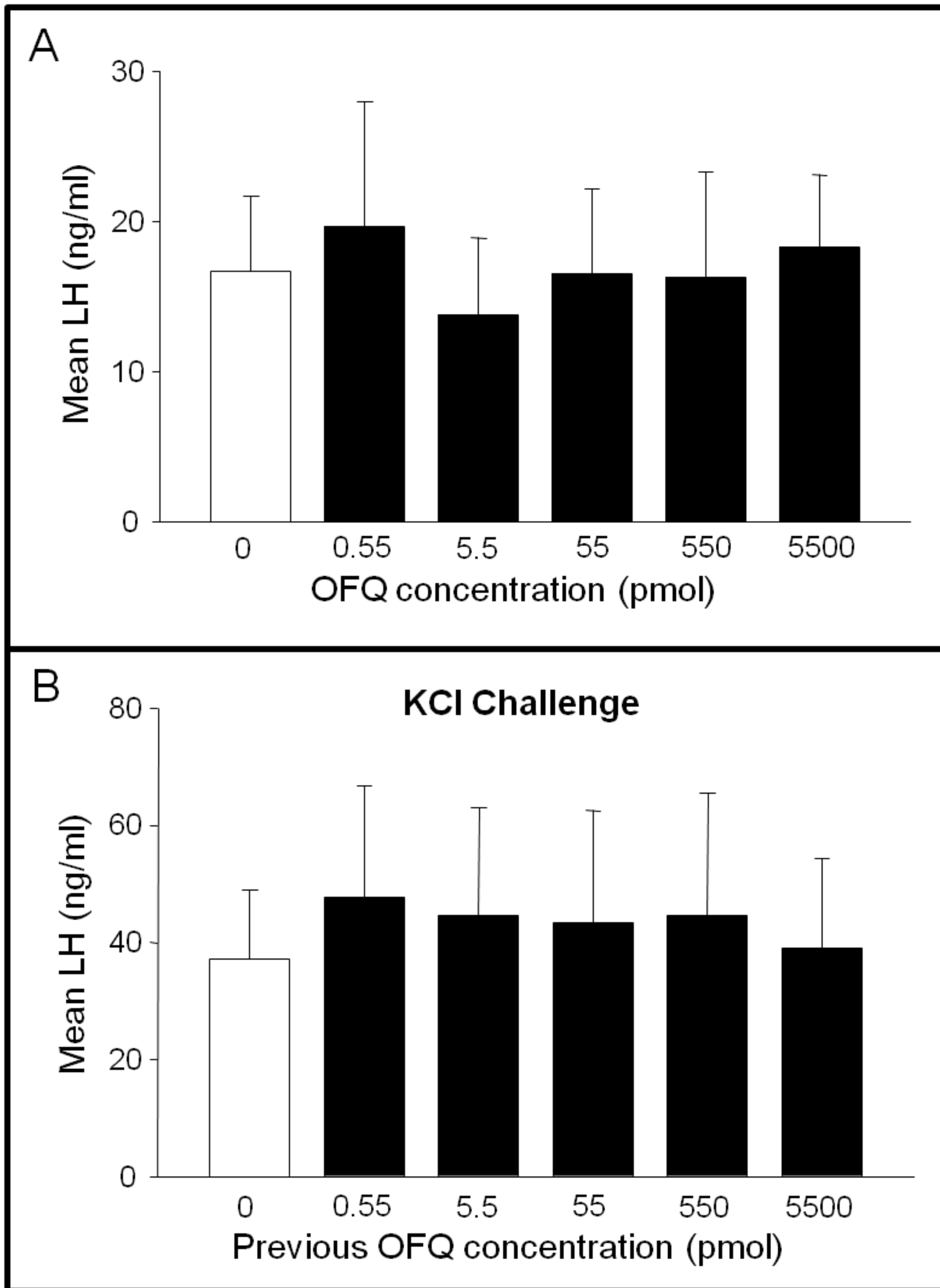
Figure 5. Mean LH pulse amplitude (± SEM; Panel A) and mean GnRH pulse amplitude (± SEM; Panel B) for saline infused OVX ewes (n = 5). Mean LH pulse amplitude (± SEM; Panel C) and mean GnRH pulse amplitude (± SEM; Panel D) for OFQ agonist, [Arg14,Lys15] OFQ, infused OVX ewes (n = 5). Measurements prior to infusion are represented by open bars. Significance ($p<0.05$) within treatment group is indicated by differing superscripts.

Figure 6. Mean LH pulse frequency (± SEM; Panel A) and mean GnRH pulse frequency (± SEM; Panel B) for saline infused OVX ewes (n = 5). Mean LH pulse amplitude (± SEM; Panel C) and mean GnRH pulse frequency (± SEM; Panel D) for OFQ agonist, [Arg14,Lys15] OFQ, infused OVX ewes (n = 5). Measurements prior to infusion are represented by open bars. Significance ($p<0.05$) within treatment group is indicated by differing superscripts.

Figure 7. Mean GnRH baseline (± SEM) for OVX ewes (n = 5/group) with infusion of saline (gray bars) or OFQ agonist ([Arg14Lys15] OFQ; black bars). Measurements prior to infusion measurements are represented by open bars. Significance ($p<0.05$) within treatment group is indicated by differing superscripts.

FIGURES

Figure 1



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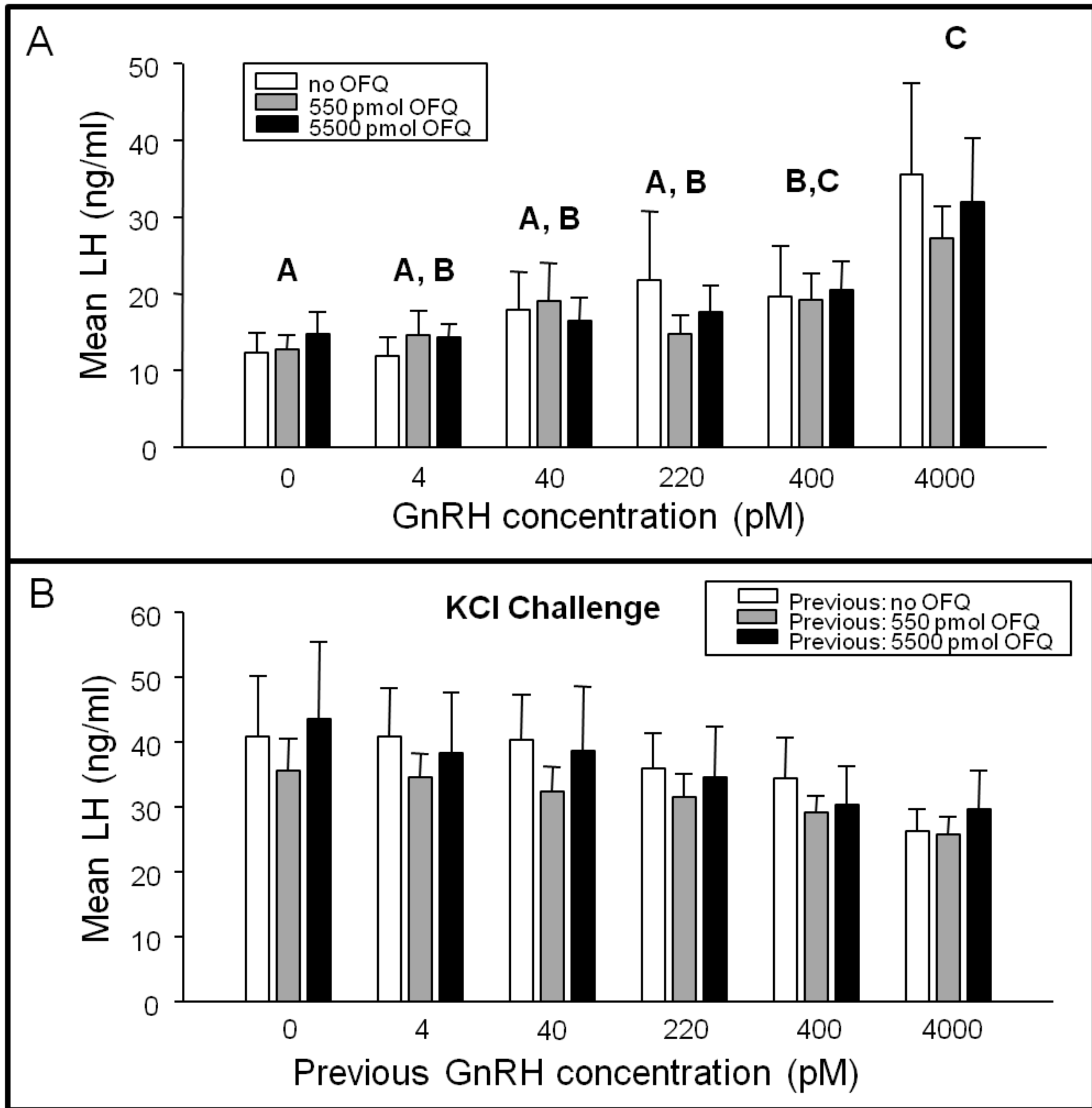


Figure 3

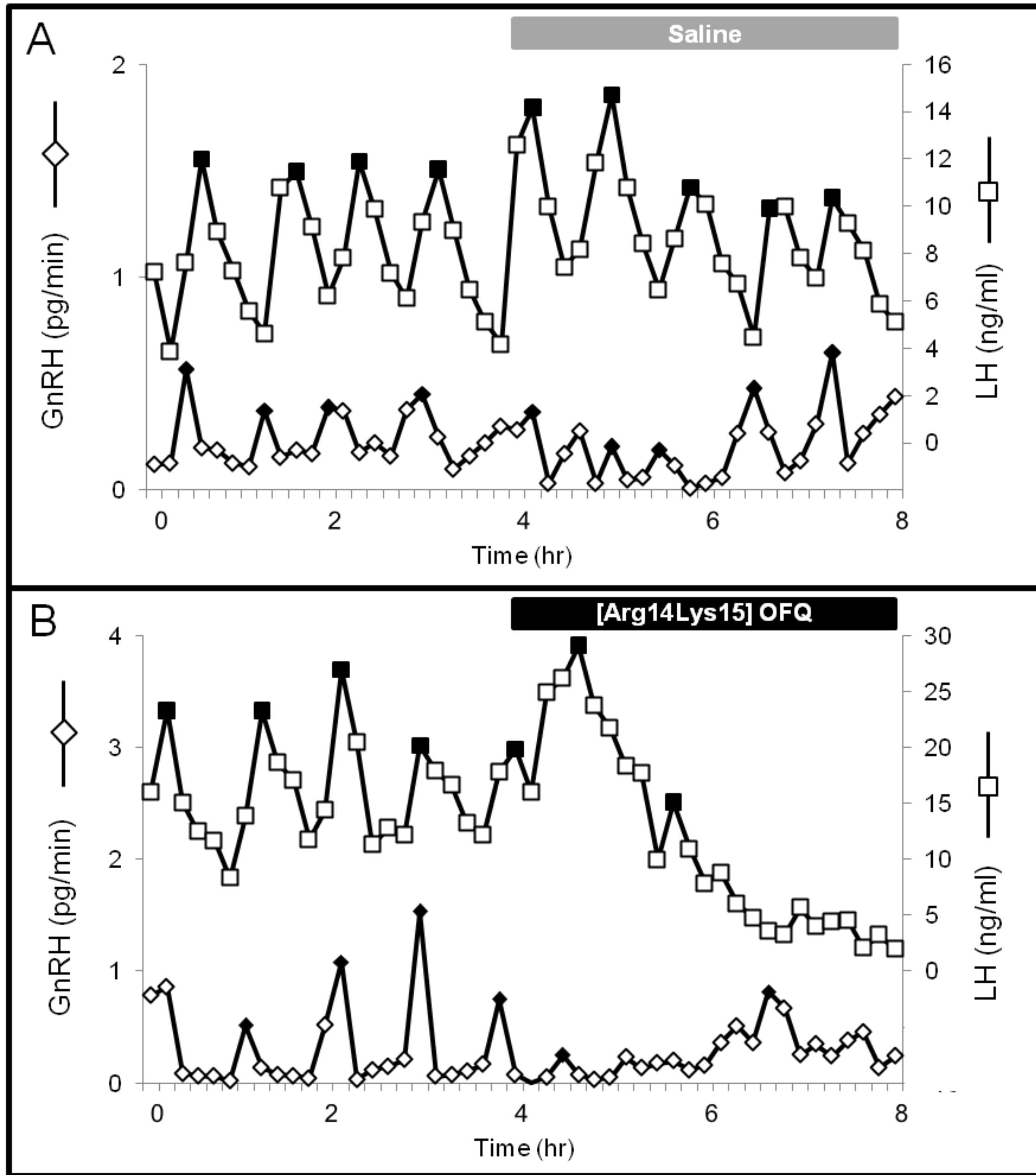


Figure 4

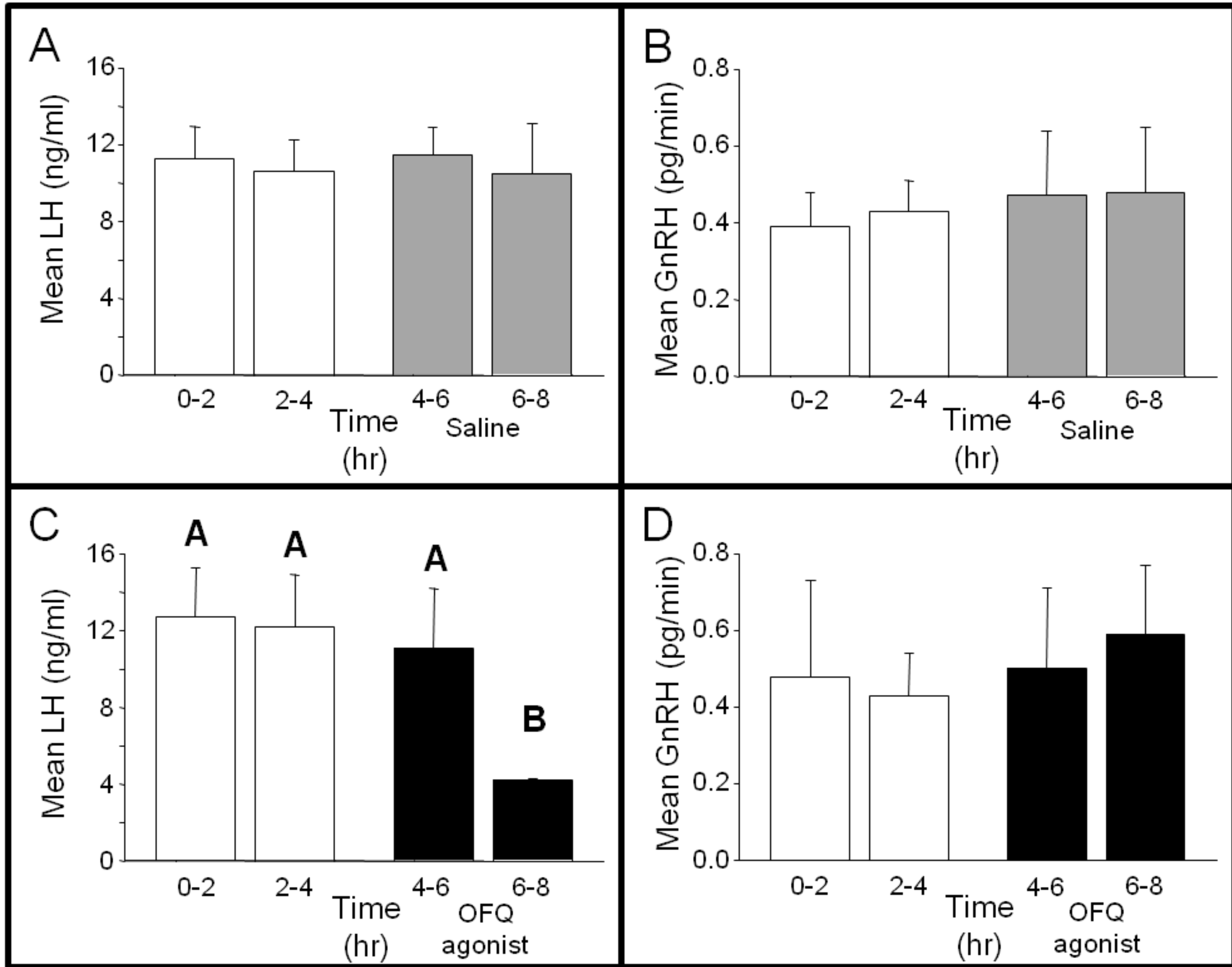


Figure 5

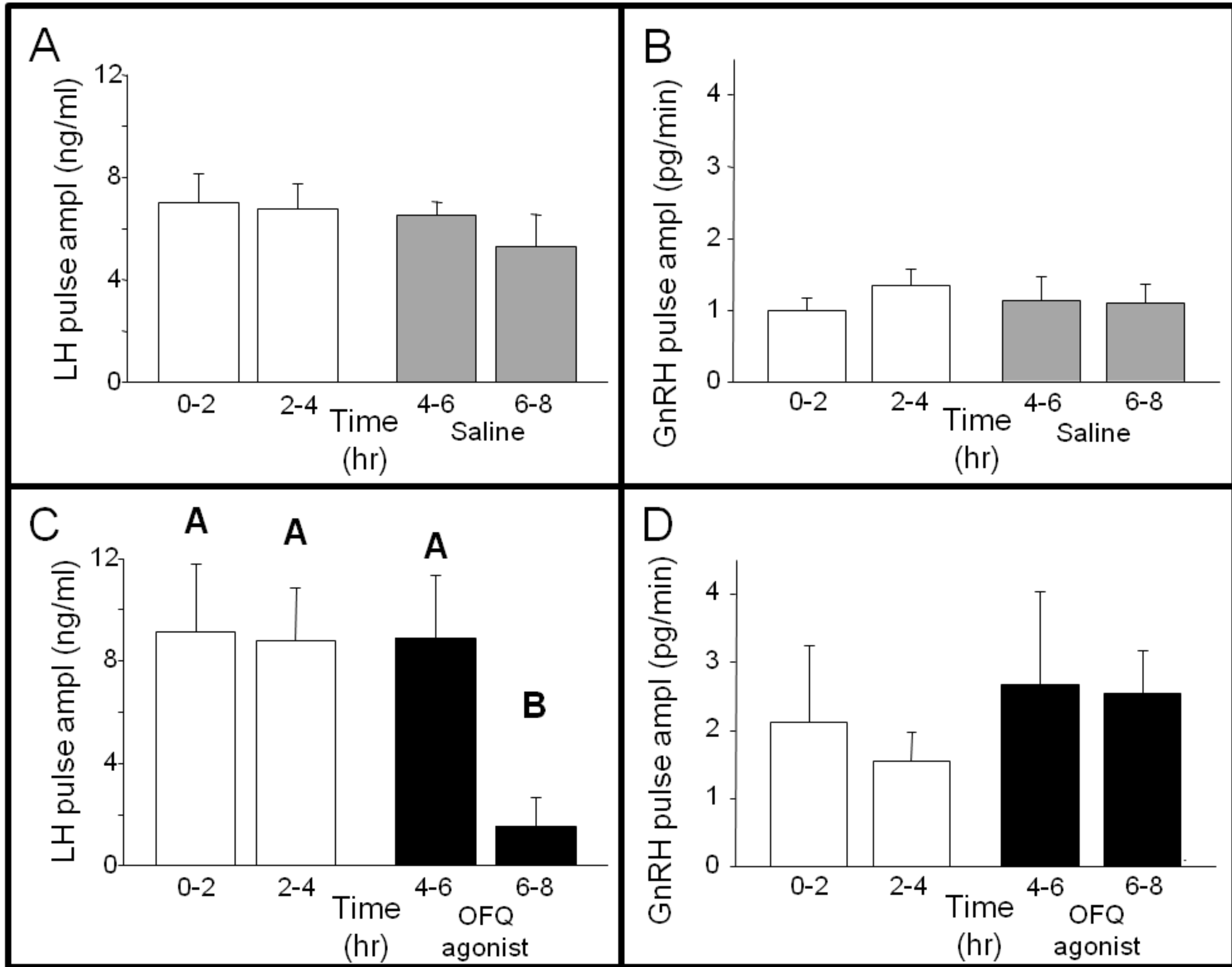


Figure 6

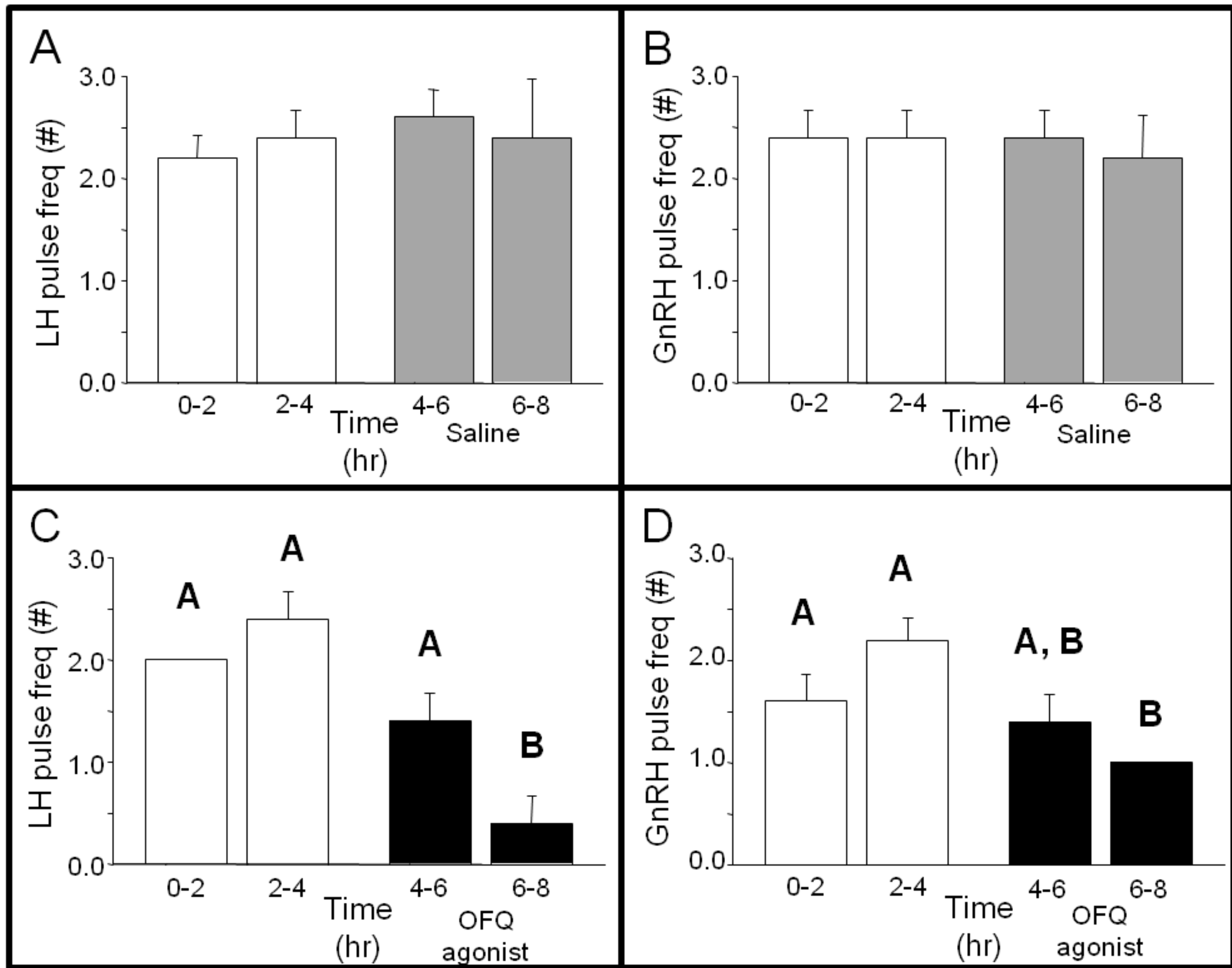
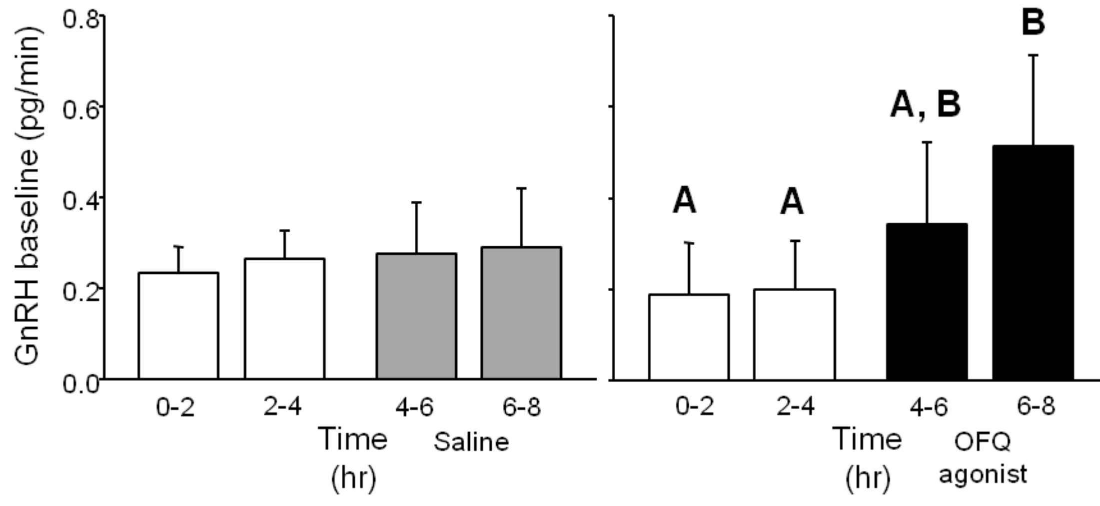


Figure 7



CHAPTER 4

Evidence that Orphanin FQ Mediates Progesterone Negative Feedback in the Ewe.

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Running Heading: OFQ mediates progesterone negative feedback in sheep

ABSTRACT

Gonadotropin-releasing hormone (GnRH) neurons are the final common pathway for neuroendocrine control of the reproductive axis. During the estrous cycle of the ewe, episodic release of GnRH, and subsequently luteinizing hormone (LH), is under the control of steroid negative feedback. Since GnRH neurons are devoid of the appropriate steroid receptors, this feedback must occur indirectly through input from other neurons. Neurons that release orphanin FQ (OFQ), a member of the opioid family, may be involved. OFQ has been shown to exist within many areas of the hypothalamus and when given centrally OFQ inhibits episodic LH secretion in ovariectomized sheep. Based on these data, we hypothesized that OFQ mediates the inhibitory effects of estradiol and/or progesterone. To test our hypothesis we examined OFQ neurons in the preoptic area (POA) and hypothalamus of luteal phase ewes for colocalization with estrogen receptor-α (ERα) and progesterone receptor. Furthermore, we examined if OFQ neurons in the arcuate nucleus (ARC) contain other neuropeptides. POA OFQ neurons did not contain ERα or progesterone receptors, while in contrast, a majority of OFQ neurons in the ARC contained both ERα and progesterone receptors. In addition, 50% of arcuate POMC neurons contained OFQ, but OFQ did not colocalize with kisspeptin, tyrosine hydroxylase or agouti-related peptide (AgRP). To test a role for endogenous OFQ, we examined the effects of an OFQ receptor antagonist, UFP-101, on LH secretion in steroid treated ewes in the breeding and non-breeding season. During the breeding season, eight adult ewes were ovariectomized and had a chronic guide tube inserted into the lateral ventricle. Ewes also received a 1cm long estradiol implant and 2 progesterone packets sc at the time of surgery. Following a recovery period of 10 days, we infused UFP-101 (30 nmole/hr) or saline (120 µl/hr) into the lateral ventricle and collected blood samples every 12 min via a jugular catheter for 6 hr. Treatments of either saline or antagonist were delivered to all sheep at random with three days between treatments. Progesterone packets were removed and treatments were repeated in a similar manner ten days later in ewes treated only with the sc estradiol implant with blood samples collected every 12 min for 5 hr. During the non-breeding season, six ovary-intact ewes received a chronic guide tube into the lateral ventricle. Following surgical recovery ewes were treated with saline or OFQ receptor antagonist at

random with three days between treatments with blood samples collected every 12 min for 4 hr. Ewes with luteal phase concentrations of progesterone showed a significant increase in LH pulse frequency during infusion of UFP-101 (4.5 ± 0.5 pulses/6hr) compared to saline infusion (2.6 ± 0.4 pulses/6hr). Ewes implanted with only estradiol or steroid intact anestrous ewes displayed no significant difference in LH pulse amplitude or frequency during infusion of UFP-101. Therefore, we conclude that OFQ mediates, at least in part, the negative feedback action of progesterone on GnRH/LH secretion in sheep.

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is the final neural pathway controlling luteinizing hormone (LH) secretion from the gonadotropes of the anterior pituitary. GnRH secretion is predominantly episodic and the amplitude and frequency of GnRH pulses are under steroid negative feedback from the ovary. During the estrous cycle in sheep, estradiol (E_2) inhibits GnRH pulse amplitude (1, 2), while progesterone suppresses GnRH pulse frequency (3, 4). The simplest explanation for control by ovarian steroids would be a direct inhibition of GnRH neurons, but GnRH neurons possess neither estrogen receptor-α (ERα) (5, 6) or progesterone receptor (PR) (7). Therefore, inhibition of GnRH release by estradiol and progesterone is mediated via afferent neurons that have yet to be completely identified.

Considerable work has implicated endogenous opioid peptides (EOP) in the negative feedback control of GnRH secretion in sheep. Infusion of an opioid antagonist increases LH pulse amplitude during the follicular phase and in estradiol-treated OVX ewes (8). However, the antagonist-induced increase in GnRH pulse amplitude is independent of E_2 (2), so it is unlikely that EOP mediate this effect of E_2 in ewes. In luteal phase ewes, an opioid antagonist increases GnRH pulse frequency in hypophyseal portal blood (9). Furthermore, administration of an EOP antagonist increases LH pulse frequency during the luteal phase and in progesterone-treated ovariectomized (OVX) ewes. The EOP dynorphin appears to mediate progesterone negative feedback for three reasons. One, greater than 90% of dynorphin neurons contain PR (10). Two, progesterone has been shown to stimulate dynorphin expression and release (11). Lastly, administration of a kappa receptor antagonist increases LH pulse frequency in luteal phase ewes (12). However, recent results raise the possibility that other neuronal systems may also play a role in progesterone negative feedback in the ewe (13).

Another EOP that could play a role in steroid negative feedback is orphanin FQ (OFQ). OFQ, also known as nociceptin, was discovered by two independent groups and binds to a G-coupled protein receptor, opioid receptor like-1 (ORL-1), that is not affected by classical EOP receptor antagonists (14, 15). OFQ and ORL-1 are found throughout the hypothalamus in rats (16, 17), humans (18) and sheep (19). In the ewe, greater than 90% of OFQ neurons in the preoptic area (POA) colocalize with GnRH, but

OFQ neurons not containing GnRH are present within the hypothalamus (19). Given its location, and because we (19) and others (20, 21) have shown that OFQ can inhibit LH secretion *in vivo*, we hypothesize that OFQ neurons contain ERα and PR and mediate steroid negative feedback in the ewe. To test this hypothesis, we used immunocytochemistry to examine whether OFQ neurons in the POA and hypothalamus of the ewe contain ERα and/or PR. Furthermore, given the high degree of steroid receptor colocalization with OFQ in the arcuate nucleus (ARC), we examined whether or not OFQ colocalized with other neuropeptides known to be found in steroid receptors-containing neurons in the ARC using dual immunofluorescence. Finally, to test a role for endogenous OFQ, we examined LH secretion following administration of an OFQ receptor antagonist in OVX ewes receiving progesterone and estradiol ($P+E_2$) or E_2 alone during the breeding season and in ovary-intact ewes during the non-breeding season.

MATERIALS AND METHODS

Animals

Adult ewes (> 3yrs) of mixed-breeding were housed indoors with lighting that simulated natural daylength and received a diet of alfalfa pellets with water *ad libitum*. Breeding season experiments were done with ewes showing regular estrous cycles (based on estrous detection with a vasectomized ram) between October and February; anestrous experiments were done in May. Reproductive status for anestrous ewes was confirmed by inspection of the ovaries for the absence of corpora lutea on the day of sacrifice. For blood collection, jugular catheters were inserted the day before blood samples (3ml) were taken; blood was collected into heparinized tubes and plasma was stored at -20°C. All procedures were approved by the West Virginia University Animal Care and Use Committee and followed NIH guidelines for use of animals in research.

Reagents

Antibodies: Rabbit antiserum against OFQ was obtained from Neuromics (Edina, MN) and mouse antisera against ERα and PR were purchased from DAKO (Carpinteria, CA) and Beckman Coulter/Immunotech (Brea, CA), respectively. Rabbit antiserum against proopiomelanocortin (POMC)

was purchased from Phoenix Pharmaceuticals, Inc. (Burlingame, CA), guinea pig antisera against mouse agouti-related peptide (AgRP) was obtained from Antibodies Australia (Melbourne, Australia), mouse antiserum against tyrosine hydroxylase (TH) was purchased from Millipore (Billerica, MA), and rabbit antiserum against kisspeptin was a gift from A. Caraty (Nouzilly, France). All of these antibodies have been validated for use in sheep (19, 22-25). In addition, omission of one or both primary antibodies and preabsorption with purified antigens served as controls (data not shown) in experiments 1 and 2.

ORL-1 antagonists: UFP-101 and JTC-801 (Tocris Bioscience, Ellisville, MO) were stored as recommended by the manufacturer and diluted to 250 nmoles/ml in 0.9% sterile saline the day before infusions.

Surgical Procedures

All procedures were performed using sterile technique under gas anesthesia (oxygen + 3% isoflurane). Ovariectomies (OVX) were performed by midventral laparotomy and a chronic lateral cerebroventricle cannula was inserted using a modification of our standard neurosurgical approach (26). After exposure of the top of the skull, an initial mark was positioned 5mm rostral and 3.5 mm lateral to bregma after which a 1cm hole was drilled at this mark to expose the dura. Four stainless steel screws were inserted in the skull surrounding the hole in order to anchor the dental acrylic. A small hole was burned in the exposed dura by brief cauterization, and a 16 gauge needle, that had been cut to a length of 1.25 inches and equipped with detachable tubing filled with sterile water, was lowered into the brain until the ventricle was pierced and flow was observed (depth of 18-22 mm). Then 1ml of a radio-opaque dye, Omnipaque 350 (Iohexol; Winthrop, NY), was slowly injected and the dorsal/ventral position of the needled adjusted to the middle of the lateral ventricle, if necessary, based on a lateral X-ray. The needle hub was plugged to prevent loss of cerebrospinal fluid and was covered with a plastic protective cap cemented in place using dental acrylic. All ewes were treated pre- and post-operatively with Penicillin Procaine G (Webster Veterinary Supply), Dexamethosone (Webster Veterinary Supply), and FluMeglumine (Webster Veterinary Supply) as previously described (26) and allowed at least 10 days for recovery.

Tissue Collection and Processing

Tissue for immunocytochemistry was collected as described previously (27). Briefly, ewes (n=3) in the mid-luteal phase of the estrous cycle were heparinized (20,000 U) and killed using an iv overdose of sodium pentabarbitol (Euthasol, Webster Veterinary, Devens, MA). Heads were removed and perfused via the carotid arteries with four liters of 2% paraformaldehyde in 0.1 M phosphate buffer (PB, pH=7.4) containing 0.1% sodium nitrite. Blocks of tissue containing the POA and the hypothalamus were then removed and stored in 2% paraformaldehyde for 24 hrs at 4°C and transferred to 20% sucrose until sectioned. Frozen coronal sections (40μm) were cut with a freezing microtome and stored in cryopreservative until the time of immunocytochemical staining.

Experiment 1: Colocalization of OFQ with ERα or PR.

Dual- immunocytochemistry for OFQ and ERα or PR was done in free floating sections as previously described (19, 25, 28); all incubations were at room temperature. On Day 1, a series of every sixth section from the POA through the ARC was washed 12x15 min in 0.1M phosphate buffered saline (PBS) and then placed in 1% H_2O_2 for 10 min followed by 4x5 min washes in PBS. Tissue was then incubated for 1 hr with 0.4% Triton-X (Sigma-Aldrich, St. Louis, MO) in 20% normal goat serum (NGS) in PBS and then incubated with 1:1000 ERα antiserum or 1:50 PR antiserum for 17 hrs and 40 hrs, respectively. Thereafter, tissue was rinsed with PBS 4x5 min and biotinylated goat anti-mouse antibody (Vector Laboratories, Burlingame, CA) at 1:500 and Vectastain ABC-elite (Vector Laboratories) at 1:500, both in 0.4% Triton-X in 4% NGS, were applied sequentially for 1 hr each with 4x5 min washes of PBS between incubations. Sections were then placed in a 3, 3'-diaminobenzidine tetrahydrochloride (DAB)-nickel solution (10 mg DAB [Sigma-Aldrich] and 2 ml of 2% nickel sulfate in 50 ml PB with 20 μl 30% H_2O_2 added just before incubation) for 10 min. Sections were washed in PBS 4x5 min and then incubated for 10 min in 1% H_2O_2 followed by 4x5 min washes in PBS. After incubation for 40 hrs with 1:2500 OFQ antiserum in 0.4% Triton-X in 4% NGS, tissue was rinsed with PBS 4x5 min and biotinylated goat anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) at 1:500 and Vectastain ABC-elite (Jackson ImmunoResearch) at 1:500, both in 0.4% Triton-X in 4% NGS, were applied sequentially for 1 hr each

with 4x5 min washes of PBS between incubations. Sections were then incubated in DAB without nickel enhancement, washed 4x5 min in PB, and mounted on Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA). Sections were dehydrated using a series of increasing alcohol baths and coverslipped using DPX Mounting Medium (Electron Microscopy Sciences, Hatfield, PA).

Experiment 2: Colocalization of OFQ with AgRP , TH, POMC and kisspeptin.

When the primary antisera were of the same species, immunofluourescence was performed using biotin tyramide amplification and conjugated second antibody detection to visualize the first and second antigen, respectively (23), otherwise amplification was not needed. For AgRP and TH visualization with OFQ, a series of tissue sections from the middle ARC was washed in 0.1M PBS, incubated in 0.1% Triton-x containing 4% and 20% normal goat serum, respectively, followed by a 17 hr incubation in PBS containing 4% normal goat serum with rabbit anti-OFQ (1:2500) and guinea pig anti-AgRP (1:1000) or mouse anti-TH (1:2000) at room temperature. The next day after washing tissue 4x5 min in PBS (from here on tissue was covered with tin foil to prevent light exposure), secondary antibody for OFQ (goat anti-rabbit Alexa 555; 1:100) and AgRP (goat anti-guinea pig Alexa 488; 1:100) or TH (goat anti-mouse Alexa 488; 1:100) in 0.4% Triton-x and 4% normal goat serum in PBS was applied to tissue for one hour. For POMC and kisspeptin, a series of sections through the middle ARC was washed in 0.1M PBS, incubated in 0.4% Triton-x in 20% normal goat serum for an hour, followed by a 17 hr incubation in rabbit anti-OFQ (1:20000) or rabbit anti-kisspeptin (1:200,000) at room temperature. The following day tissue was washed in 0.1M PBS 4x5 min, followed by one hour incubation in biotinylated goat anti-rabbit (1:500) in 0.4% Triton-x in 4% normal goat serum in PBS. Tissue was washed 4x5 min followed by a 1 hr incubation in ABC-elite solution (1:500) for 1 hr, then washed 4x5 min in PBS. Tissue was then incubated in biotinylated tyramine (1:250) for 10 min followed by 4x5 min washes in PBS. Then Alexa 555-streptavidin (red; 1:100 for OFQ) or Alexa 488-streptavidin (green; 1:100 for kisspeptin) in PBS. Tissue was then washed 4x5 min in PBS followed by a 17 hour incubation with rabbit anti-POMC (1:16000) or rabbit anti-OFQ (1:1000) in 0.4% Triton-x with 4% normal goat serum containing PBS. The following day tissue was washed 4x5 min in PBS and then incubated with goat anti-rabbit Alexa 488

(green; 1:100 for POMC) or goat anti-rabbit Alexa 555 (red; 1:100 for OFQ) in 0.4% Triton-x with 4% normal goat serum containing PBS for 30 min. After a final wash 4x5 min in PBS, all tissue was mounted on glass slides, coverslipped using Gelvatol mounting medium and stored in the dark at 4C.

Experiment 3a: Effects of ORL-1 antagonists in OVX+P+E_2 ewes.

Eight breeding season ewes were OVX and a guide tube was inserted into one lateral ventricle. At the time of OVX, ewes were given a 1cm E_2 implant sc (29) and two progesterone packets sc (30). Starting approximately two weeks later, ewes received a lateral ventricle infusion of 0.9% saline (120 µl/hr), UFP-101 (30 nmoles/hr), or JTC-801 (30 nmoles/hr). Doses of antagonists were based on data from preliminary experiments (data not shown). The day before infusion, one jugular vein was catheterized and ewes were placed in a narrow pen so that they could lie down, but not turn around; an infusion line was inserted just before the start of blood collections that extended from the tip of the guide tube to a portable microinfusion pump strapped to the back of each animal. Blood was collected every 12 min for 6 hrs with infusion starting immediately prior to and continuing throughout the collection period. Each ewe received all three treatments in random order, separated by 3 days between each treatment. Gentamicin (Webster Veterinary Supply; Devens, MA) was given im prophylactically at the end of each icv infusion.

Exp 3b: Effects of an ORL-1 antagonist in OVX+E_2 ewes.

At the end of experiment 3a, progesterone packets were removed, but E_2 implants were left in place. Two weeks later, all sheep received a lateral ventricle infusion of saline (120 µl/hr) or UFP-101 (30 nmoles/hr) with 3 days between treatments in a crossover design. Since results did not differ between antagonists, we chose to only administer UFP-101 for experiment 3b. Blood samples were collected every 12 min for 5 hr with infusion starting immediately prior to and continuing throughout the collection period.

Exp 4: Effects of an ORL-1 antagonist in ovary-intact, anestrous ewes.

Starting approximately two weeks after insertion of a lateral ventricle guide tube, 6 ovary-intact, anestrous ewes were infused with saline (120 µl/hr) or UFP-101 (30 nmoles/hr), into the lateral ventricle

with 3 days between treatments in a crossover design. Blood samples were collected every 12 min for 4 hrs with infusion starting immediately prior to and continuing throughout the collection period.

Data Analysis

Immunocytochemistry: Hemisections from a series of every sixth section from the POA and hypothalamus were used for immunocytochemical detection of OFQ, ERα and PR. OFQ was visualized using unenhanced DAB as the chromogen (brown reaction product), while ERα and PR were visualized using nickel-enhanced DAB (blue-black reaction product). Dual-labeled cells were defined as those in which a blue-black nucleus was surrounded by a brown cytoplasm as seen in the same plane of focus. Immunopositive cells were counted manually using a light microscope. A series of sections through the middle ARC was used for immunfluourecent detection of OFQ with AgRP, TH, POMC or kisspeptin. Immunoflurescent cells were visualized by confocal z-stacks of 1µm thick optical sections and were captured using a Zeiss LSM 510 (Hornwood, NY) confocal microscope at 63x magnification.

Assays: LH concentrations were measured as previously described (Whisnant and Goodman, 1988) in duplicate with a RIA using 100–200 µl of plasma and reagents provided by the National Hormone and Peptide Program (Torrance, CA). LH assay sensitivity averaged 0.12 ng/tube (NIH S24) with intra- and interassay coefficients of variation being 15.6% and 28.9%, respectively.

Statistics: Pulses were identified using previously described criteria (Goodman and Karsch, 1980) and pulse frequency, pulse amplitude, and mean LH concentrations determined for each treatment period. Pulse frequency for Exp 3a was analyzed using Friedman Repeated Measures Analysis of Variance on Ranks. Pulse frequency for Exp 3b and 4 was analyzed using Wilcoxon signed ranks test. Mean LH and LH pulse amplitude were analyzed using a one-way ANOVA in Exp 3a. A paired t-test was used to analyze mean LH and LH pulse amplitude in experiments 3b and 4. Differences were considered to be significant at $p<0.05$.

RESULTS

Experiment 1: Do OFQ neurons in the ovince hypothalamus possess ERα or PR?

OFQ, ERα, or PR immunoreactivity was examined in several hypothalamic areas and the POA as previously described (6, 7, 19). OFQ immunoreactive cells were seen in all areas examined with the most dense staining occuring in the POA, periventricular nucleus, anterior hypothalamic area (AHA) and rostral to middle ARC (Fig 1A, B, and C). ERα (Fig 1A, B, and D) and PR (Fig 2A, B, and D) immunoreactive nuclei were seen in all areas examined with the greatest density in the POA. In the rostral, middle, and caudal levels of the ARC, between 80-100% of OFQ cells colocalized ERα (Fig 1E), and 60-80% colocalized PR (Fig 2E). In the AHA, 75% of OFQ positive cells coexpressed ERα (Fig 1E) while 60% coexpressed PR (Fig 2E). In the ventromedial nucleus, 30% and 44% of OFQ positive cells contained ERα (Fig 1E) and PR (Fig 2E), respectively. In contrast, less than 2% of OFQ cells in the POA or PVN colocalized ERα or PR.

Experiment 2: Does OFQ colocalize with POMC, kisspeptin, AgRP or TH in the ARC of sheep?

Since the highest percent colocalization of OFQ and ERα/PR was within the ARC, we tested the hypothesis that OFQ would colocalize with other ARC neuropeptides. Approximately 50% of POMC neurons in the middle ARC also contain OFQ (Fig 3A-C). OFQ did not colocalize with kisspeptin (Fig 3D), AgRP (Fig 3E) or TH (Fig 3F) in the ovine ARC.

Experiment 3a: Can infusion of OFQ receptor antagonists increase LH secretion in OVX+P+E_2 ewes?

Given that OFQ can inhibit LH secretion in OVX ewes (Foradori et al., 2007), we tested the hypothesis that infusion of an OFQ receptor antagonist increases LH secretion in OVX ewes treated with with luteal phase concentrations of progesterone and E_2. Figure 4 displays the pulse profiles from one ewe that received saline (Fig 4A), UFP-101 (Fig 4B) and JTC-801 (Fig 4C). Mean LH (Fig 5A) and LH pulse amplitude (Fig 5B) were not significantly increased with infusion of UFP-101 (1.93 ± 0.22 and 1.28 ± 0.21 ng/ml, respectively) or JTC-801 (2.26 ± 0.37 and 1.30 ± 0.22 ng/ml, respectively) compared with saline infusion (2.00 ± 0.50 and 1.01 ± 0.17 ng/ml, respectively). LH pulse frequency (Fig 5C) for UFP-101 (4.50 ± 0.53 pulses/6hrs) and JTC-801 (4.86 ± 0.28 pulses/6hrs) was significantly increased compared to infusion of saline ($2.62 \pm .40$ pulses/6hrs).

Experiment 3b: Does UFP-101 increase LH secretion in OVX+E_2 ewes?

Since we observed similar results with UFP-101 and JTC-801 in experiment 3a, we conducted experiment 3b and 4 using only UFP-101. OVX+E_2 ewes infused with UFP-101 did not differ significantly from saline-infused OVX+E_2 ewes with regard to mean LH (Fig 6A; 4.06 $\pm$ 0.62 ng/ml and 5.14 $\pm$ 0.69 ng/ml, respectively), LH pulse amplitude (Fig 6B; 3.82 $\pm$ 1.11 ng/ml and 3.03 $\pm$ 1.01 ng/ml, respectively) or LH pulse frequency (Fig 6C; 4.12 $\pm$ 0.24 pulses/5hrs and 3.25 $\pm$ 0.56 pulses/5hrs, respectively).

Experiment 4: Does UFP-101 increase LH secretion in ovary-intact anestrous ewes?

In ovary-intact anestrous ewes, mean LH (Fig 7A) during infusion of UFP-101 (1.86 $\pm$ 0.37ng/ml) did not differ significantly from that during infusion of saline (1.35 $\pm$ 0.11ng/ml). Infusion of UFP-101 had no significant effect on frequency (Fig 7C; 3.59 $\pm$ 1.84 pulses/4 hr) compared to saline-infusion (1.67 $\pm$ 0.37 pulses/4 hr). Given the paucity of LH secretion of ovary-intact anestrous ewes during a 4hr sampling period, we were unable to statistically compare mean LH pulse amplitude between UFP-101 infused ewes (Fig 7B; 1.17 $\pm$ 0.34 ng/ml) and saline infused ewes (0.69 $\pm$ 0.04 ng/ml).

DISCUSSION

These data provide evidence for an important role of OFQ in the negative feedback control of GnRH/LH secretion in the ewe. Our observation that most OFQ neurons in the ARC contain ERα and PR fits with the idea that OFQ could mediate hypothalamic actions of ovarian steroids on LH secretion. Furthermore, administration of an OFQ receptor antagonist into the lateral ventricle of OVX+P+E_2 ewes increased LH pulse frequency. In contrast, no effect was observed in OVX+E_2 or ovary-intact anestrous ewes, which solidifies a role for endogenous OFQ in mediating progesterone negative feedback.

The distribution of OFQ-containing neurons throughout the POA and hypothalamus herein is similar to that seen previously in sheep (19), as well as in rats (16) and humans (18). Also, the distribution of ERα- and PR-containing cells also matches previous reports in the ewe (7, 31). Our finding that OFQ-containing neurons in the POA do not express ERα or PR is consistent with an observation that, within the POA, OFQ is coexpressed predominantly in GnRH neurons (19) and that GnRH neurons do not express either steroid receptor (5, 6). Therefore, steroid negative feedback most

likely is not exerted directly through OFQ neurons located in the POA. In contrast, a relatively high percentage of OFQ neurons in various hypothalamic regions contain ERα and PR, with the highest colocalization in the ARC. This high percentage of ARC OFQ neurons containing ERα and PR (ERα, 80-100%; PR, 60-80%) is in contrast to many other steroid receptor containing neural populations in the ARC such as those containing AgRP (ERα, 3-10%; PR, 15%), dopamine (ERα, 15%; PR, 20%) and POMC (ERα, 15-20%; PR, 28%) neurons (6, 32-34), but quite similar to the kisspeptin population of the ARC (ERα and PR, 95%;(35)).

Given the variety of steroid receptor containing neurons in the ARC, we investigated if ARC OFQ neurons coexpressed other neuropeptides that are also colocalized with ERα and PR. Within the ARC, we did not observe colocalization of OFQ with AgRP, dopamine (using TH as a marker) or kisspeptin. However, we did observe that approximately 50% of ARC POMC neurons coexpress OFQ. Based on this, the percentage of OFQ neurons containing steroid receptors is slightly higher than might be expected based on reports in the rat POMC/ERα/PR colocation (15%; (36)) and sheep (15-28%; (6, 34)). This difference most likely reflects the different antibodies utilized in each experiment because we used an antibody against the preprohormone, while others have used markers for cleaved products of POMC (*i.e.,* β-endophin and α-MSH). Furthermore, since close appositions from POMC neurons have been observed on GnRH neurons throughout the ovine hypothalamus (12) and the high degree of colocalization of POMC with OFQ, it is possible that this opioid could have direct input to GnRH neurons.

The use of opioid antagonists has helped define a role for EOP in mediating steroid negative feedback on GnRH/LH secretion in several species. Administration of naloxone, a nonspecific EOP receptor antagonist, increased LH secretion in the follicular and luteal phase of both women (37) and sheep (38). Furthermore, although ineffective in OVX ewes, WIN-444431, a long-acting nonspecific opioid antagonist, increases LH secretion in OVX ewes treated with E_2 and progesterone (8). Since these initial studies it has been shown that dynophin is a key factor for progesterone negative feedback in the ewe (12). However, we recently reported that local administration of progesterone to the caudal ARC, targeting dynorphin neurons, was not sufficient to suppress LH secretion in OVX ewes, while local

administration of a PR antagonist, RU486, to the ARC increased LH secretion in ewes that received peripheral progesterone treatment (13). Therefore, we suggested that dynorphin alone is not sufficient to mediate all the effects of progesterone on GnRH/LH secretion and that other neurons, presumably within the medial basal hypothalamus because of the local action of RU486, are also likely to contribute to progesterone negative feedback.

Evidence of a hypothalamic role for OFQ in control of GnRH secretion continues to grow. The first evidence of this was reported in the guinea pig where OFQ hyperpolarized GnRH neurons within the medial basal hypothalamus (39). Since then it has been shown *in vivo* that central administration of OFQ inhibits LH secretion (20, 40), GnRH concentrations in push-pull perfusates (20, 40) and GnRH pulse frequency in hypophyseal portal blood (41). OFQ-positive neurons containing ERα and PR could mediate the effects of steroid negative feedback on GnRH/LH secretion. We observed an increased LH pulse frequency with infusion of an OFQ receptor antagonist into the lateral ventricle of OVX ewes with luteal phase concentrations of E_2 and progesterone. Furthermore, an alternative source of steroid production has emerged with the hypothesis that estradiol stimulates central production (neurosteroidogensis) of progesterone (42, 43), which could account for actions of estradiol at the level of the brain. However, we did not observe a significant effect of the OFQ receptor antagonist on LH secretion in OVX + E_2 ewes or intact, anestrous ewes. Therefore, we conclude it is unlikely that OFQ is involved in estradiol-negative feedback, either directly through ERα or indirectly through neurosteroidogenesis, but can mediate steroid negative feedback of peripheral progesterone on GnRH/LH secretion in the ewe.

ORL-1 mRNA is detectable in the medial preoptic area, SON, PVN, VMN and ARC (18), but limited data exists for steroid effects on OFQ or its receptor. One study in rats reports an increase in ORL-1 mRNA following treatment of rats with either E_2 or E_2 + P, while changes in peptide were only observed in E_2 + P treated rats (44). Therefore, it might be possible that estradiol increases ORL-1 expression while progesterone increases OFQ expression. This would account for the increase in LH pulse frequency in ewes with luteal phase concentrations of progesterone and estradiol given that the

combination of these steroids results in increased endogenous OFQ release that is blocked by our infusion of UFP-101. However, experiments with steroid effects on OFQ and ORL-1 expression in species other than rat remain to be done. Furthermore, given the colocalization of OFQ and GnRH in the POA (19) and POMC in the ARC (herein) and since OFQ has been shown to inhibit both GnRH and POMC neurons in the medial basal hypothalamus of guniea pigs (39), we propose an autocrine action of OFQ. This would help explain the sychronized GnRH pulsatile secretion in imortalized GnRH cells (45), GnRH neurons from nasal explants (46), and GnRH neurons *in vivo*; however, it remains to be determined which neurons (*i.e.,* GnRH and/or POMC neurons) contain ORL-1. In summary, we show that OFQ neurons colocalize with both ERα and PR in multiple areas of the hypothalamus as well as with POMC neurons in the ARC. Furthermore, we show that antagonizing the receptor for OFQ increases LH pulse frequency in progesterone-treated, but not estradiol-treated, ewes. Thus, our data support a role for OFQ in progesterone negative feedback on LH secretion in the ewe.

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FIGURE LEGENDS

Figure 1: Panel A and B: Photomicrographs of OFQ immunoreactive cells (brown cytoplasm) and ERα (blue-black nuclei) in the ovine ARC. Inset of Panel A is shown at higher magnification in Panel B. Panel C: Mean (± SEM) number of OFQ positive cells in the POA and various hypothalamic areas of mid-luteal phase ewes. Panel D: Mean (± SEM) number of ERα positive nuclei in the POA and various hypothalamic areas of mid-luteal phase ewes. Panel E: Percentage of OFQ neurons that colocalize with ERα in the POA and various hypothalamic areas. Red arrows indicate OFQ neurons colocalized with ERα. 3V = third ventricle. Produced in collaboration with our colleagues at The University of Michigan.

Figure 2: Panel A and B: Photomicrographs of OFQ immunoreactive cells (brown cytoplasm) and PR (blue-black nuclei) in the ovine ARC. Inset of Panel A is shown at higher magnification in Panel B. Panel C: Mean (± SEM) number of OFQ positive cells in the POA and various hypothalamic areas of mid-luteal phase ewes. Panel D: Mean (± SEM) number of PR positive nuclei in the POA and various hypothalamic areas of mid-luteal phase ewes. Panel E: Percentage of OFQ neurons that colocalize with PR in the POA and various hypothalamic areas. Red arrows indicate OFQ neurons colocalized with PR. 3V = third ventricle. Produced in collaboration with our colleagues at The University of Michigan

Figure 3: Panel A-F: Confocal images (1 μm optical section) through the ovine ARC processed for dual-immunoflourescence detection of OFQ (red) and either AgRP, kisspeptin, TH, or POMC (green). Single-labeled neurons of OFQ (Panel A) and POMC neurons (Panel B) are identified using arrowheads and merged image for dual-labeled OFQ/POMC neurons (Panel C) with colocalized neurons indicated with arrows. Merged images in bottom row show OFQ and kisspeptin (Panel D), AgRP (Panel E) or TH (Panel F). Produced in collaboration with our colleagues at The University of Michigan

Figure 4: Panel A-C: LH profiles from Experiment 2 of ewe #258 that was OVX and treated sc with progesterone and estradiol. LH profiles during infusion of saline (Panel A), UFP-101 (Panel B) and JTC-801 (Panel C) are shown with pulses identified using a closed circle.

Figure 5: Group means (± SEM) for mean LH (Panel A), LH pulse amplitude (Panel B) and LH pulse frequency (Panel C) for saline (open bars), UFP-101 (black bars) and JTC-801 (grey bars) infused OVX+P+E ewes (n = 8). Significant difference ($p < 0.05$) from saline group is indicated by *.

Figure 6: Group means (± SEM) for mean LH (Panel A), LH pulse amplitude (Panel B) and LH pulse frequency (Panel C) for saline (open bars) and UFP-101 (black bars) infused OVX+E ewes (n = 7).

Figure 7: Group means (± SEM) for mean LH (Panel A), LH pulse amplitude (Panel B) and LH pulse frequency (Panel C) for saline (open bars) and UFP-101 (black bars) infused ovary-intact anestrous ewes (n =6).

FIGURES

Figure 1

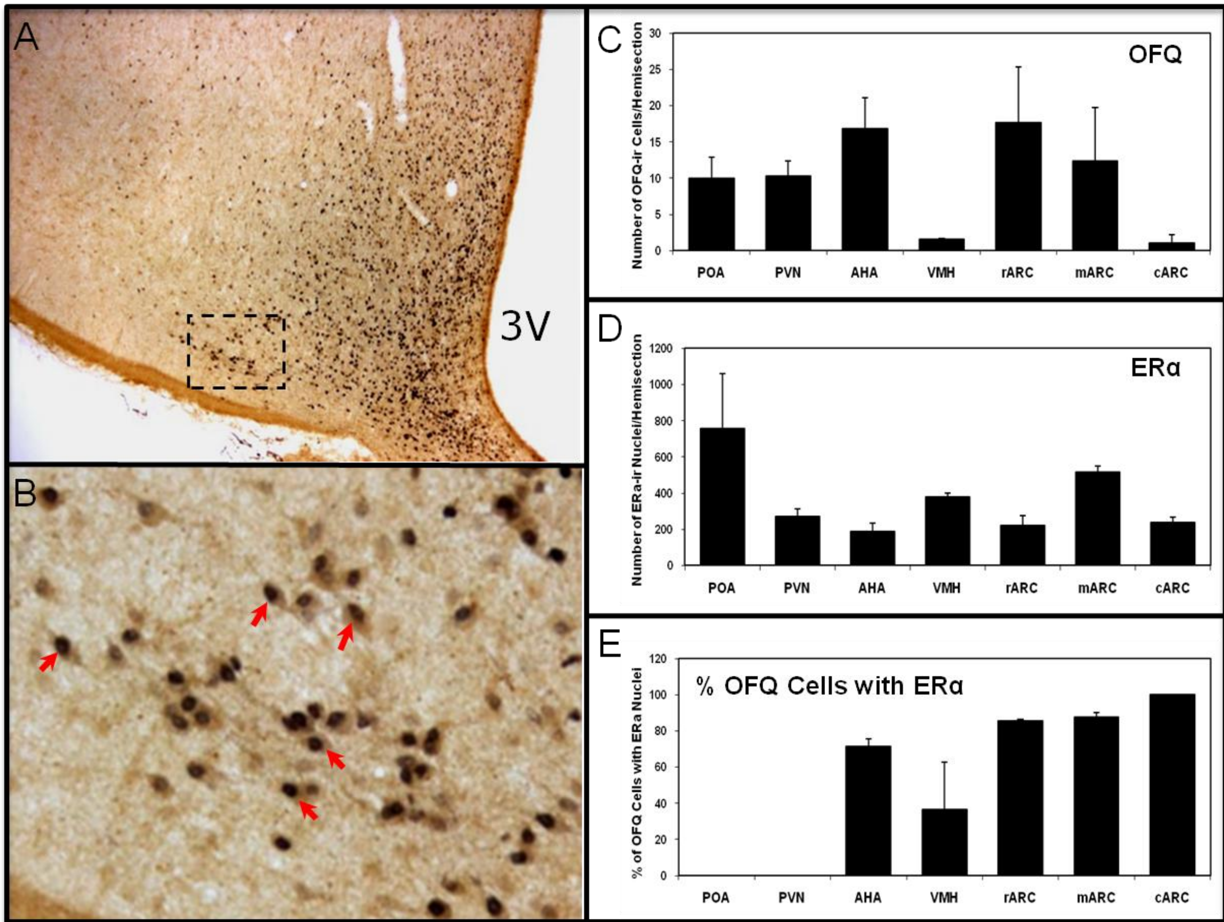


Figure 2

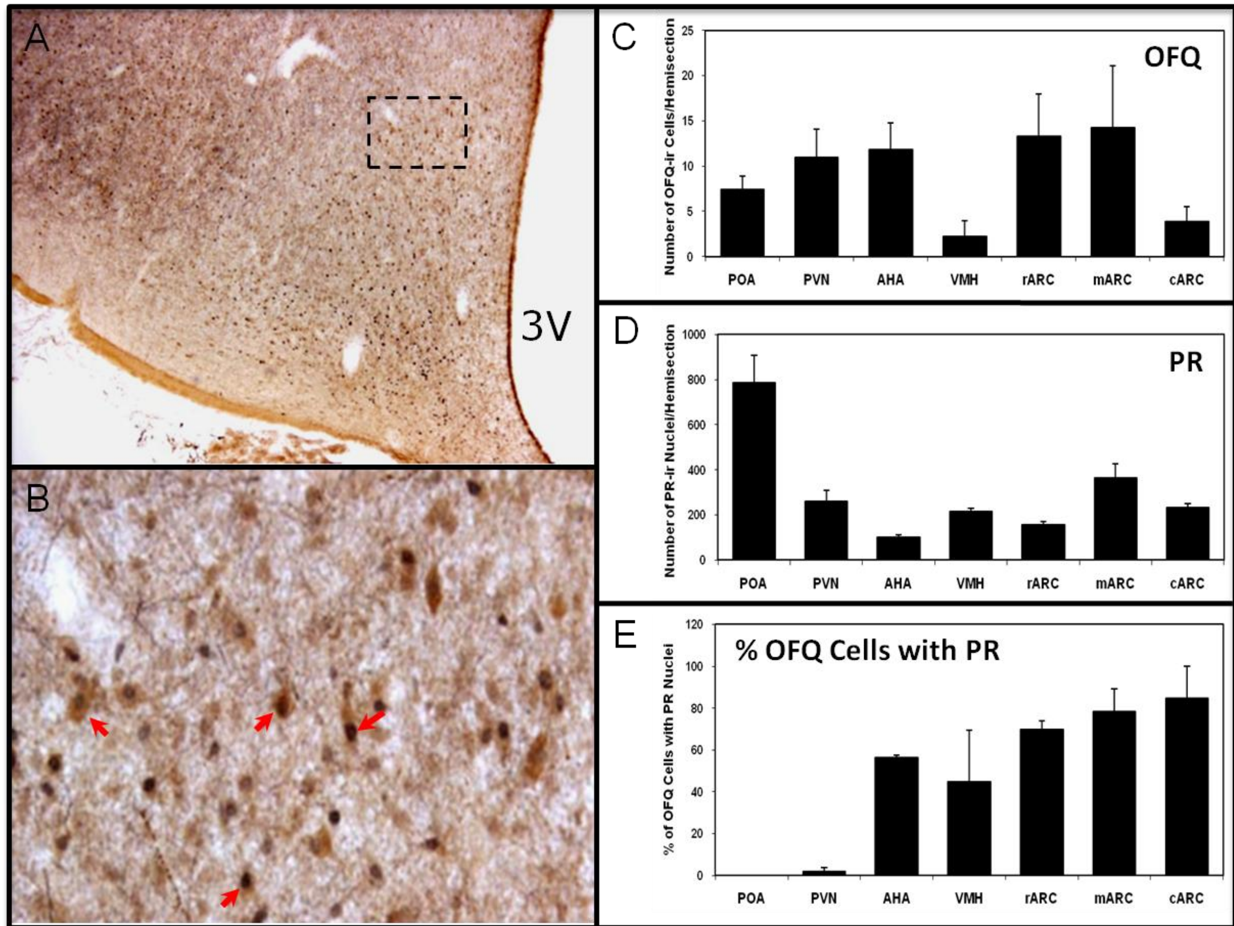


Figure 3

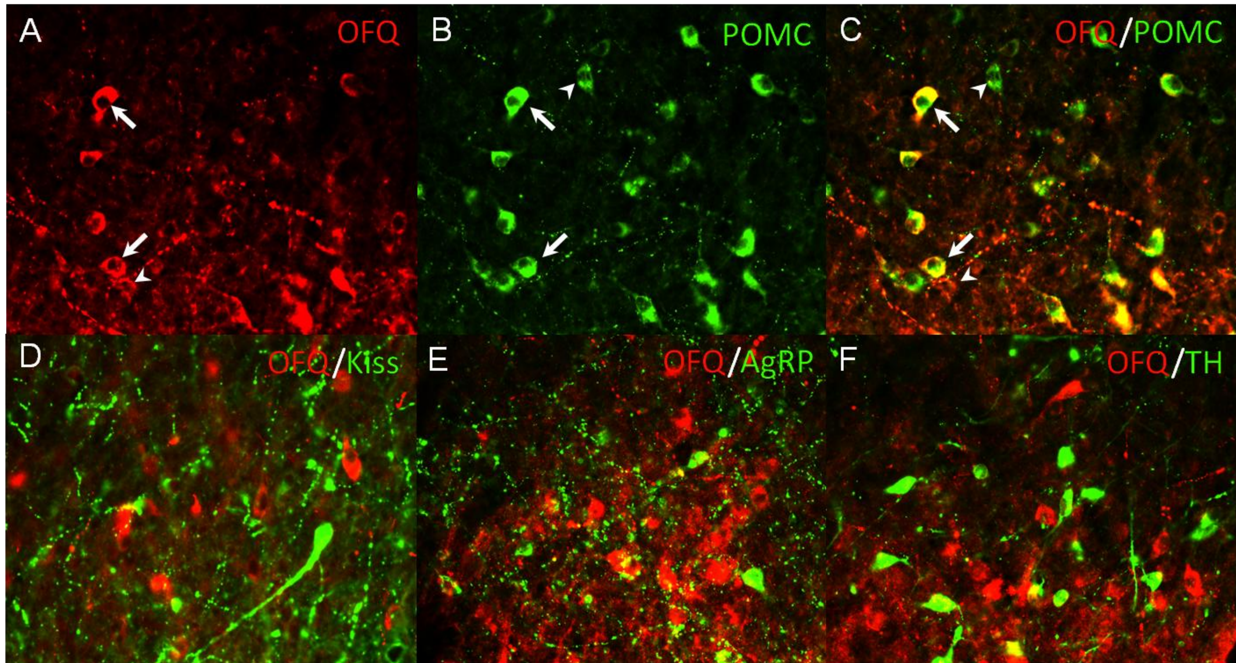


Figure 4

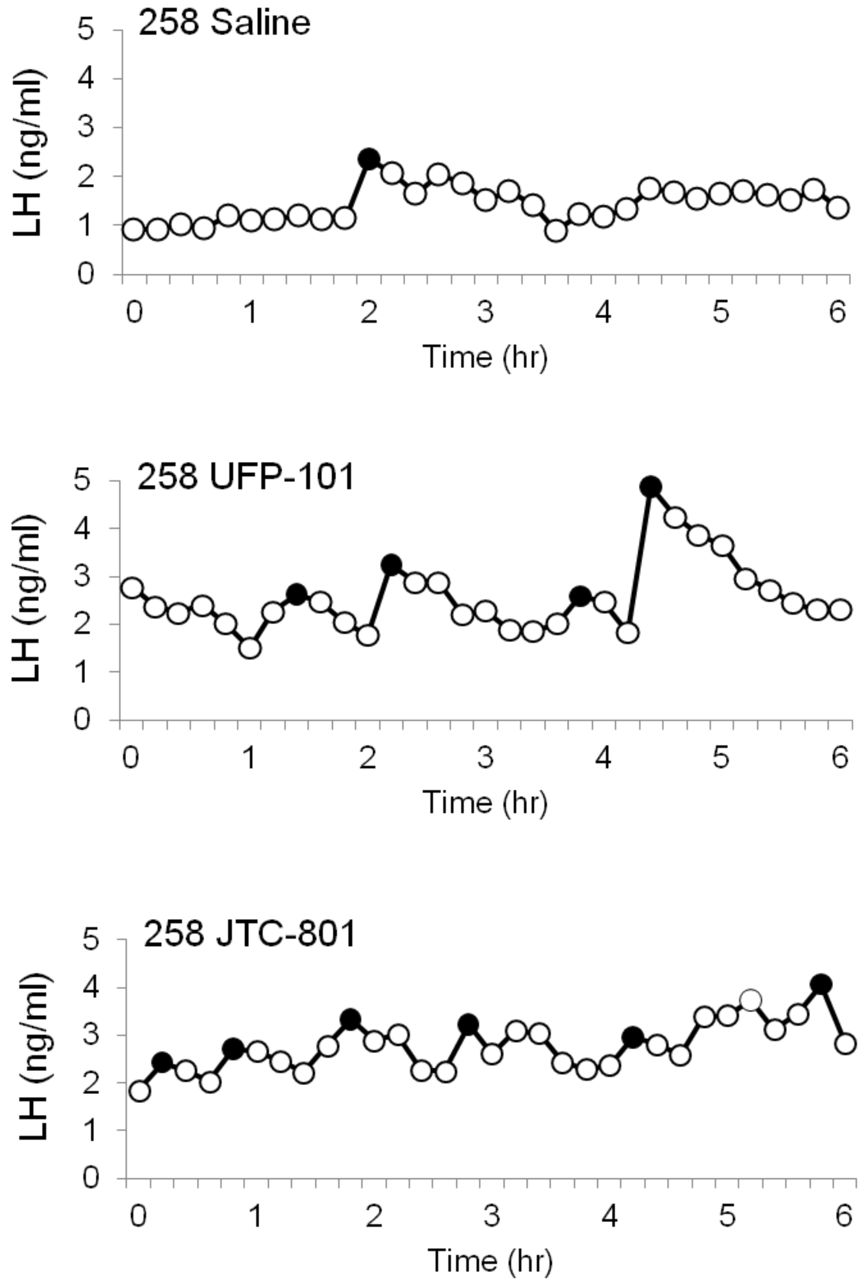


Figure 5

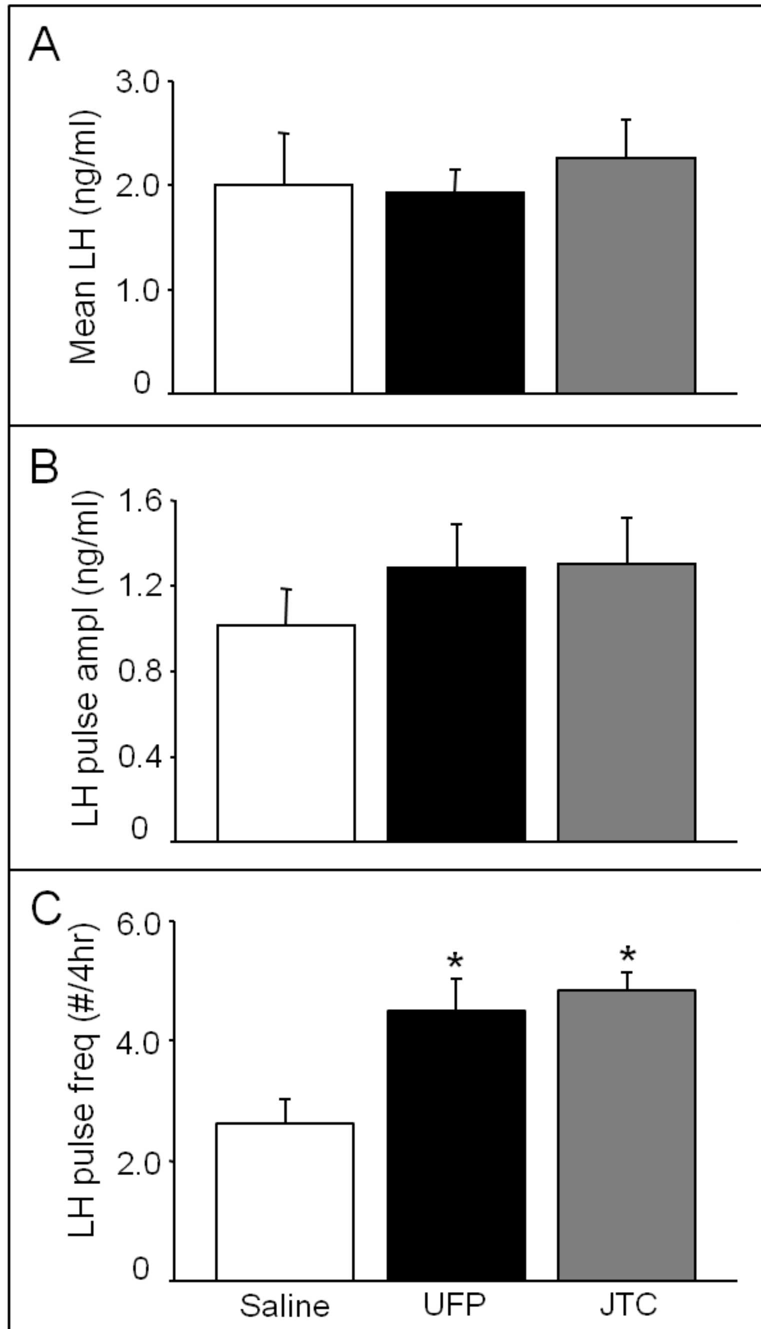


Figure 6

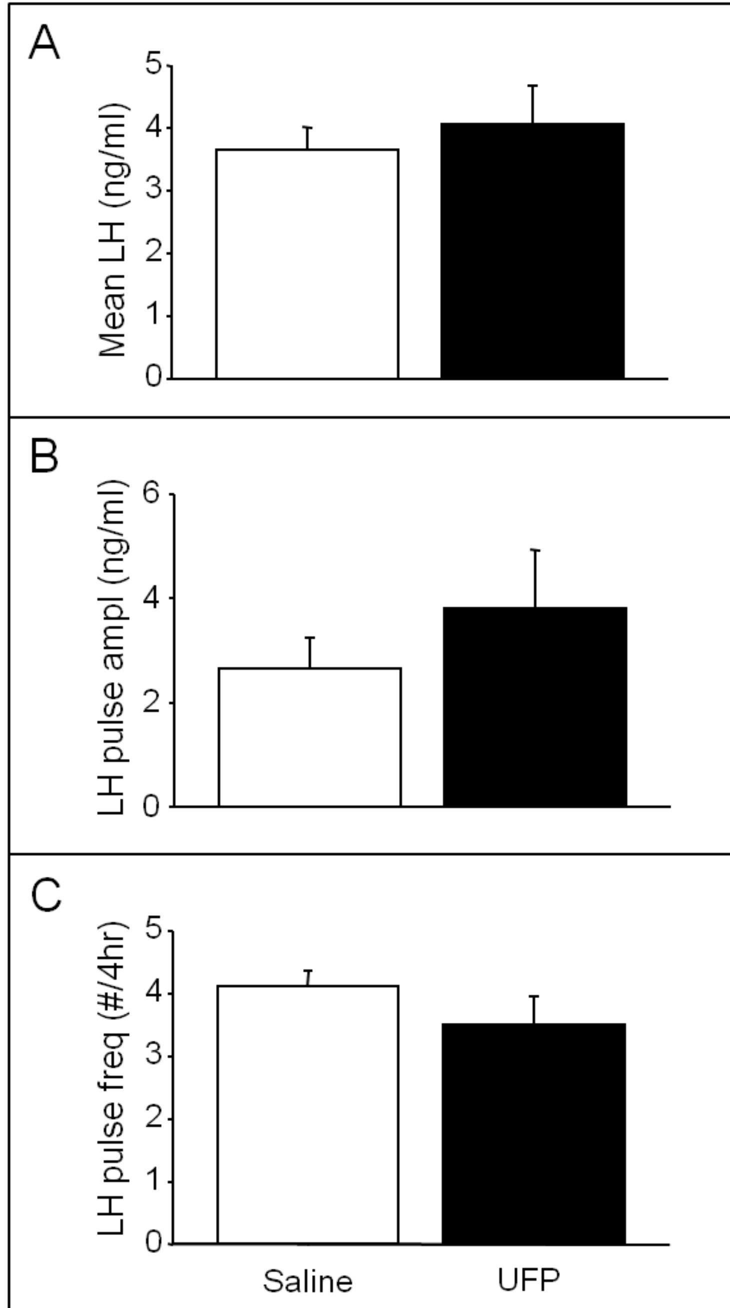
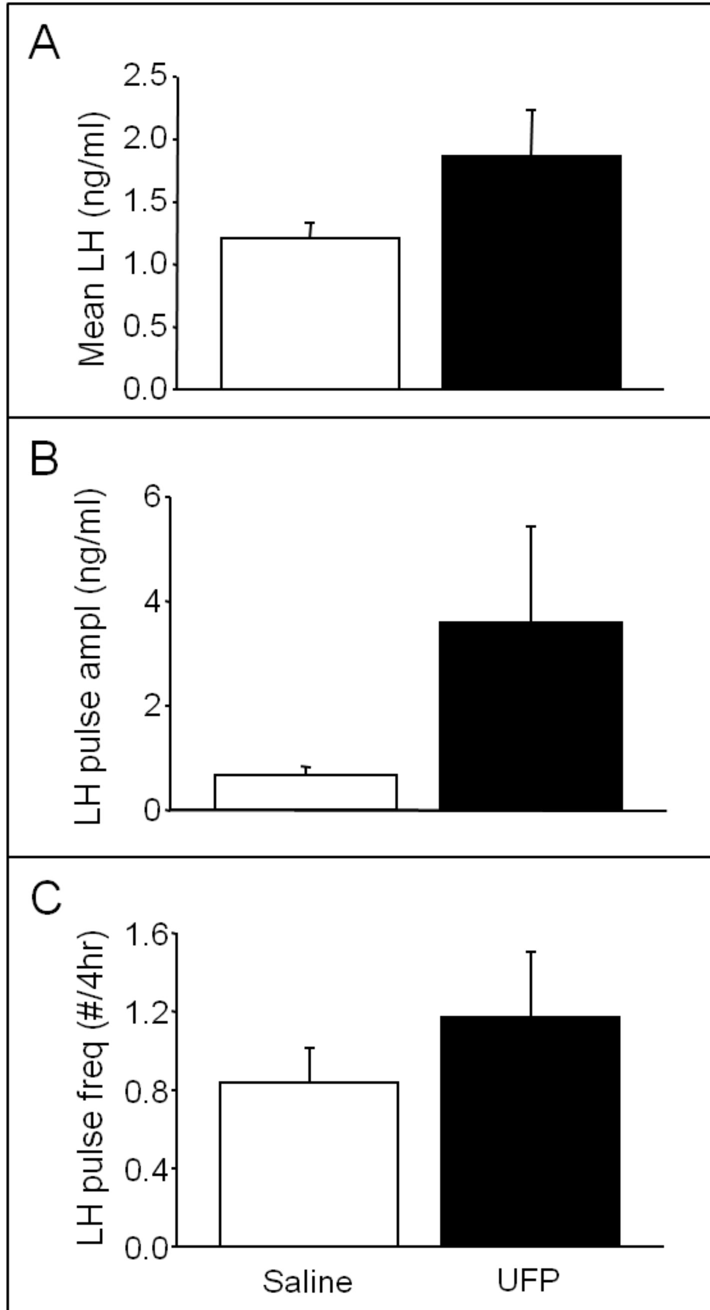


Figure 7



CHAPTER 5

GENERAL DISCUSSION

The results presented in the preceding three chapters provide strong evidence of a role for kisspeptin, NKB and OFQ in the control of GnRH/LH secretion in the ewe. Therein we showed a greater number of kisspeptin, but not NKB, positive cell bodies in the ARC in postpubertal compared to prepubertal ewes. In addition, it appears that the site of action for OFQ on LH secretion is at the hypothalamus and not at the anterior pituitary. Lastly, we demonstrated that OFQ, at least in part, mediates progesterone negative feedback in the ewe.

KISSPEPTIN AND NKB IN PUBERTY

As previously mentioned, the pubertal increase in GnRH/LH secretion is due to lessening of estradiol negative feedback allowing GnRH/LH pulse frequency to increase. In parallel with this increase in LH pulse frequency, we showed that the number of ARC kisspeptin positive neurons is higher in postpubertal ewes compared to prepubertal ewes. In mice, ablation of ARC kisspeptin neurons at 20 d of age (prior to the appearance of kisspeptin cells in the AVPV) completely abolished ovulatory cycles (1), which supports the idea that ARC kisspeptin is essential for puberty. Moreover, my observation that POA kisspeptin cells were lightly and inconsistently stained in the postpubertal ewe, leads me to conclude that this population of kisspeptin cells is unlikely to contribute to puberty in the female sheep. Furthermore, with the inclusion of age-matched male sheep in this study, we demonstrate that this change in cell number is due to a pubertal event and not to age-related changes independent of reproduction. Also, we demonstrated that kisspeptin-positive close contacts on GnRH neurons in the mPOA are greater in postpubertal ewes compared to prepubertal ewes. Changes in kisspeptin contacts in the mPOA closely mirrored the changes we observed in cell numbers in the ARC, which leads us to postulate that kisspeptin neurons in the ARC project to GnRH neurons in the POA. Immunocytochemistry for kisspeptin, dynorphin and GnRH confirm this idea by showing that 50-70% of GnRH neurons in the POA receive arcuate derived kisspeptin input (2). Together, our current working model for puberty (Figure 1) is that estradiol inhibits kisspeptin expression before puberty, but following growth and maturation, estradiol inhibition of kisspeptin lessens, allowing for an increase in GnRH/LH secretion. Therefore, kisspeptin is a gatekeeper to puberty onset in the ewe.

In contrast to kisspeptin, the number of NKB positive neurons did not differ between prepubertal and postpubertal ewes, but did increase following steroid removal in both groups. Upon further examination of the photomicrographs, we observed greater NKB fiber density in the ARC of postpubertal ewes compared to prepubertal ewes. This increase in fiber density most likely reflects increased activity of the NKB system in the postpubertal ewe. Furthermore, we are the first to demonstrate in prepubertal females that senktide can stimulate LH secretion. Given the absence of this effect in the presence of acyline, the stimulation is not at the pituitary, but occurs centrally to stimulate GnRH secretion. Since when compared to postpubertal ewes, prepubertal ewes have a lower NKB fiber density and their LH response to senktide is intact, we suggest that NKB expression is merely inhibited by estradiol in the prepubertal ewe (Figure 1).

However, a few unresolved questions still remain. One, what is the relationship between kisspeptin and NKB during puberty? It is possible that NKB cell numbers increased prior to the prepubertal time point we examined. Given recent data with kisspeptin receptor knockout mice that demonstrates NKB actions are upstream of kisspeptin (3), an increase in NKB expression may occur prior to and drive the increase in kisspeptin expression at the time of puberty. This still allows for kisspeptin to be the gatekeeper in ovine puberty, but also includes an active role for NKB. Furthermore, although we focused solely on stimulatory afferent neurons, what might the inhibitory afferent neurons be that could inhibit GnRH/LH secretion in the prepubertal ewe? One possibility that remains to be investigated is an inhibitory role for dynorphin prepubertally that lessens at the time of puberty. Another candidate is gamma-aminobutyric acid (GABA) given that a $GABA_A$ receptor antagonist increases LH secretion in prepubertal, but not in the adult, monkeys (4). Therefore, the increase in GnRH/LH secretion in the ewe may be due to an increase in kisspeptin cell numbers, an increase in NKB activity and a decrease in dynorphin and/or GABA inhibition. In addition, leptin is a key signal for puberty onset, it remains to be determined how leptin can act on the kisspeptin/NKB system to alter GnRH/LH secretion (5).

Figure 1

Prepubertal

Kisspeptin and NKB

?

GnRH

E

ovary

Growth/
Energy Metabolism

Pubertal

Kisspeptin and NKB

?

GnRH

ovary

Figure 1: Current working model for neuroendocrine pubertal development in the ewe. Potent estradiol negative feedback in the prepubertal ewe reduces kisspeptin and NKB input to GnRH neurons. As the ewe grows and matures, estradiol negative feedback lessens allowing kisspeptin and NKB input to increase resulting in an increase in GnRH/LH secretion.

SITE OF ACTION FOR OFQ

Previous reports demonstrate that central administration of OFQ inhibits LH secretion in the ewe (6) and in rodents (7, 8). We reported herein that various concentrations of OFQ alone applied directly to anterior pituitary cells have no effect on LH release. When challenged with increasing doses of GnRH, anterior pituitary cells respond with increasing concentrations of LH. However, incubation starting before with OFQ did not alter GnRH-induced LH release from these cultured anterior pituitary cells. Therefore, we conclude OFQ most likely does not act directly at the pituitary to inhibit LH secretion.

Since collection of hypophyseal portal blood in an unanesthetized animal can only be accomplished in sheep, our unique experimental model allows us to address the question of whether or not OFQ acts hypothalamically to inhibit GnRH secretion. Infusion of saline into the third ventricle had no effect on GnRH or LH secretion in OVX ewes. However, when OVX ewes received an infusion of OFQ agonist into the third ventricle, GnRH and LH secretion were suppressed. The suppression in LH secretion was due to a reduction in GnRH pulse frequency in the final two hours of infusion of the OFQ agonist. However, an unanticipated increase in GnRH baseline occurred during the final 2 hours of infusion and might be due to asynchronous GnRH release where low amplitude GnRH pulses are too low to detect in 10 min sampling intervals. Therefore, we plan to address this in a subsequent experiment by decreasing portal blood sampling intervals to 2 min to examine GnRH pulse dynamics. Nonetheless, OFQ can clearly act at the hypothalamus to decrease GnRH pulse frequency into hypophyseal portal blood.

ROLE FOR ENDOGENOUS OFQ

It is now accepted that dynorphin is an important mediator of progesterone negative feedback in the ewe since administration of a κ-receptor antagonist increases LH pulse frequency in luteal phase ewes (9). However, given the central suppressive effect of an OFQ agonist on GnRH/LH secretion, a role for endogenous OFQ could exist to mediate steroid negative feedback on GnRH neurons as well. We first investigated whether or not OFQ neurons in the hypothalamus contain steroid receptors. We observed colocalization of both ERα and PR with OFQ neurons in multiple areas of the hypothalamus including the

ARC. POA OFQ neurons did not colocalize with steroid receptors, which is consistent with the finding that most POA OFQ cells are also GnRH neurons and that GnRH neurons are devoid of steroid receptors. Also, we show that OFQ does not colocalize with kisspeptin neurons in the ARC, but does colocalize with POMC neurons, which has also been observed in the rat (10). Furthermore, we tested this idea of a physiological role using an OFQ receptor antagonist, UFP-101, in the presence of ovarian steroids in breeding season ewes. When infused into the lateral ventricle of OVX+ P +E_2 ewes, an OFQ receptor antagonist increased LH pulse frequency compared to saline infused animals. However, LH secretion in OVX+E_2 breeding season ewes was unaffected by infusion of UFP-101. Similarly, no effect of UFP-101 was observed in ovary-intact anestrous ewes, in which progesterone levels are undetectable and low levels of estradiol inhibit LH pulse frequency. From this we conclude that OFQ does not act during the non-breeding season to mediate steroid negative feedback of estradiol. However, since the antagonist increased LH secretion only in the presence of progesterone, we conclude that OFQ mediates, at least in part, progesterone negative feedback in the breeding season ewe. The current working model, including the anatomical and functional data presented herein, for progesterone negative feedback in the ewe is illustrated Figure 2.

Nonetheless, some unresolved issues still remain. First, although in rat and humans (11) a general distribution of ORL-1 exists, it is uncertain which neurons in the POA and hypothalamus contain this opioid receptor. Second, it would be beneficial to characterize the expression of OFQ and ORL-1 in the POA and hypothalamus across the estrous cycle to understand how changes in this opioid system might result in changes in GnRH/LH secretion. Third, although both dynorphin and OFQ can mediate progesterone negative feedback, whether they act in series or in parallel remains to be investigated. Furthermore, since OFQ colocalizes with GnRH and POMC, but also inhibits these neurons in the medial basal hypothalamus, it is possible that OFQ might serve an autocrine function. Thus, we have only just begun to scratch the surface of how OFQ can mediate progesterone negative feedback on GnRH/LH secretion.

Figure 2

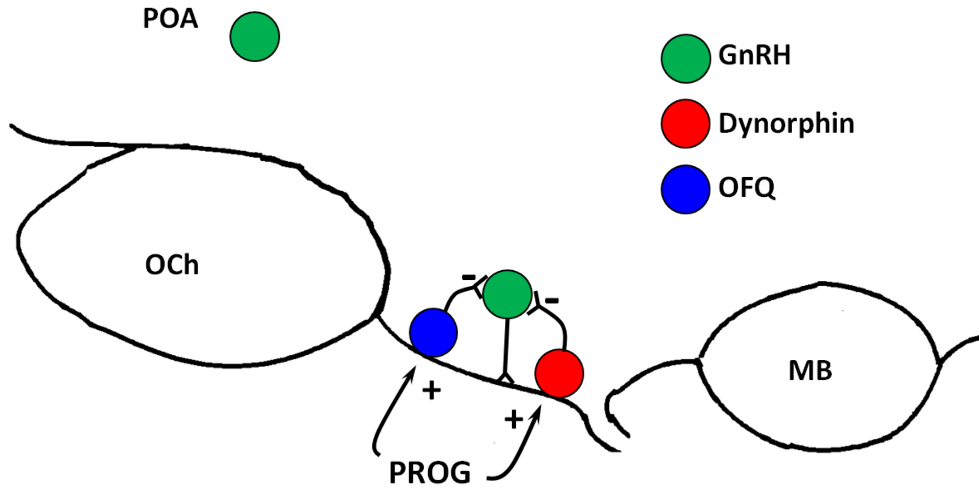


Figure 2: Current working model for OFQ and dynorphin mediating progesterone negative feedback. Progesterone stimulates dynorphin and OFQ secretion within the MBH to inhibit GnRH secretion.

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APPENDIX

SUPPLEMENTAL FIGURE LEGENDS

Figure 1. LH profiles from individual prepubertal ewes that were treated with acyline and 100µg senktide. Arrows indicate time of IV injection of senktide.

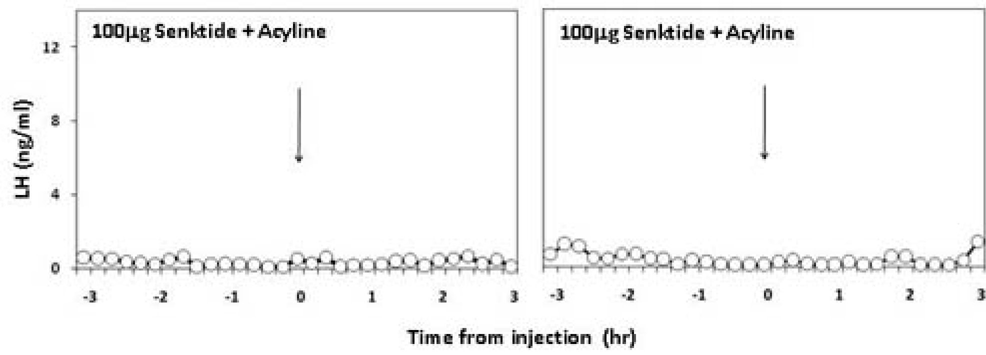
Figure 2. Photomicrographs of kisspeptin-positive neurons in the caudal portion of the ARC from representative young and older males that were intact (A, B) or gonadectomized (C, D). (young ram [YR]; young wether [YW]; older ram [OR]; older wether [OW]). Bar, 200µm.

Figure 3. Photomicrographs of kisspeptin immunoreactivity in the POA for an older (>3yrs) adult OVX ewe (A, B) and younger (11 months) EF ewe (C, D). 4x magnification (A, C). 10x magnification (B, D). Bar, 200µm.

Figure 4. Photomicrographs of NKB-positive neurons in the caudal portion of the ARC from representative young and older males that were intact (A, B) or gonadectomized (C, D). (young ram [YR]; young wether [YW]; older ram [OR]; older wether [OW]). Bar, 200µm.

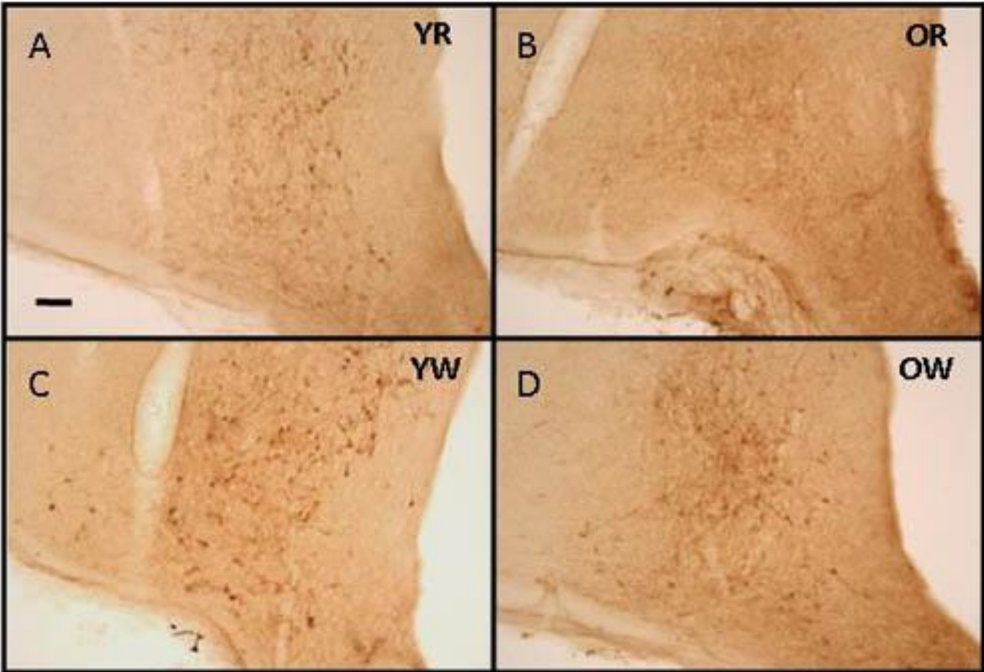
SUPPLEMENTAL FIGURES

FIGURE 1



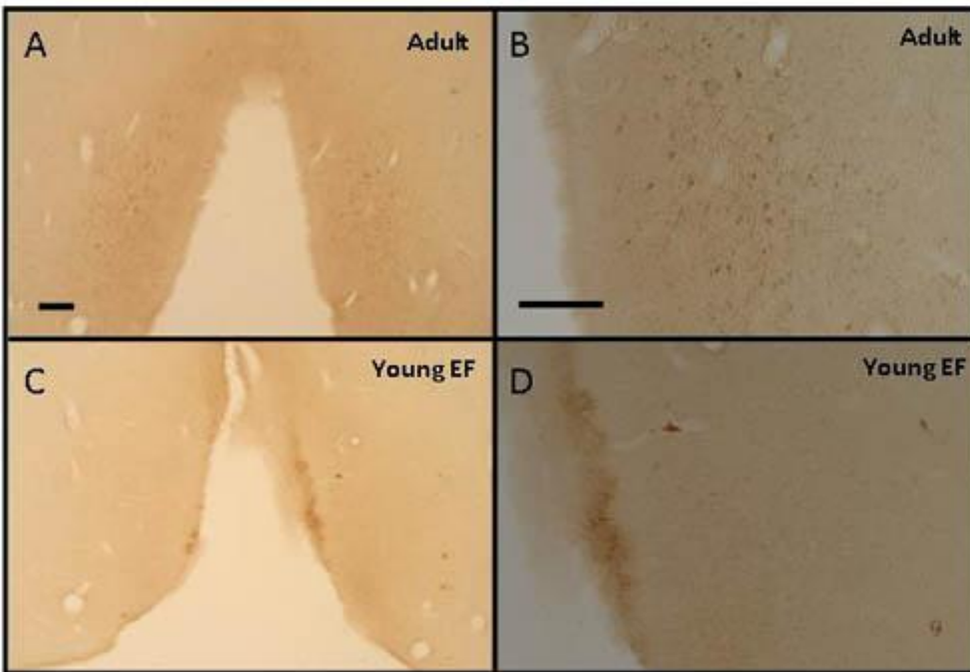
SUPPLEMENTAL FIGURES

FIGURE 2



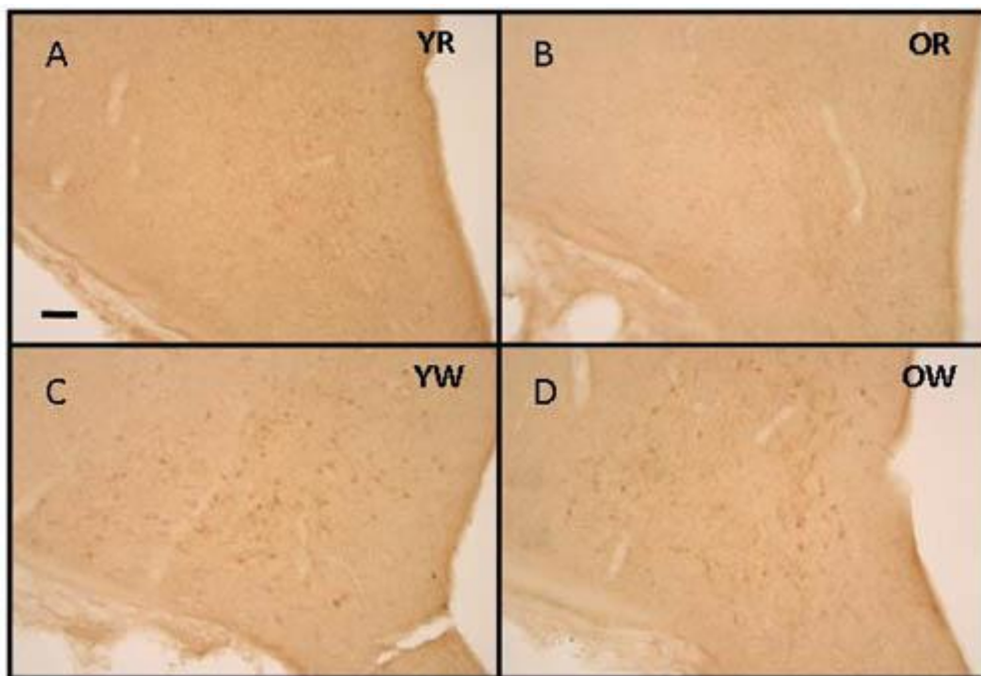
SUPPLEMENTAL FIGURES

FIGURE 3



SUPPLEMENTAL FIGURES

FIGURE 4



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DOCTORAL DISSERTATION: Evidence of a Role for Three Neuropeptides that Mediate Steroid Negative Feedback on Gonadotropin Releasing Hormone/Luteinizing Hormone Secretion in the Ewe: Kisspeptin, Neurokinin B and Orphanin FQ

Estradiol and progesterone inhibit tonic GnRH and LH release. Since GnRH neurons are devoid of steroid receptors, this inhibition most likely occurs through afferent neurons that have yet to be identified. My dissertation work focuses on the three neuropeptides which may play a role in mediating steroid negative feedback in sheep, kisspeptin and neurokinin B (NKB) in the context of puberty onset, and orphanin FQ with regard to seasonal breeding.

Puberty in the ewe is marked by an increase in GnRH/LH secretion which results from a lessening of estradiol-negative feedback. Kisspeptin and NKB are coexpressed in the same subsets of neurons in the arcuate nucleus of the hypothalamus and are critical for normal timing of puberty. Given that greater than 95% of kisspeptin/NKB neurons colocalize with estrogen receptor-α and progesterone receptor in the ewe, we examined changes in kisspeptin and NKB positive neurons in the arcuate of pre- and post-pubertal ewes in the presence and absence of sex steroids. We observed a greater number of kisspeptin, but not NKB, positive neurons in the arcuate nucleus of postpubertal ewes compared to prepubertal ewes, which corresponded with changes in LH secretion. Also, we showed an intravenous injection of senktide, an NKB receptor agonist, stimulates LH secretion in prepubertal ewes. These results support a role for kisspeptin and NKB in ovine puberty.

Sheep breed during the shorter days of autumn and are reproductively active in the relatively longer days of summer. This seasonal swing in fertility is primarily due to photoperiod-induced changes in sensitivity to estradiol-negative feedback. We used an OFQ receptor antagonist to examine a role of endogenous OFQ in controlling LH secretion in ewes during the breeding and non-breeding season. We observed an increase in LH pulse frequency in ewes with luteal concentrations of progesterone and estradiol, but no effect on LH secretion in ewes treated with estradiol alone or in intact, anestrous ewes. In addition to our work, icv administration of OFQ has been shown to inhibit LH secretion in the rat and ewe and OFQ localizes in the external zone of the median eminence. Given its effect on LH and its location, we investigated whether OFQ acts at the pituitary to inhibit LH release and/or at the hypothalamus to inhibit GnRH secretion. OFQ had no direct influence on ovine anterior pituitary cells in cell culture. However, we did observe a decrease in GnRH secretion in hypophyseal portal samples following infusion of an OFQ agonist into the third cerebroventricle of ovariectomized ewes. From this we conclude a role for OFQ in mediating, at least in part, progesterone-negative feedback in the ewe at the level of the hypothalamus.

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Thyroidectomy (ovine)

Microinjections or microinfusions of neuropeptides or receptor agonists and antagonists into the third or lateral cerebroventricle (ovine)

Stereospecific injections of tract-tracing agents into the ovine hypothalamus

Ovariectomy (ovine)

Insertion of jugular catheters (ovine)

Venipuncture (ovine)

Dissection of murine and ovine hypothalamus

Single- and dual-immunocytochemistry (ICER, GnRH, Kisspeptin, NKB, Dyn, TH, cFos)

Single- and dual-immunofluorescence (GnRH, Kisspeptin) using confocal microscopy

Radioimmunoassay (OFQ, GnRH, LH, Prolactin, Progesterone)

In vitro culturing of dispersed anterior pituitary cells (bovine and ovine)