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The Role of Kisspeptin and KNDy Cells in the Reproductive Neuroendocrine System

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Graduate Program in Neuroscience
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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THE ROLE OF KISSPEPTIN AND KNDY CELLS IN THE
REPRODUCTIVE NEUROENDOCRINE SYSTEM

by

Christina M. Merkley

Graduate Program in Neuroscience

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
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London, Ontario, Canada

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ABSTRACT

The gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus represent the final common output in the central control of reproduction. GnRH secretion is modulated indirectly by steroid feedback action of gonadal steroids on afferent interneurons. In recent years, the neuropeptide, kisspeptin, has emerged as a key mediator of steroid feedback onto GnRH neurons and the reproductive system. Kisspeptin neurons located in the preoptic area (POA) mediate estradiol (E2) positive feedback leading to the preovulatory surge, and kisspeptin cells in the arcuate nucleus (ARC) have been shown to mediate the negative feedback actions of E2 on pulsatile GnRH secretion. ARC kisspeptin neurons are distinguished from those in POA by their extensive reciprocal connections with each other, and their co-expression of two other neuropeptides, neurokinin B (NKB) and dynorphin. Because of this co-expression, the ARC kisspeptin neurons have been termed “KNDy” (Kisspeptin/NKB/Dynorphin) cells. Using sheep and mice as neuroendocrine models, the goal of this dissertation was to investigate the functional organization of KNDy cells and their projections to GnRH neurons, in order to gain insight into their role as mediators of steroid feedback. First, we showed that, contrary to expectations based on rodent work, KNDy neurons in the sheep are activated during both positive and negative feedback of E2 suggesting that they are common mediators of both GnRH pulses and the GnRH surge. Next, using KNDy cell peptides as markers, we showed that KNDy neurons in the sheep send direct neuronal projections to a majority of GnRH cell bodies spread over multiple regions, as well as input to GnRH terminals in the median eminence. We then explored the potential plasticity of synaptic inputs onto KNDy and GnRH neurons across the ovine estrous cycle, and found a significant increase in the total number of inputs to KNDy neurons, as well as KNDy inputs to GnRH cells, at the time of the preovulatory GnRH surge. Performing similar experiments in transgenic mice, we tested the hypothesis that plasticity in these inputs is regulated, in part, by E2. Finally, we began to investigate the possibility that NKB released by KNDy cells might play a functional role in the GnRH surge, by examining internalization of the NKB receptor, NK3R, across the estrous cycle. Taken together, these findings provided novel information on the roles of kisspeptin and KNDy neurons in steroid feedback control of GnRH secretion, and set the stage for future experiments to

explore the mechanisms for steroid feedback in individual neurons, the functional role of synaptic plasticity in this system, and the role of KNDy peptides in regulating the reproductive neuroendocrine system across the estrous cycle.

Keywords: Kisspeptin, KNDy, estrous cycle, estradiol, neural activation, gonadotropin-releasing hormone (GnRH), reproduction, luteinizing hormone, glutamate, neurokinin B, dynorphin, confocal microscopy, immunohistochemistry, immunofluorescence, plasticity, neuroendocrinology, hypothalamic-pituitary-gonadal axis.

CO-AUTHORSHIP

All contents of the current dissertation were carried out in collaboration with Michael N. Lehman and Lique M. Coolen. A large majority of this work (chapters 2-5) is also in collaboration with Robert L. Goodman, long-time collaborator of Drs. Lehman and Coolen. Chapter 2 was co-written by Christina M. Merkley and Michael N. Lehman with inputs by Robert L. Goodman and Lique M. Coolen. Chapter 3 was written by Christina M. Merkley and Katrina L. Porter, with intellectual inputs by Stan M. Hileman, Robert L. Goodman, Michael N. Lehman and Lique M. Coolen. Animal procedures and tissue collection (sections 3.2.1-2.2.4) were conducted at West Virginia University under the supervision of Robert L. Goodman. Experimental procedures and data analyses (section 3.2.5-3.2.6) were performed by Christina M. Merkley for Experiment 1, and Katrina L. Porter for Experiment 2. Chapter 4 was written by Christina M. Merkley, with intellectual inputs by Robert L. Goodman, Lique M. Coolen and Michael N. Lehman. Animal procedures and tissue collection (sections 4.2.1-4.2.2) were carried out at West Virginia University under the supervision of Robert L. Goodman. All experimental procedures, confocal microscopy, and data analysis (sections 4.2.3-4.2.5) were performed by Christina M. Merkley. Chapter 5 was written by Christina M. Merkley, with intellectual inputs by Robert L. Goodman, Lique M. Coolen and Michael N. Lehman. Animal procedures (sections 5.2.1-5.2.4) were conducted at West Virginia University, while experimental procedures, confocal microscopy and data analysis (sections 5.2.5-5.2.7) were performed out by Christina M. Merkley with inputs by Lique M. Coolen. Chapter 6 was written by Christina M. Merkley, with intellectual inputs by Michael N. Lehman. Animal procedures (sections 6.2.1- 6.2.2) were carried out at the University of Texas Southwestern Medical Center under the supervision of Carol F. Elias. Experimental procedures, confocal microscopy and data analysis (sections 6.2.3-6.2.6) were performed by Christina M. Merkley. Chapter 7 was written by Christina M. Merkley with intellectual inputs by Michael N. Lehman. Animal procedures (sections 7.2.1-7.2.3) were conducted at Texas A&M University under the supervision of Marcel Amstalden. Experimental procedures, confocal microscopy and data analysis was conducted by Christina M. Merkley (sections 7.2.4-7.2.5) with inputs by Lique M. Coolen.

In addition to the work described above, Christina M. Merkley has taken part in animal work at the Sheep Research Facility at the University of Michigan, Ann Arbor, Michigan, and has contributed to numerous research studies at this facility. This includes perfusions and brain extractions, blood sampling, blood processing, fetal tissue collection surgery, fetal organ preservation, and lambing (ear tagging, weighing and blood sampling).

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LIST OF ABBREVIATIONS

3V, third ventricle
ABC, avidin-biotin-horseradish peroxidase complex
AC, anterior commissure
AGRP, agouti-related peptide
AHA, anterior hypothalamic area
AR, androgen receptor
ARC, arcuate nucleus
AVPV, anteroventral periventricular nucleus
BNST, bed nucleus of the stria terminalis
BSA, bovine serum albumin
CSF, cerebral spinal fluid
DMN, dorsomedial hypothalamus
Dyn, dynorphin
E2, estrogen/estradiol
eGFP, enhanced green fluorescent protein
EM, electron microscopic
ER α , estrogen receptor- α
ER β , estrogen receptor- β
EOP, endogenous opioid peptide
FSH, follicle stimulating hormone
FX, fornix
GABA, gamma aminobutyric acid
GAD, glutamic acid decarboxylase
GDX, gonadectomy/gonadectomised
GFP, green fluorescent protein
GnRH, gonadotropin-releasing hormone
H₂O₂, hydrogen peroxide
HPG, hypothalamic-pituitary-gonadal
ICC, immunocytochemistry

IR, immunoreactivity
IV, intravenous
ISH, *in situ* hybridization
Kiss1R, kisspeptin receptor
KNDy, kisspeptin/neurokinin B/dynorphin
KOR, kappa opioid receptor
LH, luteinizing hormone
LHA, lateral hypothalamic area
ME, median eminence
MT, mammillothalamic tract
MBH, mediobasal hypothalamus
NGS, normal goat serum
NK3R, neurokinin 3 receptor
NKB, neurokinin B
NMDA, N-methyl-D-aspartate
NPY, neuropeptide Y
OC, optic chiasm
OT, optic tract
OVX, ovariectomy/ ovariectomized
P4, progesterone
PB, phosphate buffer
PBS, phosphate buffered saline
PeN, periventricular nucleus
PGF_{2α}, prostaglandin F_{2α}
POA, preoptic area
POMC, pro-opiomelanocortin
PR, progesterone receptor
PVN, paraventricular nucleus
PVpo, periventricular preoptic nucleus
RCh, retrochiasmatic area
RFRP, RF-amide related peptide

RP3V, rostral periventricular area of the third ventricle

SCN, suprachiasmatic nucleus

SON, supraoptic nucleus

T, testosterone

TAC3, tachykinin 3

vGlut, vesicular glutamate transporter

VMH, ventromedial hypothalamus

CHAPTER 1: GENERAL INTRODUCTION

1.1. Introduction

1.1.1. The Reproductive Axis

The dynamic control of reproductive function involves interactions among the brain, the pituitary gland, and the gonads (Figure 1.1). The primary hierarchical component in the hypothalamic-pituitary-gonadal (HPG) axis are hypothalamic neurons which synthesize and release the decapeptide, gonadotropin-releasing hormone (GnRH) (1). In mammals, GnRH neurons are scattered across rostral-caudal levels of the preoptic area and hypothalamus (2) and projections from these neurons to the median eminence (ME) serve as the final common output in the central control of reproduction (3, 4). At the ME, GnRH is secreted into the hypophyseal portal blood vessels and carried to the anterior pituitary where it acts on gonadotropes to stimulate the release of gonadotropin hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH). The pattern of endogenous GnRH secretion is intimately correlated with LH secretion (5), thereby, changes in the patterns of GnRH release are reflected in the pattern of LH secretion (3, 6). The gonadotropins, LH and FSH, are essential for normal fertility in both males and females, and act on somatic cells in the gonads to stimulate sexual maturation and gametogenesis (spermatogenesis in males and oogenesis in females)(7). Activation of somatic receptors induces the synthesis and release of gonadal steroid hormones, testosterone (T) in males and estrogen (E2) and progesterone (P4) in females (6, 7). These gonadal hormones participate in the dynamic regulation of the HPG axis through hormonal feedback loops that modulate GnRH and LH secretion across the estrous cycle (menstrual cycle in humans) (Figure 1.1).

1.1.2. Modes of GnRH Secretion and their Control by Steroid Hormones

Considerable progress has been made towards understanding the pattern of endogenous GnRH release through the development of a unique blood sampling technique in sheep that can sample hypophyseal portal blood to directly measure GnRH release (5, 8). In other species in which GnRH release cannot be directly measured due to the small size of the portal vessels, LH secretion is often used as a surrogate to provide

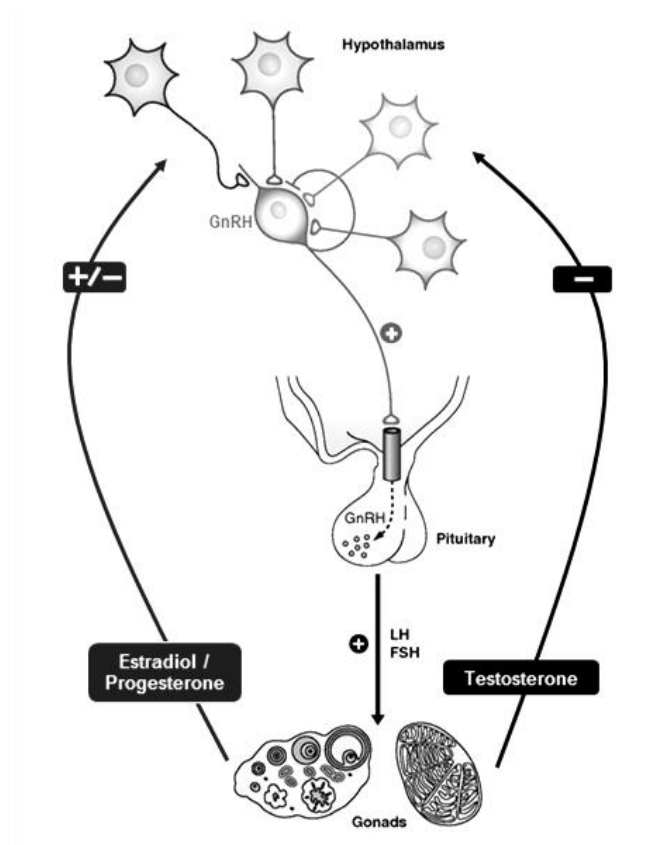


Figure 1.1. The hypothalamic-pituitary-gonadal (HPG) axis. GnRH acts on the pituitary gland to stimulate the release of gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). These gonadotropins, in turn, act on receptors located in the gonads (ovaries in females and testes in males), where they stimulate the synthesis and release of gonadal hormones. In females, estradiol and progesterone exert both negative and positive feedback actions at the level of the hypothalamus to regulate GnRH secretion. In males, testosterone secreted from the gonads negatively regulates the reproductive axis at the hypothalamic level. Hormonal feedback also occurs at the pituitary level (not shown). Various afferent interneurons (shown in different colors) participate in conveying steroid signals and other information to GnRH neurons. GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle stimulating hormone. Modified from Pinilla et al. (9).

information on the pattern of GnRH release, as the release of GnRH and LH are synchronous, with the former driving the latter (5, 6). This section summarizes the major modes of GnRH and LH secretion, and how they are regulated by steroid feedback.

Tonic GnRH Secretion and Negative Feedback

There are two modes of GnRH secretion: tonic and surge secretion. Tonic secretion of GnRH is episodic, occurring in a pulsatile pattern throughout most of the estrous cycle in females and the reproductive life span of males (6, 10, 11). In females, the frequency and amplitude of pulses varies across the estrous cycle and is modulated by the feedback actions of ovarian steroids (6, 12)(See below). Sufficient pulsatile secretion of GnRH is obligatory for proper reproductive function (10, 11, 13), and pituitary responsiveness to GnRH is dependent on intermittent (and not constant) release of GnRH (10, 14). Furthermore, the synchronous release of GnRH comprising each pulse is proposed to be driven by a coordinated network of cells termed, the GnRH pulse generator (13).

In males, testosterone acts to inhibit GnRH (15) and LH (16, 17) pulse frequency and amplitude. In rodents, these effects appear to be mediated via both estrogen and androgen receptors (18, 19). In females, E2 and P4 act to suppress GnRH pulse frequency and amplitude (12, 20-22) during the estrous cycle (Figure 1.2), and these effects are mediated through estrogen receptor- α (ER α) and progesterone receptor (PR)-dependent mechanisms (22-24). In sheep, progesterone levels increase late in follicular phase and are elevated throughout luteal phase, where they act to suppress GnRH/LH pulse frequency (6, 25, 26). After luteolysis, P4 levels rapidly decline and E2 levels increase steadily throughout follicular phase, which results in high frequency, low amplitude pulses in follicular phase (6, 25, 26).

Surge Secretion of GnRH

The second mode of GnRH secretion occurs at the end of follicular phase in females and is characterized by a surge-like release of GnRH. The GnRH surge is characterized by prolonged and elevated GnRH and is closely followed by a surge in LH, as a result of the positive feedback actions of estradiol late in follicular phase (27, 28)

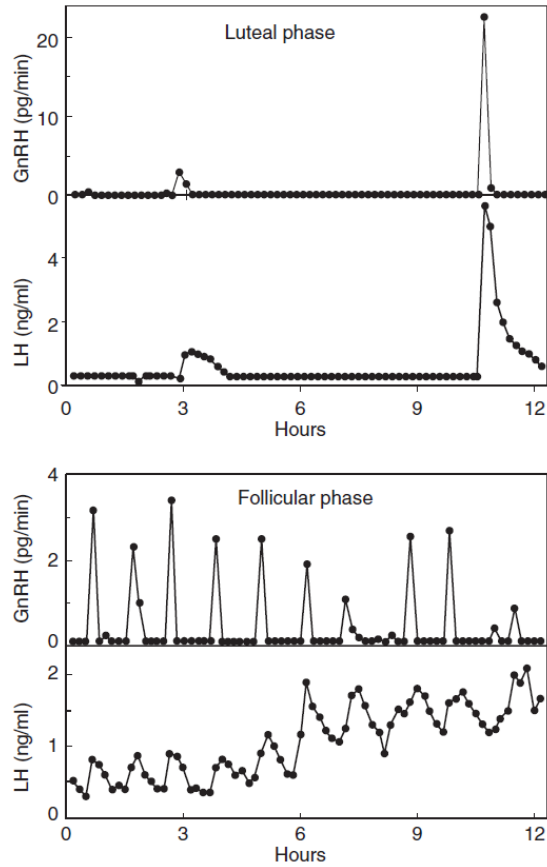


Figure 1.2. Pulsatile GnRH (pg/min) and LH (ng/ml) release during luteal and in late follicular phase of the estrous cycle in the ewe. GnRH was sampled from hypophyseal portal blood and LH sampling from jugular blood. Low frequency, high amplitude GnRH and LH pulses are characteristic of luteal phase, while high frequency, lower amplitude GnRH and LH pulses are present in late follicular phase, after progesterone withdrawal (x axis, hours post progesterone withdrawal). GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone. From Goodman & Inskeep (6), describing data from Moenter et al. (26).

(Figure 1.3). The GnRH surge is sexually differentiated and only present in females (29, 30), where it induces a subsequent surge in LH that is the key event responsible for triggering ovulation (4).

In rodents, the site of E2 positive feedback is thought to be in the anteroventral periventricular preoptic region (AVPV) (31) and this feedback occurs via an ER α -dependent mechanism (32). Consistent with this, the AVPV is sexually differentiated in rats, with more steroid-responsive neurons in females than males (33). By contrast, in sheep, E2 implants in the preoptic region are unable to induce a GnRH/LH surge; instead the site of E2 positive feedback in sheep is believed to be the mediobasal hypothalamus (MBH) (34, 35). In primates, the primary site of E2 positive feedback also appears to be the MBH (36); thus there are significant species differences between rodents and sheep or primates in the sites of E2 regulation of the GnRH surge. Despite these differences, in each of these species GnRH neurons are activated at the time of the surge (37-40), and this activation may occur via a common mediator in both species. The physiological mechanisms that underlie the switch from E2 negative to E2 positive feedback remain largely elusive.

1.2. Steroid Feedback Control of GnRH Neurons: Role of Kisspeptin

Steroid feedback onto GnRH neurons is likely relayed via an indirect route, as GnRH neurons do not colocalize ER α (41-44), PR (45, 46) or androgen receptors (AR; (47, 48)). There is evidence that estrogen receptor- β (ER β) is localized within GnRH neurons (49-51), however it does not appear to play a critical role in reproduction and gonadotropin secretion (18, 32, 52, 53). Thus, considerable efforts have been dedicated towards identification of the steroid-sensitive afferent interneurons that relay feedback effects onto GnRH neurons. To date, a large number of neuropeptide and neurotransmitter systems have been shown to alter GnRH and gonadotropin release, and provide direct neuronal input to GnRH neurons (54, 55). One of the neuronal phenotypes that has received a lot of attention in the last decade, and elicits profound physiological effects on GnRH and reproduction, is kisspeptin (9).

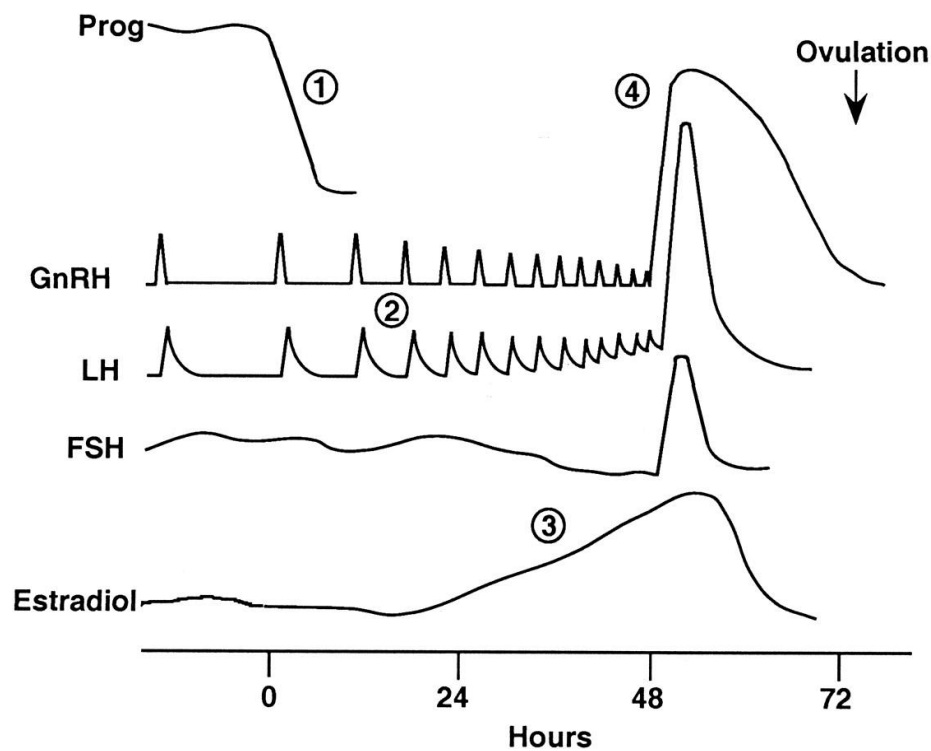


Figure 1.3. Schematic illustrating the hormonal secretory profiles across the follicular phase in the ewe. Circulating progesterone rapidly declines after luteolysis (1) followed by an increase in GnRH and LH pulse frequency concomitant with a decrease in amplitude (2). Rising E2 levels (3) give rise to the GnRH and LH surges (4) which trigger ovulation. Prog, progesterone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone. From Battaglia et al. (36).

1.2.1. Kisspeptin

Significant advancements in our understanding of the neural mechanisms controlling GnRH secretion in the last decade have derived from the discovery of kisspeptin, and its essential physiological role in reproduction. Kisspeptin is a key upstream regulator of GnRH that is well poised to integrate central and peripheral signals (9, 56, 57).

1.2.2. Kisspeptin and Reproduction

Kisspeptins are a family of RF-amide related peptides (RFRP), coded by the *Kiss1* gene, that are derived from the proteolytic processing of a common precursor to form biologically active peptides (58, 59) (Figure 1.4). Originally identified for its role as a tumor metastasis suppressor gene (60), *Kiss1* was later shown to be the peptide ligand for the G-protein coupled receptor, GPR54 (58, 59, 61), now commonly referred to as Kiss1R (62). The initial product of the *Kiss1* gene encodes a 145 amino acid peptide which is cleaved to form the 54 amino acid carboxy terminal amidated peptide known as kisspeptin or kisspeptin-54 (58, 59, 61). This undergoes further proteolytic processing to yield shorter kisspeptin peptides (kisspeptin-14, -13, -10), that share a carboxy terminus and show similar binding affinities to Kiss1R (58). Although the ligand/receptor relationship had been characterized, the role of kisspeptin in reproduction was not known. Its critical role was first illustrated when two independent studies in humans reported that mutations in Kiss1R resulted in idiopathic hypogonadotropic hypogonadism, characterized by impaired pubertal maturation, underproduction of gonadotropins, and isolated infertility (63, 64). Genetic studies in mice have shown that mutations in Kiss1R (64-67) or *Kiss1* (67, 68), produce the same phenotype of reproductive deficits, and extend findings in humans that kisspeptin/Kiss1R signaling plays an indispensable role in reproduction.

Kisspeptin neurons are located in two well-conserved populations in a variety of mammalian species: the arcuate nucleus (ARC) and the preoptic region (69-73). In addition to these, smaller populations of kisspeptin cells have been reported in other

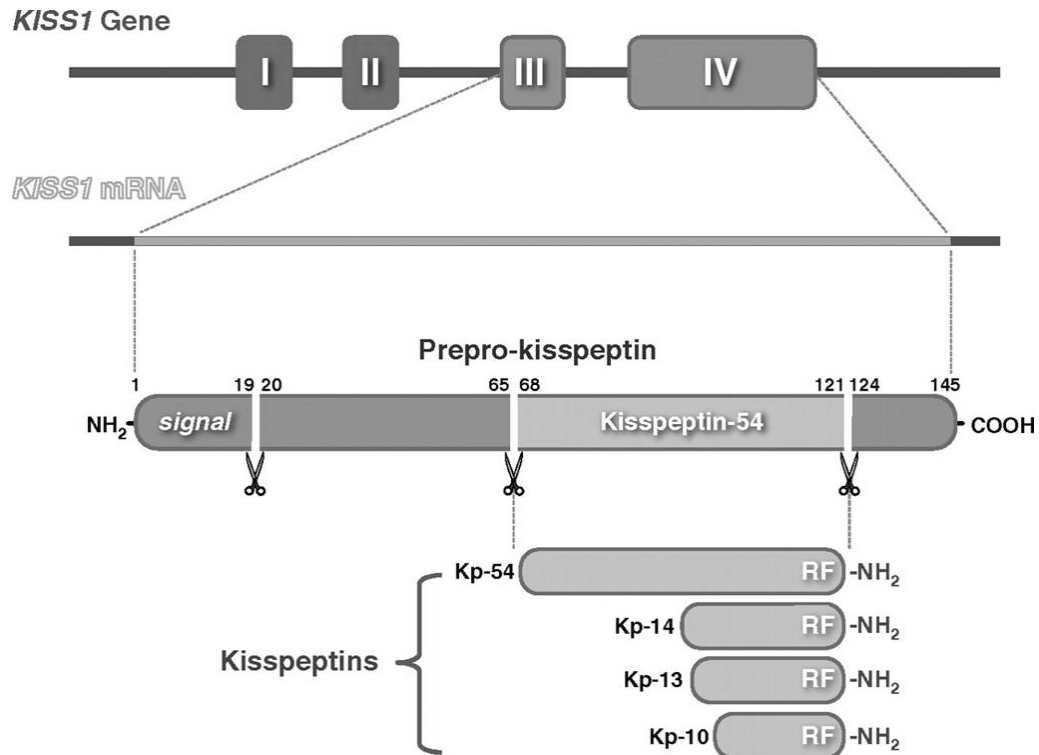


Figure 1.4. Generation of kisspeptins from the *Kiss1* gene. Different kisspeptin peptides are generated from the proteolytic processing of the primary translation product, which is a 145 amino acid protein in humans. This yields a 54 amino acid amidated peptide (kisspeptin-54 or shown as Kp-54). Further proteolysis yields shorter kisspeptin peptides (Kp-14, Kp-13, Kp-10) that commonly share a 10 amino acid C-terminal region which is sufficient to activate GPR54. From Pinilla et al. (9).

hypothalamic regions and in the amygdala (69, 74-78). A detailed summary of the distribution of kisspeptin cells in the mammalian brain is given in Chapter 2.2.

1.2.3. Stimulatory Effects of Kisspeptin on Gonadotropin Secretion in Mammals

Shortly after the discovery of its role in reproduction (63, 64), kisspeptin was revealed to be an extraordinarily potent secretagogue for gonadotropin secretion in a variety of mammalian species including humans (79, 80), monkeys (81, 82), sheep (83, 84), goats (85, 86), rats (87-91), mice (76, 84), horses (92), cows (93) and pigs (94). The stimulatory effects of kisspeptin are present in both males and females (79-81, 84, 86, 88), and appear quite resilient to physiological state such as estrous cycle phase (83, 95), lactation (95), pregnancy (95), metabolic state (96, 97) and aging (98). Finally, the reliably stimulatory effects of kisspeptin are present when given centrally or systemically (99), and reinforces the view that the actions of kisspeptin are critical for reproduction.

1.2.4. Actions of Kisspeptin on GnRH Neurons

Consistent with the idea that kisspeptin-stimulated LH secretion is mediated via central action on GnRH neurons, kisspeptin has been shown to mediate GnRH release in the sheep (84) and monkey (100) (Figure 1.5). Support for the idea that the effects of kisspeptin are mediated via action on GnRH neurons, can also be found in studies using GnRH antagonists, which have been shown to block the kisspeptin-mediated increase in LH (76, 82, 87, 88, 90). This may take place directly, as the majority of GnRH neurons have been shown to express Kiss1R (84, 87, 101-103), coupled with electrophysiological evidence that kisspeptin can depolarize and increase firing rates of GnRH neurons *in vitro* (101, 104-107). Alternatively, this may be via an indirect mechanism, as Kiss1R are expressed by other neurons and thus the effects of kisspeptin may be mediated by other neurotransmitter systems which are afferent to GnRH cells (106, 108-110). Finally, kisspeptin fibers and terminals make direct contacts onto GnRH cell bodies, as well as their axon terminals in the ME, in a variety of species (see Chapter 2). In summary, there is compelling evidence for direct modulation of GnRH neurons by kisspeptin.

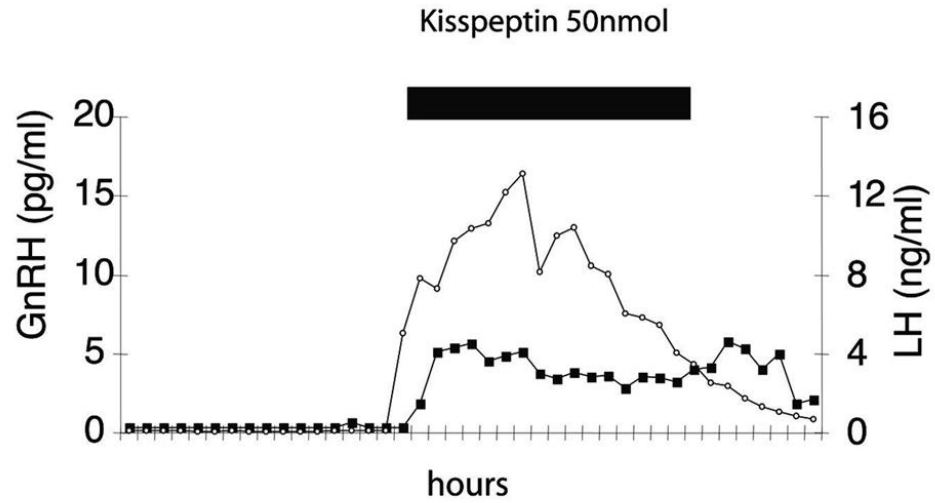


Figure 1.5. Stimulatory effects of kisspeptin administration (50 nmol over 4 hours) on GnRH (*filled squares*) and LH (*open circles*) secretion in sheep. GnRH (pg/ml) was measured in cerebral spinal fluid (CSF). From Messenger et al. (84).

1.2.5. Kisspeptin Neurons Mediate Steroid Feedback

Current evidence suggests that kisspeptin neurons mediate the negative and positive feedback effects of ovarian steroids onto GnRH neurons (9). Kisspeptin cells express the gonadal steroid receptors which are thought to be essential for steroid feedback control of GnRH (ER α , PR and AR) (38, 40, 69, 73, 111-114)(described in Chapter 2), and therefore are positioned to be mediators. In addition, Kiss1 mRNA levels are regulated by gonadal steroids (38, 73, 89, 113); briefly reviewed below.

1.2.5.1. Roles of the ARC Kisspeptin Population in Steroid Feedback

Negative Feedback

As mentioned above (Section 1.1.2), episodic GnRH release throughout the majority of the estrous cycle is under negative feedback regulation by sex steroids (12, 15, 20-22). Kisspeptin neurons of the ARC are associated with the negative feedback regulation of GnRH, as gonadectomy (GDX; or low circulating sex steroids) increases, and steroid replacement decreases, Kiss1 mRNA in a variety of species including humans (115), monkeys (115, 116), sheep (113), rats (38, 87), mice (73, 89, 114), and pigs (117). This effect appears to be mediated by ER α in females (73, 89), and by contributions from both ER α and AR in males (114). Although there is evidence that ER α mediates the negative regulation of Kiss1 mRNA, it has only recently been demonstrated that these effects are largely mediated by ER α in kisspeptin neurons, using transgenic mice to delete ER α exclusively within *Kiss1* cells (118, 119). Kisspeptin antagonist delivery in ovariectomized sheep and prepubertal monkeys inhibits pulsatile GnRH and LH release, respectively (103, 120) providing further evidence of the role of kisspeptin in the control of GnRH and LH pulses.

Positive Feedback

In sheep, there is evidence to support the role of ARC kisspeptin neurons in positive, as well as negative feedback. In late follicular phase, Kiss1 mRNA increases in the middle and caudal ARC (121, 122). This suggests that these neurons may be important in generating the preovulatory surge (121), and is consistent with the MBH being the site of positive feedback in the sheep (35). However studies in sheep using Fos

as a marker of neuronal activation to determine whether ARC kisspeptin cells were activated during positive feedback yielded inconclusive results (122, 123), with one study showing increased activation after a surge-producing bolus of E2 (122), and the other showing a lack of activation in ARC kisspeptin cells during the preovulatory surge (123). Therefore, ARC kisspeptin neurons appear to play a role in positive feedback in sheep, but their activation across the estrous cycle and during the preovulatory surge is unclear. Furthermore, it is currently unknown whether the same or different subsets of ARC kisspeptin cells mediate negative and positive feedback. This will be addressed in Chapter 3.

1.2.5.2. Role of Preoptic Kisspeptin Neurons in Steroid Positive Feedback

As noted above, the site which E2 exerts positive feedback to induce a preovulatory surge in rodents is located in the preoptic region (31), and there is now substantial evidence to support a role for anteroventral periventricular (AVPV) kisspeptin neurons in the regulation of E2 positive feedback (38, 73). Kiss1 mRNA is decreased in the AVPV following GDX, and is increased with steroid replacement (T in males and E2 in females) (38, 73, 111, 114). Similar increases in Kiss1 mRNA are shown in the preoptic area (POA) of sheep in late follicular phase (122) and in the periventricular nucleus of ovariectomized (OVX) pigs replaced with E2 (117). AVPV kisspeptin cells in rodents express Fos during the surge, indicating their neural activation (38, 40, 111). These data support a role for these rostrally-located kisspeptin neurons in E2 positive feedback. Consistent with this, kisspeptin antibody or antagonist delivery to the POA in rodents has been successful in blocking the preovulatory surge (111, 124, 125). In sheep, kisspeptin antagonists only partially block the E2-induced surge suggesting that other peptides/transmitter may be involved (103). Further, there is dispute as to whether POA kisspeptin neurons in the sheep mediate positive feedback (122, 123). Studies using Fos expression in kisspeptin cells are mixed, with one study showing Fos in POA kisspeptin cells during the LH surge (123), and the other showing a lack of Fos in the same neurons (122). Taken together with the *in situ* hybridization work showing an increase in Kiss1 mRNA in the POA during late follicular phase (122), the role of the POA kisspeptin cells in sheep has not been resolved.

1.3. The KNDy (Kisspeptin/Neurokinin B/Dynorphin) Neurons of the ARC

A distinguishing anatomical feature of the ARC kisspeptin population is the presence of two additional neuropeptides, neurokinin B (NKB) and dynorphin, each of which play an important role in steroid feedback control of GnRH and LH secretion (70)(Figure 1.6). Colocalization of the three peptides was first identified in the sheep (70), and was later identified in a number of other species (70, 71, 126-128). Because there is almost 100% co-localization of these three peptides in the ARC, this population has been termed, KNDy (Kisspeptin, Neurokinin B/Dynorphin) cells (129). For a detailed summary of the anatomical features of the KNDy cells including their steroid receptor coexpression and projections within the ARC and to GnRH, see Chapter 2.

1.3.1. Role of Neurokinin B in KNDy Cells and Reproduction

Since the early 1990s, NKB had been known to play a role in steroid negative feedback (130, 131), but this peptide had been ignored by most reproductive neuroendocrinologists until 2009, when patients bearing mutations in the gene encoding NKB (*TAC3* in humans) or its receptor TACR3 in humans were shown to display hypogonadotropic hypogonadism and infertility (132, 133), a similar phenotype to that seen in cases of mutations in the *Kiss1* gene or its receptor (63, 64). This data suggested that NKB, like kisspeptin, had a stimulatory role in reproduction. In monkeys and rodents, OVX stimulated NKB mRNA expression (130, 134), and E2 replacement suppressed NKB mRNA in the ARC (135-137). This led to the hypothesis that ARC NKB plays a role in negative feedback, in a manner similar to kisspeptin, in which OVX and E2 replacement increase and decrease *Kiss1* mRNA, respectively (38, 73, 113). However, data in sheep have been inconclusive with respect to the participation of NKB in negative feedback (138, 139).

By contrast, there is growing evidence that NKB in the sheep may be involved in events leading to the preovulatory GnRH/LH surge. In sheep, the effects of senktide, an NK3R agonist, vary depending on phase of the estrous cycle: stimulating LH in the follicular phase, with no effect in luteal phase (140). Because senktide also exerts stimulatory effects in anestrus ewes, the stimulation of LH via NK3R does not appear to depend on high E2 levels (140). These authors propose that the actions of P4 during

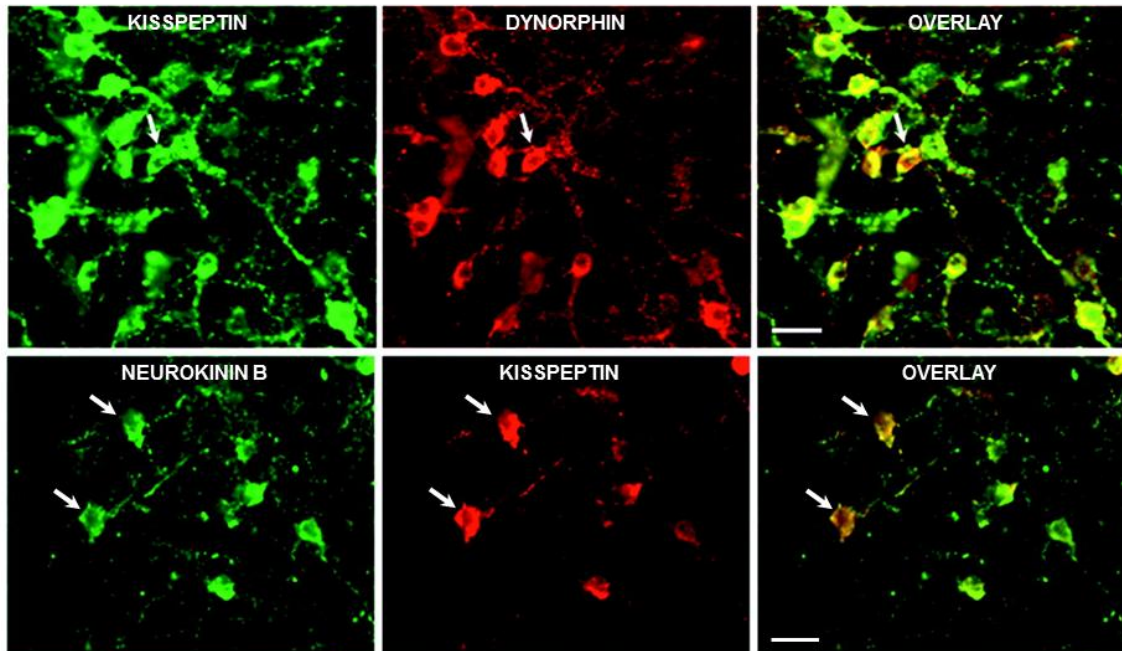


Figure 1.6. KNDy peptide co-localization in the ARC of sheep. Confocal microscopic images in the arcuate (ARC) stained either for kisspeptin (*green*) and dynorphin (*red*) (upper panels), or for neurokinin B (NKB; *green*) and kisspeptin (*red*) (lower panels). White arrows indicate examples of cells that coexpress both peptides (kisspeptin/dynorphin or kisspeptin/neurokinin B). Scale bar, 20 μm . Modified from Goodman et al. (70).

luteal phase to suppress LH secretion, may block the stimulatory effects of NKB (or NK3R activation) (140). Interestingly, central administration of NKB in E2 and P4 treated OVX goats, evokes an LH pulse (128). This suggests that although LH pulses are highly suppressed under these hormonal conditions, NKB is still able to stimulate a pulse (128), a finding different from sheep in which senktide had no effect during the luteal phase when pulses are also inhibited (137). This may be attributed to a species difference, or perhaps the use of NKB rather than senktide. Similar to the stimulatory actions of senktide in sheep, in juvenile male monkeys, senktide stimulates LH secretion (127, 141). The actions of senktide on LH release in rodents, however, appear to be dependent on hormonal milieu. In OVX mice and rats treated with low E2, senktide suppresses LH secretion (142-144). However, in male mice (145), intact female mice and rats (137, 146), and OVX mice with physiological (proestrus levels) of E2 (137), senktide exerted stimulatory actions on LH secretion.

In general, ARC KNDy neurons express NK3R (126, 147, 148), whereas GnRH neurons do not (147, 149), although in rats, a small subset of GnRH neurons do co-localize NK3R (150, 151). Therefore, the mechanism by which NKB regulates GnRH release is thought to be indirect, perhaps involving the action of NKB on KNDy neurons (126, 129). However, NKB expression and/or release across the estrous cycle or under different hormonal conditions has not been systematically characterized in any species. Internalization of NK3R and other G-protein coupled receptors can be used as a marker of receptor activation through ligand binding to the receptor (152-155), to give insight into endogenous NKB release. This will be the focus of Chapter 7.

1.3.2. Role of Dynorphin in Steroid Feedback

Dynorphin (Dyn) is an endogenous opioid peptide (EOP) that is highly co-expressed with PR in the ARC (112, 156), and is thought to mediate the negative feedback effects of progesterone on GnRH pulse frequency, acting via the kappa opioid receptor (KOR) (156-158). Evidence from sheep, goats and mice using Dyn or KOR receptor antagonists have demonstrated an inhibitory action of Dyn on LH secretion (126, 128, 158). Consistent with a role for ARC Dyn in mediating negative feedback, OVX sheep (157) and postmenopausal women (159), show decreased mRNA for the precursor

peptide, preprodynorphin, in the ARC (or infundibular nucleus in humans). KOR is present in a subset of ARC KNDy neurons in mice (126), and GnRH neurons appear to be devoid of KOR (160, 161), which suggests that the action of Dyn to suppress LH secretion may be, at least in part, via KNDy neurons (162).

1.3.3. Projections of the KNDy neurons

Identifying the projections of KNDy neurons is an important step in our understanding the circuitry underlying control of GnRH secretion. A number of anatomical studies have identified the projections of each of the individual KNDy peptides within the hypothalamus and to GnRH cell bodies and terminals in the ME (72, 74, 124, 129, 158, 163-165). However, because there are other populations of cells, a limitation of these studies is that it is not clear whether these terminals are derived from the KNDy neurons, or other single-labeled populations that may project to GnRH. Since co-expression of the three peptides is exclusively seen in the KNDy population, dual- and triple-label immunocytochemistry for two or more KNDy peptides has been used as an approach to delineate projections that can be traced back to a KNDy origin. Most of the data in humans, monkeys and rodents have identified projections from KNDy neurons to GnRH terminals in the ME (71, 103, 127, 166, 167). By contrast, there have been very few studies of KNDy projections to GnRH cell bodies, with the exception of a study in humans by Molnar et al. (2012) in which they found very few KNDy (Kisspeptin/NKB) labeled inputs to GnRH cell bodies (168). In sheep, the projections of the KNDy network to GnRH neurons in the hypothalamus or terminals had not been characterized. This will be the focus of Chapter 4.

An interesting feature of the KNDy population is that they appear to comprise an interconnected network, with KNDy-labeled terminals in close apposition to ARC KNDy cell bodies in rodents and sheep (148, 166, 169). Coupled with evidence for autoregulatory NKB and Dyn receptors in KNDy neurons (126, 147, 148), these interconnections may play an important role in the coordination of this population during pulsatile secretion, or during the preovulatory surge. In addition, ARC KNDy cells colocalize the vesicular glutamate transporter, vGlut2 in rats and mice (163, 170), suggesting that they are glutamatergic. Based on this evidence for interconnections

among the KNDy network, and the co-localization of glutamatergic markers within this population, glutamate release onto the KNDy neurons may be an additional signal to coordinate this population (129) and that KNDy projections may constitute at least some of the glutamatergic inputs to kisspeptin cells. This will be addressed in Chapters 5 and 6. Furthermore, changes in steroid feedback to modulate kisspeptin expression in response to E2 or across the estrous cycle (73, 113, 121, 122) may lead to changes in the KNDy reciprocal circuitry, or to alterations in KNDy cell projections to GnRH neurons. This hypothesis will be tested in Chapters 5 and 6.

1.4. Goal

Our overarching goal is to delineate the functional organization of KNDy cells and their connections to GnRH neurons, including possible morphological changes that occur during the estrous cycle and may underlie the ability of KNDy cells to mediate negative and positive feedback control of GnRH secretion. All studies except Chapter 6 utilize sheep as the animal model. Use of sheep as a neuroendocrine model has many advantages: because of their large size, large volumes of blood can be frequently sampled over long periods of time, from the hypophyseal portal vessels and peripheral circulation, in fully conscious and unanesthetized animals (171). In addition, estrous cycles are analogous to ovarian cycles of other spontaneously ovulating species, and similar in hormone milieu to the human female menstrual cycle (6).

1.4.1. Objectives

1. To test the hypothesis that KNDy neurons are involved in both pulsatile and surge secretion of LH, using cFos to identify neuronal activation in response to removal of E2-negative feedback and at the time of the LH surge (Chapter 3).
2. To test the hypothesis that KNDy neurons provide direct input to GnRH cell bodies using multiple-label immunocytochemistry and confocal microscopy (Chapter 4).

3. To test the hypotheses that there are a) increased inputs to both KNDy and POA kisspeptin neurons and b) increased glutamatergic kisspeptin inputs to GnRH neurons during the follicular phase of the estrous cycle (Chapter 5).
4. To test the hypothesis that ER α signaling in kisspeptin neurons is critical for E2-mediated changes in presynaptic inputs onto KNDy and preoptic kisspeptin neurons, using transgenic Kiss1-GFP mice and transgenic mice with ER α deletion in Kiss1 cells (Chapter 6).
5. To test the hypothesis that there is an increase in NKB-induced activation of NK3 receptors in the ARC during the follicular phase of the estrous cycle using visualization of NK3R endocytosis as a marker of endogenous NKB binding to the receptor (Chapter 7).

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CHAPTER 2

Anatomy of the kisspeptin neural network in mammals

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2.1. Introduction

Kisspeptin is one of a family of RFamide-related peptides (RFRP) that is now recognized as an essential endogenous regulator of the GnRH neuroendocrine system (1). Kisspeptin and its related peptides are ligands for the orphan G protein-coupled receptor 54 (GPR54, now called Kiss1R), mutations of which produce hypogonadotropic hypogonadism and a delayed or absent puberty in humans (2, 3) and mice (3). Kisspeptin was subsequently shown to be an extraordinarily potent stimulator of GnRH/LH secretion in a variety of species (4-7), and because of the presence of Kiss1R in GnRH neurons (5, 8) and the ability of GnRH antagonists to block the effects of kisspeptin (7), early work quickly suggested that this influence was conveyed directly upon the GnRH neuroendocrine system.

Because of its key role in reproduction, there has been avid interest in identifying the location of kisspeptin neurons, and characterizing the neural circuitry by which kisspeptin acts to stimulate GnRH release and regulate reproductive neuroendocrine function (1). An understanding of the functional role of kisspeptin signalling in the brain depends on the anatomical framework within which kisspeptin acts, i.e., knowing the location of neuronal cell bodies that synthesize the peptide, and their afferent/efferent connections. The primary aim of this review is to update our current understanding of the anatomical organization of the kisspeptin network; in this context, we would note that there has been one previous, excellent review of the neuroanatomy of the kisspeptin system (9). However, in light of the recent addition of information from a wider variety of mammalian species, we viewed it as timely and worthwhile to re-evaluate the range of data reported to see where consistent patterns might emerge concerning the organization of the kisspeptin neural network. In addition, we review anatomical evidence of steroid receptor colocalization in kisspeptin neurons, findings supporting the existence of direct connections between kisspeptin and GnRH neurons, and recent evidence of phenotypic heterogeneity among subsets of kisspeptin cells which may contribute to their physiological functions. Finally, we end with a consideration of current gaps in this knowledge and some suggestions of future studies to fill those gaps.

2.2. Distribution of kisspeptin cells and fibers in the mammalian brain

The location of kisspeptin cell bodies in the mammalian brain has been examined by two primary techniques: *in situ* hybridization (ISH) to detect cells expressing *Kiss1* mRNA transcripts, and immunocytochemistry (ICC), using either fluorescent or histochemical detection methods, to visualize kisspeptin peptide (Table 2.1). Initially, the use of ICC to detect kisspeptin-positive cell populations and fibers was confounded by the use of antibodies that cross-reacted with other members of the RFRP peptide family (10). More recently, an antibody generated by Caraty and colleagues targeted against the C-terminal end of kisspeptin has been shown to be specific in a number of species both by careful preabsorption controls (11-13) and the use of *Kiss1* knockout mice as negative controls (11). Studies using other kisspeptin antibodies have performed similar controls (14-16). Thus in our analysis of the location of kisspeptin cells and fibers (Tables 2.1 and 2.2) we have omitted ICC studies that utilized antibodies which have been shown to cross-react with other RFRP peptides (e.g., from Phoenix Pharmaceuticals) and where appropriate controls for such cross-reactivity are lacking.

The most consistent population of kisspeptin neurons identified across different mammalian species is the group located in the arcuate (ARC) nucleus (infundibular nucleus in humans). To date, this cell group has been identified, either by ISH or ICC, in humans, monkeys, sheep, rats, mice, hamsters, goats and horses (see Table 2.1 for references). In rodents, this population appears to be distributed throughout all rostral-caudal levels of the ARC (17, 18), whereas in sheep, primates, goats and horses, they are located primarily at middle and caudal levels of the nucleus (12, 13, 16).

In addition to the arcuate population, kisspeptin cell bodies have also been identified in the preoptic region by ISH or ICC in humans, monkeys, sheep, rats, mice and hamsters (Table 2.1). There are species differences in the precise location, and neurochemical phenotype, of kisspeptin cells in this region. In mice, rats and hamsters, preoptic kisspeptin cells are located in the anteroventral periventricular nucleus (AVPV) and extend as a continuum into the adjacent periventricular preoptic nucleus (PeN) (17-19); as discussed in more detail below, a subset of AVPV kisspeptin cells colocalize tyrosine hydroxylase (20), galanin (21), which are present in other AVPV cells as well. In the sheep, monkey and human, kisspeptin cells are located at similar rostral-caudal

<i>Species</i>	<i>ARC</i>	<i>Preoptic Region</i>		<i>ME</i>	<i>DMH</i>	<i>VMH</i>	<i>Medial Amygdala</i>	<i>BNST</i>	<i>ISH Reference</i>	<i>ICC Reference</i>
		<i>POA</i>	<i>AVPV^a</i>							
Human	+++	+							(22)	(34)
Monkey	+++	++		+				+	(7, 22, 23)	(16, 35)
Sheep	+++	++			+	+			(24-27)	(12, 13, 26, 27, 36)
Rat	+++		++						(5, 19, 20, 28, 29)	(29, 37, 38)
Mouse	++		++		+		+	+		(11, 39)
Hamster	++		++						(8, 17, 18,	(14, 40)
Goat	+++								30, 31)	(15, 41)
Horse	+++				+				(32, 33)	(42, 43)

Table 2.1. Distribution of kisspeptin/*Kiss1* cells in the mammalian nervous system.

+++, large (50-150); ++, moderate (15-50); +, few (<15 or numbers not reported);

^a Includes cells in PeN; ^b A. Caraty antibody used for ICC detection

Species	ARC	POA	AVPV ^a	Int. ME	Ext. ME	PVN	DMH	SON	BNST	Lat. Septum	Other	Ref
Human	++	++	++	++	+	+	+			+	+ ^b	(34)
Monkey	++	+		++	+							(16, 35)
Sheep	++	+		++	+		+					(12, 13, 26)
Rat	++	+	+	++	+	++	+	+	+	+	+ ^c	(28, 29, 37, 38)
Mouse	++		+	++		+	+	+	+	+	+ ^d	(11, 39)
Hamster	++											(40)
Goat	++	+		++	+							(15, 41)
Horse	++	+		++								(42)

Table 2.2. Distribution of kisspeptin fibers in the mammalian nervous system.

++, dense fibers; +, moderate or few fibers; PVN, paraventricular nucleus; Int.ME, internal zone of the median eminence; Ext. ME, external zone of the median eminence.

^a Includes fibers in the median preoptic nucleus, AVPV, and PeN; ^b Other regions include: VMH, lateral hypothalamic area (LHA); ^c Other regions include: suprachiasmatic nucleus, septohypothalamic area, medial septum; ^d Other regions include: medial amygdala, periaqueductal gray, locus coeruleus.

levels in the preoptic area, although they are not directly adjacent to the third ventricle and appear to be more scattered than kisspeptin cells in the AVPV (12, 13, 23, 34). Furthermore, in the sheep, there appears to be no clear homolog to the AVPV, since the other neurochemical cell types that comprise this nucleus (e.g., dopamine, galanin (44)) are not present as a well-defined cell group in the periventricular preoptic region (Lehman, unpublished observations). Whether the kisspeptin cells of the AVPV in rodents, and of the preoptic area (POA) in sheep and primates, are homologous to each other remains an open question and awaits use of additional markers. For the purpose of this review, we will refer to these subsets of neurons independently as the AVPV and POA populations, and consider both of them as cell groups in the ‘preoptic region’ (Table 2.1). It should be noted, however, there are a few species examined to date in which the presence of an AVPV, POA or other preoptic kisspeptin population has yet to be confirmed. For example, in goats, kisspeptin cells were not observed in the preoptic region despite the presence of large numbers of cells in the ARC in the same brains (15, 41). However, these studies were performed using castrated male animals, and since kisspeptin expression in the AVPV and POA appears to be dependent on the presence of gonadal steroids (see below), these cells may not have expressed sufficient amounts of peptide to be detectable in castrated males. In the horse mare, one study (43) did identify POA neurons but used an antibody that has been questioned with regards to specificity (13); another study in the female horse which used the more specific Caraty antibody (42, 43) failed to detect POA kisspeptin neurons. Hence the question of whether a kisspeptin cell population is present in the preoptic region of all mammals remains to be determined.

Total kisspeptin cell number appears to differ between the ARC and preoptic region populations, with greater numbers of cells seen in the ARC than POA in humans (22) and sheep (25), in the ARC than AVPV in rats (19, 20, 28) based on ISH. It should be noted that the gonadal steroid, estradiol, has, in general, an opposite effect on each of these populations, stimulating *Kiss1* mRNA and peptide in the preoptic region¹ and

¹ An exception to this is in the monkey, where quantitative PCR showed no difference in *Kiss1* mRNA levels in the POA between ovariectomized and gonadally-intact female rhesus monkeys.

inhibiting it in the ARC (18-20, 26). Thus differences between the number of preoptic region and ARC kisspeptin cells detected in these studies might simply be a reflection of the hormonal status of the animals used. However, even when the influence of steroidal milieu is taken into account (e.g., comparing kisspeptin cell number in the ARC of ovariectomized animals with preoptic region cell number in OVX animals treated with estradiol), the absolute number of detectable kisspeptin cells is higher in the arcuate nucleus than the preoptic region. For example, the POA of estradiol-treated OVX sheep during the breeding season contains approximately 100 *Kiss1*-expressing cells (26); by contrast, the ARC of OVX sheep contains more than 400 cells (26). Similarly in the rat, the number of *Kiss1*-mRNA expressing neurons in the AVPV of estradiol-treated OVX females contains approximately 120 cells (20) whereas the ARC of OVX females without steroid treatment contains approximately 200 *Kiss1* cells (20). Thus, it appears that the ARC kisspeptin cell population contains consistently greater numbers of cells than the kisspeptin population in the preoptic region, even though the level of kisspeptin expression in these cells is influenced by gonadal hormones.

In addition to neuronal populations in the ARC and preoptic region, there are a few additional, smaller populations of kisspeptin cells that have been reported, and are variable among species. Perhaps the most controversial of these is a small group of scattered kisspeptin-immunoreactive neurons in the dorsomedial hypothalamus (DMH), that is seen in the brains of sheep (12), mice (11, 39) and horse mares (42), but not in the rat (20, 38) or hamster (14). Although the genuine nature of kisspeptin localization in these cells was originally questioned because of their detection by non-specific antibodies that cross-reacted against RFRP3 cells in this region, as well as the failure to detect them by ISH, recent ICC studies have confirmed their presence in mice, sheep and horses, using the highly-specific Caraty antibody. The inability to detect these cells by ISH may be due to either their few number and scattered distribution, and/or to low levels of mRNA expression. In addition to kisspeptin cells in the DMH, in sheep and horses, a few kisspeptin-immunoreactive cells have also been reported in the ventromedial hypothalamic nucleus, however, in the sheep, these were detected using ISH and have not been confirmed with ICC using the Caraty antibody (24), and in the horse (43), were detected with an antibody questioned for its specificity (43). In the monkey, a small

number of kisspeptin cells extend from the ARC population directly into the median eminence (16), and in the human, kisspeptin neurons have been identified in the infundibular stalk (34). Finally, there is strong evidence from ISH studies in the mouse that a distinct population of kisspeptin cells exists outside the hypothalamus, in the medial amygdala (17, 30) and BNST (17). Kisspeptin cells in the medial amygdala have not yet been identified in other species, however, a small number of *Kiss1* expressing cells in the BNST have been recently reported in the female rhesus monkey (23). The presence of kisspeptin-immunoreactive cell bodies in the medial amygdala or BNST have not yet been reported, although there are kisspeptin-positive fibers in both regions of the mouse (11, 39).

In addition to hormonal influences, there is evidence of clear sexual dimorphism in kisspeptin expression in all species examined to date, and gender is a factor that needs to be taken into account when evaluating the presence or absence of specific populations using either ISH or ICC. In rodents, the AVPV kisspeptin population is sexually differentiated, with females expressing a significantly greater number of *Kiss1*/kisspeptin-ir neurons than males (19, 32, 39). This dimorphism cannot be accounted for by differences in the adult hormonal milieu, because both intact and gonadectomized males and females show this sex difference, as well as gonadectomized males and females replaced with the same gonadal steroid (20, 28). Thus differences in the AVPV are likely due to the organizational influence of gonadal steroids during development (45). In contrast to the AVPV population, kisspeptin cells in the ARC of rodents show no sex difference in their number. However, in sheep, both POA and ARC kisspeptin populations show sex differences, with greater numbers of cells in ewes than rams (36). The sex difference in the ARC population also appears to be due to organizational effects of gonadal steroids since ovariectomized pubertal ewes show greater numbers of cells than castrated rams (46), but steroid replacement studies have yet to be done. In addition, sex differences in the same direction have recently been reported in the infundibular nucleus of humans with greater numbers of kisspeptin cells in females than males (34). In the human preoptic region, kisspeptin-immunoreactive neurons were consistently visualized in females, while none were seen in any of the male brains examined (34). Sex differences in kisspeptin cell number in other species, including monkeys, have not yet

been reported, nor is it known whether the differences reported are due to gender-related cell death, as in the case of the sexual dimorphic nucleus of the preoptic area (47), or due to changes in gene/peptide expression. The reason for the difference in which kisspeptin populations are sexually differentiated between rodents, and sheep and humans, may lie in the functional roles that these areas play in the preovulatory GnRH surge, which is present in females, but not males. In rodents, the AVPV has been shown as a critical region driving the preovulatory GnRH surge, as lesions of this area prevent the estradiol-induced surge (48), whereas the ARC region has been implicated as essential for the steroid-induced preovulatory surge in sheep (49) and primates (50, 51). Thus, sexual differences in the ARC (infundibular) kisspeptin population in sheep and humans may reflect the importance of this cell group in the generation of the GnRH surge, compared to rodents in which the AVPV plays the predominant role.

Axonal fiber projections arising from kisspeptin cell populations have been analyzed by ICC in a range of species (Table 2.2). Kisspeptin fibers are reported consistently in the same regions where a majority of kisspeptin cell bodies are located, namely the ARC and preoptic region, with denser kisspeptin fibers reported in the ARC than in the preoptic region for all species. Besides the ARC, the densest accumulation of kisspeptin fibers is seen in the internal zone of the median eminence (12, 34, 35, 38, 39, 41, 42); In sheep (12), monkeys (16), rats (38) and goats (41), kisspeptin fibers have also been seen in the external zone of the median eminence where GnRH fibers terminate on portal vessels. However, it is noteworthy that in each of these species, axons and terminals in the external zone are much fewer in number and density than the kisspeptin fibers in the internal zone of the median eminence. A caveat is this observation may reflect more active release (and depletion) of peptide from fibers in the external than internal zone; comparison of kisspeptin fiber staining in the external zone under different endocrine conditions, presumably reflecting different patterns of endogenous kisspeptin release, might be useful in addressing this possibility.

Thus far, the mouse, rat and human, are the only species in which kisspeptin fibers have been thoroughly mapped outside of the ARC, POA and median eminence, and studies in each of these species have used the Caraty antibody. The overall comparison reveals many areas where kisspeptin fibers are found in common in mouse,

rat, and human; these include the ARC, AVPV (including PeN), internal zone of the median eminence, PVN, DMH and lateral septum (Table 2.2). However, there are some differences: for example, in the mouse but in the rat, kisspeptin-positive fibers are seen in the paraventricular nucleus of the thalamus, medial amygdala, periaqueductal gray, and locus coeruleus (11, 38). In the rat but not the mouse, kisspeptin fibers were reported in the suprachiasmatic nucleus and septohypothalamic area (38), while in humans, kisspeptin fibers are seen within the VMH (34) while in the rat (38) and mouse (11), fibers surround the VMH but do not enter it. Since these studies used antisera with the same specificity (Caraty anti-Kp-10 #564 and 566), and since the immunocytochemical protocols were largely the same, there may be genuine species difference in the distribution of kisspeptin fibers in these regions. As discussed below, the discovery of a unique set of neuropeptide markers of the ARC kisspeptin populations (13, 41, 52) has made it possible to use multiple-label ICC to map out fiber projections specific to the ARC population, along with identification of its postsynaptic targets.

2.3. Steroid receptor colocalization in kisspeptin neurons

A substantial body of work has implicated kisspeptin neurons as primary mediators of gonadal steroid feedback control of GnRH release in mammals (53-55). One of the major pieces of evidence for this role is the high degree of colocalization of kisspeptin cells with gonadal steroid receptors, specifically those for estradiol, progesterone and testosterone (Table 2.3). In general, studies using multiple-label ISH or ICC to evaluate colocalization have revealed fairly similar pictures of the extent of colocalization in different species. For example, in the ARC, studies in rats, mice and sheep reveal a similar high degree of colocalization of estrogen receptor-alpha (ER- α), progesterone receptor (PR), and androgen receptor (AR) in kisspeptin neurons, ranging from 70-99% (Table 2.3; Figure 2.1A-C). In the sheep POA, approximately 50% of the kisspeptin neurons coexpress ER- α (12), and in the rodent AVPV, a range from 62-99% in colocalization of ER- α and PR has been reported (18, 19, 28, 56). The difference between sheep and rodents may be due to the different techniques employed (ISH in most rodent studies vs. ICC in sheep), or may reflect species differences in the functional roles that these populations may serve (i.e., the preovulatory GnRH surge). Nonetheless, there

<i>Species</i>	<i>ARC</i>				<i>POA</i>			<i>AVPV</i>		
	<i>ER-α</i>	<i>ER-β</i>	<i>PR</i>	<i>AR</i>	<i>ER-α</i>	<i>ER-β</i>	<i>PR</i>	<i>ER-α</i>	<i>ER-β</i>	<i>PR</i>
Sheep	93 ^a		86 ^b , >95 ^c	>85 ^d	50 ^a					
Rat	92 ^e , 70 ^f	11 ^f						90 ^e , 62 ^f	21 ^f	
Mouse	>99 ^g , 88 ^h	25 ^g						99 ^g , 65 ⁱ	31 ^g	67 ⁱ

Table 2.3. Percentage of kisspeptin/*Kiss1* cells colocalizing gonadal steroid receptors in female mammals. ^a Franceschini et al., 2006, ICC; ^b Smith et al., 2007, ISH; ^c Cheng et al., 2010, ICC, data from males and females; ^d Lehman, unpublished, ICC; ^e Adachi et al., 2007, ISH; ^f Smith et al., 2006, ISH; ^g Smith et al., 2005a, ISH; ^h Smith et al., 2005b, ISH; ⁱ Clarkson et al., 2008, ICC

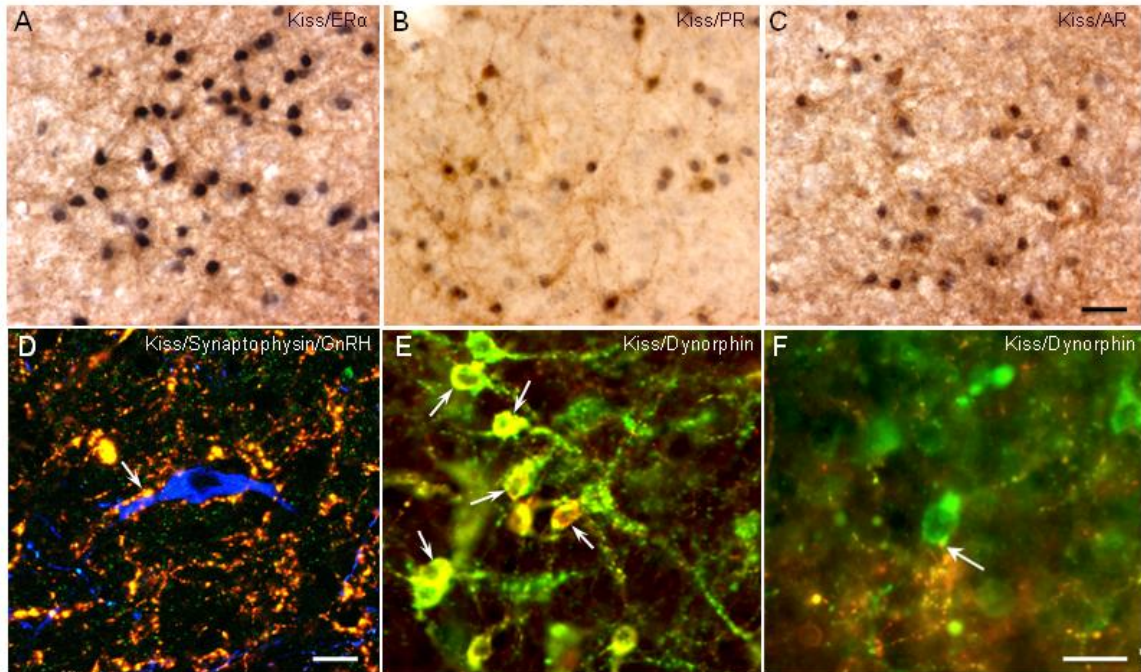


Figure 2.1. Light microscopic and confocal images showing receptor and peptide coexpression in kisspeptin cells, and kisspeptin inputs to GnRH. A-C: Colocalization of gonadal steroid receptors in kisspeptin neurons. Dual immunostained sections of sheep ARC showing high degree of colocalization of nuclear ER- α , PR and AR (*blue-black*) in kisspeptin cells (*brown*). Bar = 50 μ m. D: Kisspeptin synaptic contacts onto a GnRH neuron. Confocal optical section (1 μ m thick) of a triple-labeled section showing a terminal labelled with both kisspeptin (*red*) and synaptophysin (*green*) in direct contact with an ovine GnRH (*blue*) cell body (modified from Smith et al., 2008). Bar = 10 μ m. E-F: Phenotypic heterogeneity between ARC and preoptic kisspeptin neurons. E: Colocalization of the endogenous opoid peptide, dynorphin (*red*), in kisspeptin neurons (arrows; *green*) of the sheep ARC. F: By contrast, kisspeptin neurons (*green*) in the sheep POA do not colocalize dynorphin even though they receive input from dynorphin-positive fibers (arrow; *red*). Scale bar = 20 μ m. (modified from (13)).

is a consistent, high degree of colocalization of ER- α , PR and AR in kisspeptin cells across species, supporting this feature as a key characteristic of the kisspeptin neuronal network. By contrast, the percentage of kisspeptin cells in both the ARC and preoptic region that colocalize estrogen receptor-beta (ER β) is much less, ranging from 11-25% in the ARC and 21-43% in the AVPV (Table 2.3). Thus, the effects of estradiol on both ARC and AVPV kisspeptin populations are likely mediated primarily by ER- α , consistent with evidence that this isoform mediates physiological control of GnRH secretion by estradiol feedback (18, 57). For the most part, other nuclear steroid receptors have not yet been studied for colocalization in kisspeptin cells. One exception is the type II glucocorticoid receptor which has been shown to be present in approximately 50% of dynorphin neurons in the ARC (58); given near complete colocalization of dynorphin and kisspeptin in the ARC (see below), glucocorticoid receptors are almost certainly co-expressed in ARC kisspeptin cells as well.

2.4. Anatomical sites of interaction between kisspeptin and GnRH neurons

Given the expression of the Kiss1 receptor (Kiss1R) within GnRH neurons (5, 8, 59), as well as the demonstration of direct stimulatory effects of kisspeptin upon GnRH cell electrophysiology (8, 45, 60), it has been presumed that kisspeptin neurons must synapse directly upon GnRH neurons. Nonetheless, while a number of studies have shown contacts between kisspeptin fibers and GnRH neurons at a light microscopic level, there is currently no direct electron microscopic (EM) evidence of kisspeptin terminals synapsing directly on GnRH somas or dendrites. Perhaps the best evidence shy of EM comes from dual-label studies using the confocal microscope where optical sections of 1 micron or less in thickness can be analyzed for close associations between kisspeptin and GnRH neurons. Thus far, confocal images of kisspeptin terminals in direct apposition to GnRH cell bodies have demonstrated in monkeys, sheep, mice and horses (16, 26, 39, 42). As in the case of other kisspeptin fibers, it should be noted that the detection of close contacts with GnRH neurons depends on the level of peptide present in those presynaptic terminals, and thus may vary according to gonadal hormone levels. Studies in monkeys (16, 23), sheep (26), and mice (39), have quantified the number of kisspeptin close contacts onto GnRH neurons located in either the POA and/or mediobasal hypothalamus

(MBH). In the case of POA GnRH neurons, evidence from female mice (39) and sheep (26) show that approximately 41-55% of GnRH cells receive at least one kisspeptin positive close contact; in the female sheep this percentage is much higher (95%) for GnRH cells located in the MBH. However, in female rhesus monkeys, the percentage of GnRH cells receiving input is much lower, with approximately 5-15% of POA GnRH neurons, and 20% of MBH GnRH neurons, receiving at least one kisspeptin-positive contact (23). In addition to species and regional differences, there is also evidence for sex differences in kisspeptin inputs onto GnRH neurons. Specifically, Clarkson and Herbison (2006) showed that a greater percentage of POA GnRH neurons in female brains (40%) receive direct kisspeptin contacts than in the male brain (10%) (39). A sex difference in kisspeptin input to GnRH neurons may also be present in the monkey, where 33% of MBH GnRH neurons in the male receive one or more kisspeptin-positive inputs (16), as opposed to 20% of MBH GnRH neurons in the female (23). However, these observations are based on separate studies, and, as in the case of cell number comparisons, studies of sex difference in kisspeptin inputs need to be replicated with comparisons between gonadectomized animals, as well as gonadectomized animals with steroid replacement.

Confocal evidence of close contacts is not the same as direct EM level observations of synapses, but the additional detection of synaptic markers allows confocal multiple-label ICC to be used as a reliable proxy for the presence of synapses. For example, we have shown that direct contacts between synapsin-positive terminals and neurochemically-identified postsynaptic cells, seen under a light microscope in thin, 1 μm , sections, are always predictive of synapses when the same material is viewed at an EM level (61). Thus, analysis of 1 μm thick confocal sections that are triple-labeled for kisspeptin, synaptophysin (another synaptic marker), and GnRH, should provide strong evidence of synaptic inputs onto those cells. Using this approach, we have demonstrated that almost all kisspeptin close contacts on ovine GnRH neurons in the MBH are also synaptophysin-positive, and thus likely represent bona fide synaptic inputs (Smith et al., 2008; Figure 2.1D). This same approach should be very useful for assessing the relative contribution of kisspeptin compared to other types of inputs (e.g., GABAergic) onto

GnRH neurons, and the variation in that array of inputs with respect to sex, endocrine status, or age.

In addition to contact onto GnRH cell bodies, studies in the rhesus monkey and horse have noted the close associations of kisspeptin fibers with GnRH terminals in the external zone of the median eminence (16, 42). Anterograde tracing from neurokinin B cells in the ARC (that colocalize kisspeptin, see below) in rodents (62), as well as retrograde tracing studies from the median eminence in the sheep (53), confirms this projection, and suggest that the ARC population is a major source of this kisspeptin input onto GnRH terminals. The ability of kisspeptin to affect the release of GnRH from murine hypothalamic slices lacking GnRH cell bodies has provided evidence for the median eminence as a potential site of action for kisspeptin in its control of GnRH secretion (63). Observations of close interactions between kisspeptin and GnRH fibers in the external zone provides an anatomical substrate for this site of action; however, as noted above, the number of kisspeptin-positive fibers in the external zone of the median eminence is sparse and variable among species, especially compared with the high density of these fibers in the internal zone (Table 2.2). It may be that if kisspeptin acts upon GnRH terminals in this region it does so via paracrine signaling, with kisspeptin released in the internal zone diffusing to the external zone. The possibility of paracrine signaling within the median eminence has been noted in older work where tancytes and other glial cells elements have been postulated to mediate the transport of molecules across internal and external zones (64, 65).

2.5. Heterogeneity among kisspeptin populations

Recent evidence suggests that not all kisspeptin neurons are the same phenotypically, and, that some of these anatomical differences may underlie functional differences in the role of specific kisspeptin population in positive and negative steroid feedback controls of GnRH secretion (66, 67). In particular, there is consistent evidence in the mouse, rat, sheep, goat, and human that kisspeptin cells in the ARC, but not in the POA/AVPV, colocalize two other neuropeptides shown to be important in the control of GnRH secretion, neurokinin B (NKB) (68) and dynorphin (DYN) (69) (Figure 2.1E-F; Figure 2.2). Nearly all ARC kisspeptin neurons in these species colocalize NKB and

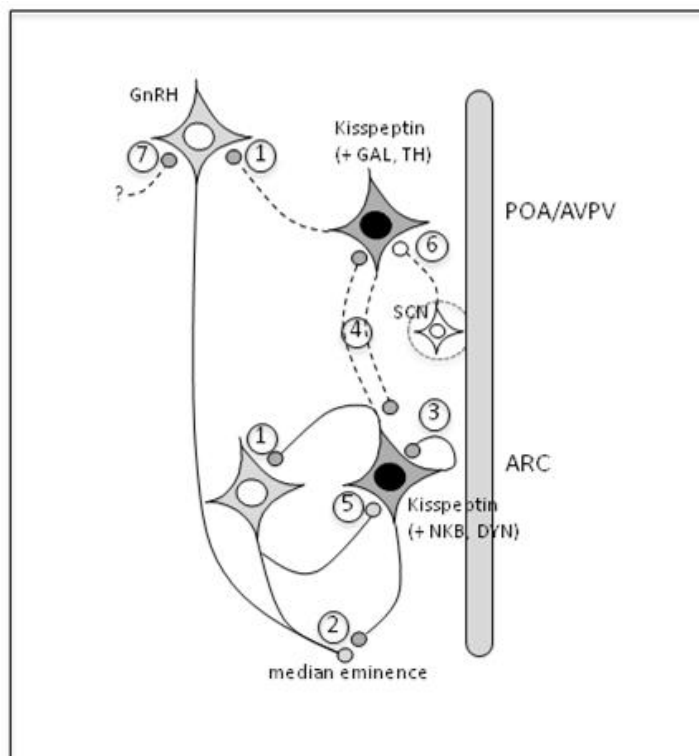


Figure 2.2. Schematic of a horizontal section (top=rostral, bottom=caudal) showing the POA/AVPV and ARC kisspeptin populations in the mammalian hypothalamus, and their potential sites of interactions with GnRH neurons. Virtually all ARC kisspeptin neurons co-express neurokinin B and dynorphin (13, 41, 52), while subsets of AVPV kisspeptin neurons in the preoptic region express either galanin (21) or tyrosine hydroxylase (20). Connections (solid lines, published data; dotted lines, unpublished data) and sites of interactions between kisspeptin and GnRH systems include: 1) Direct projections from ARC and AVPV kisspeptin cells onto GnRH cell bodies (16, 26, 37, 39, 53, 70); 2) Inputs from ARC kisspeptin cells onto GnRH terminals in the median eminence (16, 53, 70, 71); 3) reciprocal connections among ARC kisspeptin cells that could be from the same or adjacent neurons (41, 62, 71, 72); 4) Projections from ARC kisspeptin neurons to POA kisspeptin cells in the sheep (Lehman, unpublished), and from AVPV kisspeptin neurons back to the ARC (73); and 5) projections from GnRH neurons back onto ARC kisspeptin cells (16). In addition, afferents to POA/AVPV kisspeptin cells from the suprachiasmatic nucleus (SCN) are indicated (6) (74), as well as the possibility that

kisspeptin inputs to GnRH neurons in the POA or MBH may arise from other populations such as the DMH, BNST or medial amygdala (7).

Dyn (13, 53) because of this, and for convenience, we have termed this cell population, the KNDy (Kisspeptin, Neurokinin B, Dynorphin) cells (36). KNDy cells likely play multiple roles in control of GnRH secretion. Evidence from sheep and rodents suggest they are critical for conveying the negative feedback influence of estradiol and progesterone onto GnRH neurons (25, 69); in the sheep they may also play a role in the positive feedback influence of estradiol to induce the preovulatory GnRH surge (27, 53). Another interesting characteristic of KNDy cells in the ARC is that they possess extensive reciprocal connections with each other, confirmed at both light microscopic and EM levels, forming what appears to be an interconnected network (62, 71, 75). Reciprocal connections appear much less abundant among POA kisspeptin neurons in sheep (53), which, if homologous to preoptic kisspeptin cells in the rodent, correlates with the lack of effect of kisspeptin on the electrophysiological firing of AVPV kisspeptin neurons in the mouse (76). Thus, the reciprocal coupling of the ARC kisspeptin population, together with the colocalization of NKB and dynorphin receptors, may be critical in enabling these neurons to fire synchronously with each other. Observations of rhythmic, multiunit activity recorded from the location of kisspeptin neurons in the ARC is consistent with this (15), and the temporal association of these electrophysiological rhythms with GnRH/LH pulses has led to speculation that they comprise a critical component of the “GnRH pulse generator” (41, 52, 53). Observations of changes in the shape of each GnRH pulse in response to an opioid antagonist (77) and alterations in both multi-unit activity (41) and LH pulse frequency induced by treatment with opioid (Dyn) and NK3R (NKB) antagonists (78) are consistent with this hypothesis.

The presence of the unique set of KNDy neuropeptides for the ARC kisspeptin population has provided the opportunity to use multiple-label ICC to analyze the projections of this population, including its potential inputs to GnRH neurons. Thus far, studies in the rat have used a combination of dynorphin and NKB (Burke et al, 2006), as well as the combination of kisspeptin and NKB (79), to trace projections from KNDy cells. Dual-labeled projections from KNDy neurons have been reported in each of the areas shown in Table 2.2 where single-labeled kisspeptin fibers are found, albeit in lesser numbers. In addition, dual-labelled KNDy fibers are found in the external zone of the median eminence (71), and dynorphin fibers can be seen in direct contact with GnRH

terminals in the median eminence at an electron microscopic level (53). In addition, the colocalization of dynorphin and NKB in axon terminals has been used to show direct contacts of KNDy neurons onto GnRH cell bodies in the sheep (53).

While kisspeptin cells in either the POA or the AVPV do not express either neurokinin B or dynorphin (13, 52), there is evidence that a majority of AVPV kisspeptin population in the rodents colocalizes galanin, a neuropeptide implicated in female reproductive function (21), and that a subset also colocalizes tyrosine hydroxylase, a marker for dopaminergic neurons (20) (Figure 2.2). Subsets of AVPV neurons also express GABA and glutamate, and the extent to which either AVPV or POA kisspeptin neurons may colocalize these neurochemicals has not yet been examined. However, neurotensin, which is also expressed by AVPV neurons, is not colocalized with kisspeptin (80). Kisspeptin cells of the amygdala (30) have not yet been examined for the co-expression of other neuropeptides/transmitters, although based on studies of the phenotype of cells in the medial amygdala that project to the preoptic area and GnRH cells, it seems likely that some of these may colocalize the neuropeptides, cholecystokinin and/or substance P (81).

2.6. Summary and Future Directions

Key features of the kisspeptin neural network and its interactions with GnRH neurons, based on our current knowledge, are summarized in Figure 2.2. Kisspeptin cells are found consistently in two major cell populations, one located in the ARC and the other in preoptic region, in either the AVPV or POA. While the ARC population is highly conserved among species, there is variation in the location and phenotype of preoptic kisspeptin neurons. In rodents, kisspeptin cells comprise a component of the AVPV, but in sheep, monkeys and humans, they appear to be more scattered and an AVPV homologue is not evident. It may be, in fact, that the connections and neurochemical features of the AVPV and its kisspeptin cells are critical for the functional role of this population in rodents, and may underlie differences between rodents and other species in the control of the GnRH surge (48, 49, 51). In addition to the ARC and preoptic populations, there is also evidence for smaller populations of kisspeptin neurons in the DMH, BNST and medial amygdala, but it is not clear whether they are consistently seen across

species, nor has their functional role(s) been identified. Kisspeptin cells of the ARC and preoptic populations differ in their neurochemical phenotype: virtually all ARC kisspeptin cells contain both neurokinin B and dynorphin, while subsets of AVPV kisspeptin cells express galanin and/or tyrosine hydroxylase.

There are several sites of demonstrated and potential direct interactions between the kisspeptin and GnRH neuronal networks (Figure 2.2, numbers 1-2). Evidence from confocal, multiple-label studies using synaptic markers strongly supports the existence of direct synaptic connections onto GnRH cell bodies (26), some of which arise from the ARC kisspeptin population.. Transneuronal tracing studies (57) and studies using conventional tracers (82-84) have demonstrated that cells of the AVPV provide direct input to GnRH neurons; unpublished data (44) suggests that these inputs arise at least in part from AVPV kisspeptin cells, although there are likely also inputs from other neurochemical subsets of the AVPV (glutamate, GABA). In addition, to direct inputs from kisspeptin cells onto GnRH cell bodies, there is also evidence for potential inputs at the level of GnRH terminals in the median eminence. EM observations have confirmed that dynorphin terminals, presumably arising from the ARC, establish direct contacts with GnRH terminals (53). Nonetheless, evidence is still needed at an EM level to confirm that kisspeptin-positive terminals are in direct contact with GnRH terminals in the median eminence, as well as demonstration that Kiss1 receptors are present on the plasma membranes of those GnRH terminals.

A number of anatomical features support the view that kisspeptin cells form a reciprocally-innervated functional network, both within a given region and between different populations, and extending to include GnRH neurons. First, as noted above, kisspeptin cells of the ARC have extensive reciprocal connections with each other (Figure 2.2, number 3) (13, 62, 71, 75). Second, there is evidence that ARC kisspeptin cells (based on dual-labeling for kisspeptin and dynorphin) send direct projections to kisspeptin neurons in the sheep POA (Lehman, unpublished). Further, recent evidence suggests that approximately 40% of AVPV kisspeptin neurons in the mouse, in turn, project to the ARC (73), raising the possibility of reciprocal communication between these two populations (Figure 2.2, number 4). Finally, in the monkey (16) and sheep (Lehman, unpublished), GnRH fibers have been shown to contact ARC kisspeptin

neurons (Figure 2.2, number 5), providing a route for two-way communication between GnRH and kisspeptin populations. Given that GnRH neurons are interconnected morphologically, at the level of their dendrites as well as axon terminals (85), there is therefore potential for kisspeptin input to GnRH neurons, either at the level of their cell bodies or terminals, to influence the coordinated release of GnRH from many distributed neurons.

Many unanswered questions remain concerning the anatomical organization of this network. First, while some information has been obtained about the efferent connections of ARC and preoptic kisspeptin populations, projections from kisspeptin cells in the DMH, BNST and medial amygdala have not yet been examined, including the possibility that one or more of these populations also provides input to GnRH neurons. There is evidence from tract tracing studies that all three areas project to the preoptic region (82, 86, 87) and specifically to GnRH neurons (88-90). Second, the sources of afferent inputs to each kisspeptin population need to be identified. One likely source of afferents to the AVPV kisspeptin population is the suprachiasmatic nucleus (SCN), given evidence that these kisspeptin neurons are involved in circadian regulation of the GnRH surge in rodents (91) (Figure 2.2). In fact, vasopressin and vasoactive intestinal peptide-expressing terminals originating from the SCN make contacts onto AVPV kisspeptin neurons, as shown recently by anterograde tract tracing combined with ICC (74). In the future, transgenic neuron-specific tracing, both anterograde and retrograde (transneuronal), could be used for defining the inputs to each kisspeptin population as it has for leptin-responsive cell populations (92) and GnRH neurons (57, 88, 90), respectively. Third, the identity of other postsynaptic targets of kisspeptin cells, besides GnRH, needs to be examined; for example, recent evidence suggests that kisspeptin plays a role in the regulation of prolactin via direct contacts of kisspeptin fibers onto A12 dopamine cells (93). Finally, it will be important to know which cellular targets of kisspeptin cells actually express Kiss1R. While there is a considerable overlap between regions that express Kiss1R and kisspeptin fiber-immunoreactivity (59), there are some examples of apparent receptor-ligand mismatch. For example, the dentate gyrus of the hippocampus is a region which contains a large number of Kiss1R-expressing cells (59), although kisspeptin fibers have not been identified in that region. Conversely, some areas

that have contain dense kisspeptin fibers, such as the ARC, are devoid of Kiss1R (59). Whether these examples of receptor-ligand mismatch reflects the presence of other as yet unidentified receptors or ligands, or the influence of kisspeptin via extra-synaptic communication routes, remains to be seen.

In summary, converging data from a range of species suggests that the overall organization of the kisspeptin neuronal system in mammals is fairly consistent, and that direct anatomical projections to GnRH neurons, at the level of both cell bodies and terminals, are a common feature. In addition, there is growing recognition that kisspeptin is present as only one of several important peptides/neurotransmitters in this circuitry, and that the neural projections of the kisspeptin network are likely to include other neuroendocrine systems, as well as extend outside the preoptic-hypothalamic continuum. Indeed, the neurochemical and anatomical heterogeneity of the kisspeptin network is likely to be critical in defining the individual functional roles of subsets of kisspeptin neurons, and much important work remains to be done in order to define the structural framework for kisspeptin action in the brain.

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CHAPTER 3

KNDy (Kisspeptin/Neurokinin B/Dynorphin) neurons are activated during both pulsatile and surge secretion of LH in the ewe

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3.1. Introduction

Although the importance of gonadotropin-releasing hormone (GnRH) in reproduction is well known, identification of the pathways through which estradiol (E2) regulates GnRH release remains an area of continuing investigation. Ovarian steroids exert negative feedback on GnRH/LH secretion throughout the luteal and early follicular phase of the estrous cycle (1). During the breeding season of ewes, E2 serves to inhibit LH pulse amplitude while progesterone inhibits pulse frequency (1, 2); however, during anestrus (non-breeding season), E2 inhibits LH pulse frequency (3). In contrast to these inhibitory effects, the preovulatory GnRH/LH surge that occurs at the end of the follicular phase is induced by high levels of E2 secreted from preovulatory follicles (4). Estrogen receptor- α (ER α) is the key receptor in mediating regulation of GnRH by E2 (5). Because few, if any, GnRH neurons express ER α (6), the feedback actions of E2 must occur via other neurons, and recent work points to kisspeptin neurons as playing a key role in this function. Importantly, most kisspeptin neurons express ER α (7) and can directly interact with GnRH neurons, 90% of which express the kisspeptin receptor (8-10).

Kisspeptin, a member of the RF-amide family of neuropeptides, binds to its receptor, GPR54 (11, 12), and stimulates LH release in humans (13) as well as in mice (14), rats (15, 16), sheep (17), and primates (18). A mutation in GPR54 causes hypogonadotropic hypogonadism in humans (19, 20), indicating the importance of the kisspeptin-GPR54 interaction in controlling sexual maturation and reproduction. Kisspeptin has now been identified as a key regulator of GnRH and LH secretion and has been proposed to mediate both negative and positive feedback actions of E2 (21, 22).

Two anatomically distinct kisspeptin populations exist in rodents. Those in the anteroventral periventricular nucleus (AVPV) have been proposed to mediate the E2-induced GnRH/LH surge (23, 24), while kisspeptin neurons in the arcuate nucleus (ARC) are thought to be responsible for E2-induced inhibition of episodic GnRH/LH secretion (21, 24-26). Sheep do not have a distinct AVPV, but kisspeptin neurons are evident in both the preoptic area (POA) and ARC, with some anatomical differences between the two populations. Almost all kisspeptin neurons in the ARC coexpress dynorphin and neurokinin B and, hence, are referred to as KNDy neurons (27). More than 90% of these

neurons receive input from other KNDy neurons, thus forming an interconnected, reciprocal network with one another (28-30). POA kisspeptin neurons in sheep do not exhibit colocalization with dynorphin or neurokinin B, and do not exhibit the same degree of reciprocal connections with each other as seen among KNDy neurons. There also may be functional differences between these two kisspeptin-containing neural populations. The effects of E2 removal (22) and replacement (10, 22) on Kiss1 mRNA expression implicate ARC kisspeptin neurons as important mediators of E2-negative feedback. However, the role of both POA and ARC kisspeptin neurons in the preovulatory LH surge remains unclear. During late follicular phase, elevated Kiss1 mRNA expression has been observed in both the POA and some regions of the ARC (10). However, studies using Fos to analyze neuronal activation of kisspeptin neurons have been inconclusive (10, 31). The purpose of this study was to determine which population(s) of kisspeptin neurons are activated during the LH surge and in response to removal of E2-negative feedback, using Fos as an index of neural activity. Because anatomical subsets of ARC kisspeptin neurons have been implicated in the surge (10), and to assess the possibility that different ARC kisspeptin neurons might be dedicated to surge and tonic GnRH secretion, we analyzed Fos expression in rostral, middle, and caudal portions of the ARC, as has been done in previous work (10).

3.2. Materials and Methods

3.2.1. Animals

Adult blackface ewes were maintained under ambient conditions in an open barn with free access to food and water. Three to five days before any experimental procedures, ewes were moved to an indoor facility with controlled photoperiod simulating natural outdoor day length. In this facility, they had free access to water and a mineral lick and were fed a pelleted maintenance diet daily. Estrous behavior was determined using vasectomized rams. Blood samples (3-5 ml) were taken by jugular venipuncture, placed into heparinized tubes, and plasma collected and stored at -20 C. Ewes used for experiment 1 were killed during the breeding season (Oct.-Feb.), and ewes used in experiment 2 were killed during the non-breeding season (May through July). All experimental procedures involving animals were approved by the West Virginia

University Animal Care and Use Committee, and followed the National Institutes of Health guidelines for animal research.

3.2.2. Animal Protocol

Experiment 1. Which kisspeptin neurons are activated during the preovulatory LH surge?

To collect tissue from ewes at specific times of the estrous cycle, their cycles were synchronized with im injections (10 mg) of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Lutalyse; Upjohn, Kalamazoo, MI) to induce regression of the corpus luteum. Once cycles were synchronized, brain tissue was collected on d 9-10 of the luteal phase ($n=4$) or $PGF_{2\alpha}$ was again injected and two controlled internal drug releasing (CIDR) devices were inserted to maintain luteal phase progesterone concentrations (32). Ten days later, the CIDRs were removed and blood samples were collected every 4 h starting 12 h after CIDR removal until perfusion. Tissue was collected 4 h after onset of estrous behavior and, based on LH concentrations, animals were classified into pre-surge ($n=11$) and surge ($n=5$) groups. One ewe included in the surge group was estrous-synchronized with only $PGF_{2\alpha}$ (i.e., CIDRs omitted) and was killed during a clear LH surge.

Experiment 2. Which kisspeptin neurons respond to removal of E2 negative feedback?

This experiment was performed in anestrus, when E2 is the only ovarian steroid inhibiting GnRH pulse frequency (2, 3). Bilateral ovariectomies were performed via midventral laparotomy using sterile procedures on ten anaesthetized (isoflurane) ewes as previously described (33). Tissue from the short-term OVX group ($n=5$) was collected at 24 h post-OVX, and tissue from the long-term OVX group ($n=5$) was collected at least 1 month post-OVX. Tissue from ovary-intact anestrus ewes ($n=5$) was also collected. Blood samples were collected every 12 min for 4 h just before tissue collection in all three groups to determine LH pulse patterns.

3.2.3. Tissue Collection

Tissue collection and processing was performed as previously described (27). Ewes were euthanized via an iv overdose of sodium pentobarbital (~2g in 7ml saline; Sigma,

St. Louis, MO) after two iv injections of heparin (25,000 U), 10 min before and immediately before pentobarbitol. The head was removed and perfused through both carotid arteries with 6 liters of 4% paraformaldehyde containing 10 U/ml heparin and sodium nitrate. After perfusion, brains were removed, and POA/hypothalamic tissue was dissected out. The tissue was then infiltrated with 30% sucrose, and coronal sections (50 μ m thick) were cut on a freezing microtome and stored at -20 C in cryoprotectant for later processing.

3.2.4. Assays

LH was measured in duplicate aliquots of 50-200 μ l of plasma, using a previously validated RIA (33), and expressed in terms of NIH-LH-S12. The minimal detectable concentration of LH in these assays average 0.077 ng/tube; inter and intraassay coefficients of variation were 3.8% and 1.7%, respectively. Circulating progesterone was measured in duplicate aliquots of 150 μ l plasma using a commercially available solid-phase RIA kit (Coat-A-Count P₄, Diagnostic Products, Corp., Los Angeles, CA), which has been validated in sheep (34) to confirm stage of cycle.

3.2.5. Immunocytochemistry for kisspeptin and Fos

All immunohistochemistry was carried out on free-floating sections at room temperature, and sections were washed with 0.1 M PBS between incubations. For all experiments, tissue sections were first incubated in hydrogen peroxide (10 min in PBS; EMD Chemicals, Inc., Gibbstown, NJ) to occupy endogenous peroxidase sites, followed by incubation in a solution containing 20% normal goat serum (Jackson Immunoresearch Laboratories, Inc., Gibbstown, NJ) in PBS containing 0.4% Triton X-100 (Fisher Scientific, Pittsburgh, PA) for 1 h to minimize nonspecific binding.

A dual-label immunoperoxidase procedure was then performed on a series of every fifth POA and ARC section in each animal. Fos-ir nuclei were detected using a rabbit polyclonal antibody against c-Fos (1:1,000 (Exp. 1) or 1:2500 (Exp. 2); sc-52, Santa Cruz Biotechnology, Inc.) for 17 hr, followed by incubation with biotinylated goat anti-rabbit IgG (1:500; Jackson Immunoresearch Laboratories, Inc.) for 1 h and avidin-biotin-horseradish peroxidase complex (ABC-elite; 1:500; Vector Laboratories,

Burglingame, CA) for 1 h. Labelling was visualized using nickel-enhanced diaminobenzidine (Sigma) as the chromogen, producing a black/purple reaction product that is limited to the nucleus. For detection of kisspeptin, sections were incubated for 17 h in polyclonal rabbit anti-kisspeptin-10 serum (1:100,000 (Exp. 1) or 1:50,000 (Exp. 2); #564, gift from A. Caraty, Université Tours, Nouzilly, France) for 17 h, an antibody that has been previously validated for use in sheep tissue (27, 35). Following incubation in primary antiserum, tissue was labelled with biotinylated goat anti-rabbit IgG (1:500; Jackson Immunoresearch Laboratories, Inc.) and ABC-elite (1:500; Vector Laboratories) for 1 h each. Kisspeptin was visualized using diaminobenzidine as the chromogen, which produced a brown reaction product within the cytoplasm. Sections were then mounted onto Superfrost slides, air-dried, dehydrated, and coverslipped using Depex (DPX; Electron Microscopy Sciences, Hatfield, PA).

For each ewe, the numbers of single kisspeptin and dual-labeled kisspeptin/Fos cells were counted under bright field illumination in the POA (three sections per ewe) and ARC (three sections per rostral, middle and caudal subdivisions in each ewe) by an independent observer. POA sections selected for analysis were those at the rostral-caudal level of, and just posterior, to the organum vasculosum of the lamina terminalis. For selection of ARC sections, the rostral ARC was defined as that level of the nucleus anterior to the appearance of the tubero-infundibular sulcus; the middle ARC was at the level of the tubero-infundibular sulcus; and the caudal ARC was at the level of the mammillary recess of the third ventricle. To assess the possibility of functionally distinct subsets of ARC kisspeptin neurons, we analyzed all three rostral-caudal levels of the ARC to account for the possibility that there may be regional differences within the ARC in the functional activation of kisspeptin cells during the surge. To assist with analysis, bright-field images were captured using a cooled charge-coupled device camera (Microfire, Optronics, Goleta, CA) attached to a Leica microscope (DM5000B, Leica Microsystems, Wetzlar, Germany) and Neurolucida software (MicroBrightfield, Inc., Williston, VT) with fixed camera settings for all animals; a transparent grid was then placed over the printed images to aid with counting. A cell was considered dual labeled when brown kisspeptin-positive cytoplasm was seen to surround a black Fos-positive nucleus in the same plane of focus, and the percentage of dual labeling was calculated as

the total number of dual-labeled cells divided by the total number of kisspeptin-positive cells.

3.2.6. Statistical Analysis

The mean number of single-labeled kisspeptin and dual-labeled kisspeptin/Fos cells in each region (POA and rostral, middle and caudal subdivisions of the ARC) were counted for each animal, and the percentage of kisspeptin cells containing Fos was calculated. The mean and SEM were calculated for each group, and one-way ANOVAs were conducted within each region between luteal, pre-surge, and surge groups (Exp. 1), and intact, 1 d OVX, and chronic OVX groups (Exp. 2). Post hoc analyses of all pairwise comparisons were computed using Tukey tests to account for the problem of multiple comparisons. In experiment 2, LH pulses were identified using standard criteria (1) and statistically significant differences in mean LH concentrations analyzed by one-way ANOVA and in LH pulse frequencies by Kruskal-Wallis one-way ANOVA on ranks.

3.3. Results

Experiment 1: which kisspeptin neurons are activated during the preovulatory LH surge?

As expected, mean LH concentrations at perfusion in surge animals increased by seven and five fold from luteal and pre-surge groups, respectively ($P < 0.05$) (Figure 3.1B).

Anatomically, kisspeptin perikarya were localized to the POA and ARC, with the greatest numbers of cell bodies seen within the middle ARC and the fewest number of cell bodies in the POA (Figure 3.2A). In the middle ARC, pre-surge animals had significantly greater numbers of kisspeptin cells compared with luteal phase animals, but not surge animals ($P < 0.05$; Figure 3.2A). There were no significant effects of stage of cycle on kisspeptin cell numbers in any other area. In addition, we noted that labeling of kisspeptin-ir fibers appeared more intense in the pre-surge animals compared with the surge animals (Figure 3.2C).

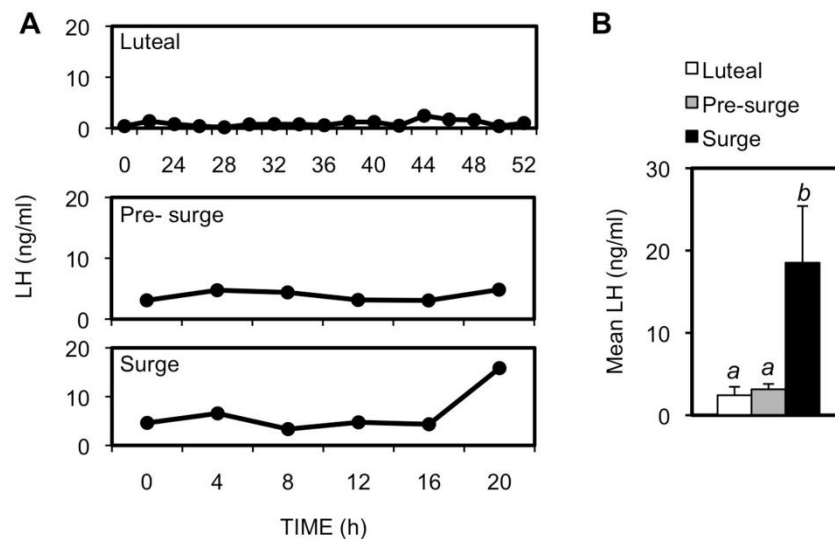


Figure 3.1. Luteinizing hormone (LH) in luteal, pre-surge and surge ovary-intact ewes. A, Representative LH profiles from ovary-intact luteal, pre-surge and surge ewes taken before perfusion. Note that on the y-axis of the presurge and surge LH profiles, time = 0 represents 12 h after CIDR removal. B, Mean LH levels at perfusion were significantly higher in the surge group compared with both luteal and presurge groups. Letters (a and b) represent significant differences from other groups, $P < 0.05$. Data are means \pm SEM.

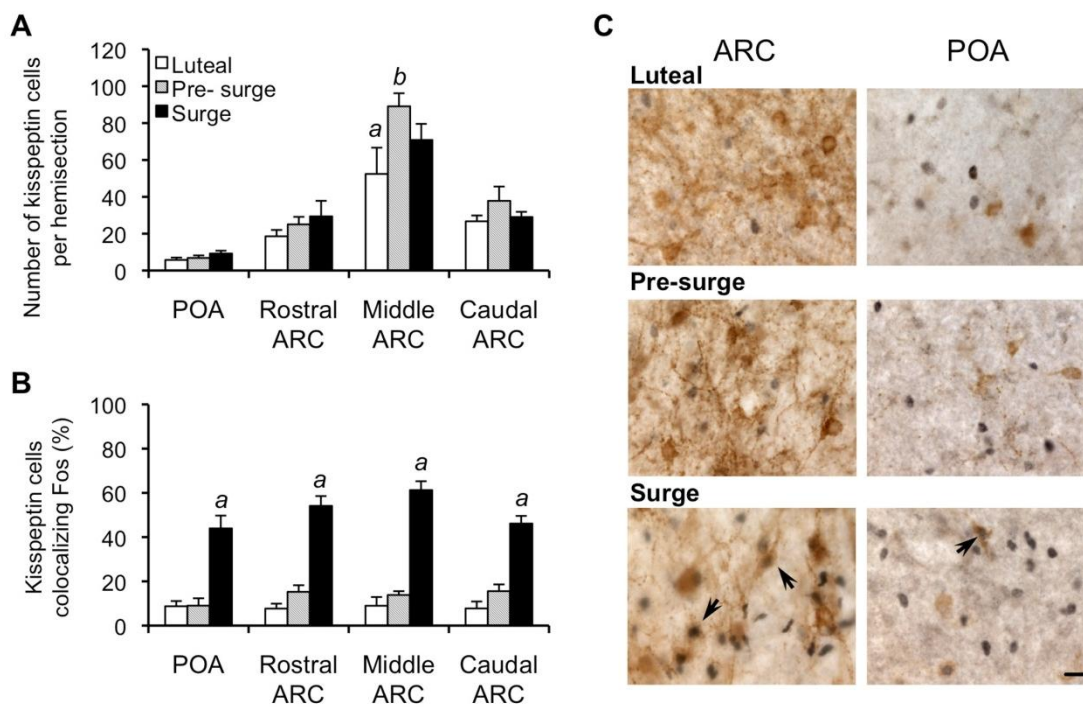


Figure 3.2. Kisspeptin cells in both the ARC and POA are activated during the LH surge. Kisspeptin cell number (A) and percent colocalization with Fos (B) in luteal, presurge and surge groups in the POA, and rostral, middle and caudal levels of the ARC. Letters (a, b, and c) represent significant differences between groups within the same area, $P < 0.05$. C, Sections immunostained for kisspeptin and Fos through the middle ARC (*left column*) and POA (*right column*) from luteal, presurge and surge animals. Arrows show examples of dual-labeled cells. *Scale bar*, 10 μm .

Surge animals showed a significantly greater percentage of kisspeptin cells colocalizing Fos compared to both luteal and pre-surge animals throughout the rostral, middle and caudal divisions of the ARC ($P < 0.05$; Figure 3.2B) and in the POA ($P < 0.05$; Figure 3.2B). The percentage of kisspeptin cells that colocalized Fos in each region of surge animals was consistent, ranging from 44 to 61% in the both ARC (rostral, middle, caudal) and POA, but because there were far fewer kisspeptin cells in the POA, the absolute number of Fos-expressing cells in this region was lower than in the ARC (Figure 3.3). Examples of single- and dual-labeled kisspeptin neurons in the ARC and POA are shown in Figure 3.2C, and their anatomical location is shown in Figure 3.3. The percentage of colocalization of kisspeptin with Fos did not differ between luteal and pre-surge animals in any area (Figure 3.2B).

Experiment 2: which kisspeptin neurons respond to removal of E2 negative feedback?

As expected, LH pulse frequency increased after OVX from 1.0 ± 0.3 pulse/4 h in intact ewes to 2.0 ± 0.3 pulses/4 h 1 d after OVX. Frequency further increased to 4.0 ± 0.4 pulses/4 h in chronically OVX animals (Figure 3.4). Mean LH concentrations also increased three and nine fold in 1-d OVX and long-term OVX ewes, respectively (Figure 3.4B).

In the ovary-intact anestrous ewes, ARC kisspeptin cells were most numerous in the caudal ARC, with moderate numbers present in the middle ARC and POA (Figure 3.5A). After more than 1 month after OVX, kisspeptin cell numbers significantly increased in both the rostral and middle ARC. A similar pattern of increased cell numbers was also observed in the 1-d post-OVX ewes ($P < 0.05$; Figure 3.5A). There were no significant effects of OVX on kisspeptin cell numbers in the POA.

E2 withdrawal significantly increased the percentage of ARC kisspeptin cells colocalizing Fos in the rostral, middle, and caudal ARC after 24 h post-OVX, and this colocalization was further increased in chronically OVX ewes ($P < 0.05$; Figure 3.5B and Figure 3.3). No significant changes in the percentage of kisspeptin/Fos colocalization were observed in the POA at either time point, and this percentage of colocalization remained low (<10%).

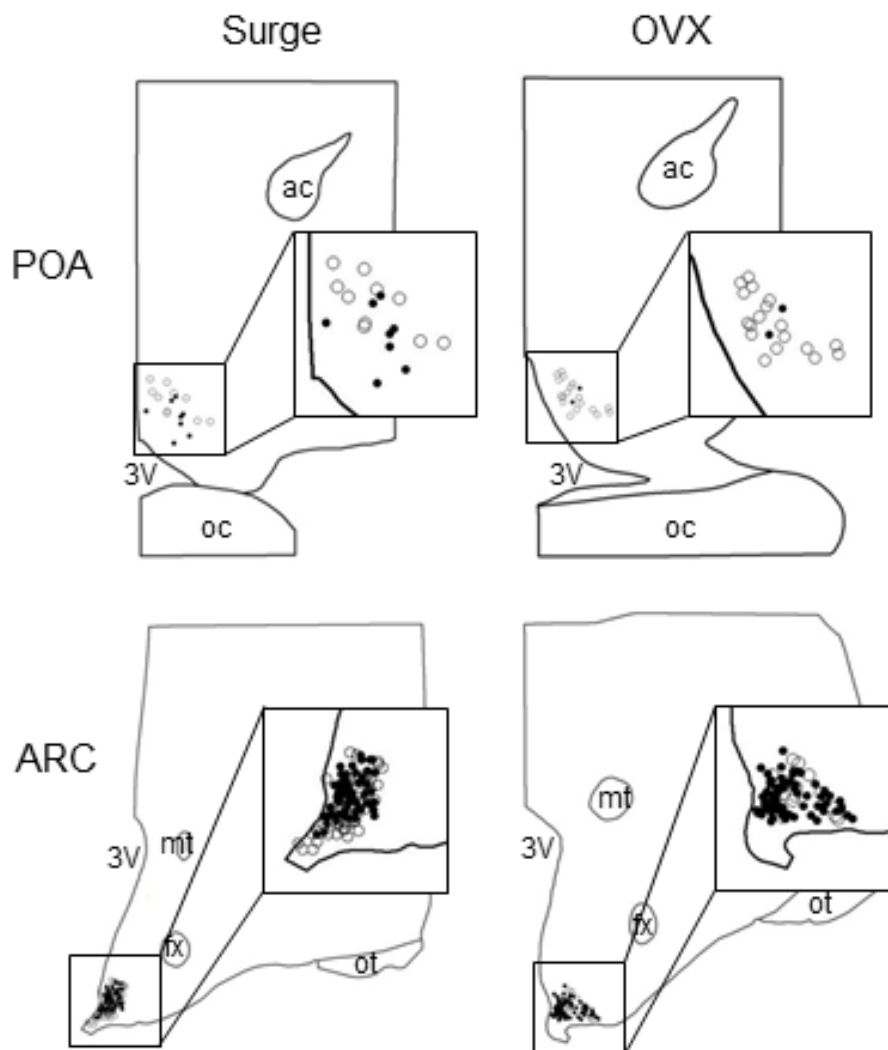


Figure 3.3. Camera lucida drawings of representative sections through the POA (*top*) and ARC (*bottom*) of sheep killed either during the LH surge (*left*) or 30 d after OVX (*right*). The location of single-labeled kisspeptin cells (*open circles*) and dual-labeled Fos/kisspeptin cells (*black circles*) are shown at low and high magnification in each of the drawings (*boxed areas*). ac, Anterior commissure; 3V, third ventricle; fx, fornix; oc, optic chiasm; ot, optic tract; mt, mammillothalamic tract.

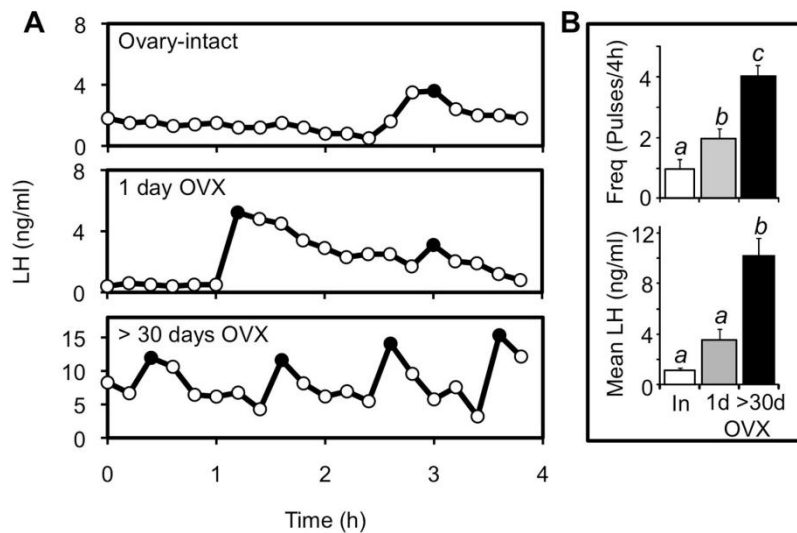


Figure 3.4. Luteinizing hormone (LH) in intact and OVX ewes. A, Representative LH profiles from ovary-intact, 1 d after OVX, and more than 30 d post-OVX ewes. *Black circles* indicate pulse peaks. Note different scale on y-axis for data from ewe OVX for more than 30 d. B, Mean pulse frequency (*top panel*) and LH concentration (*bottom panel*) in each group of ewes. Letters (a, b, and c) represent significant differences from other groups, $P < 0.05$. Data are means \pm SEM.

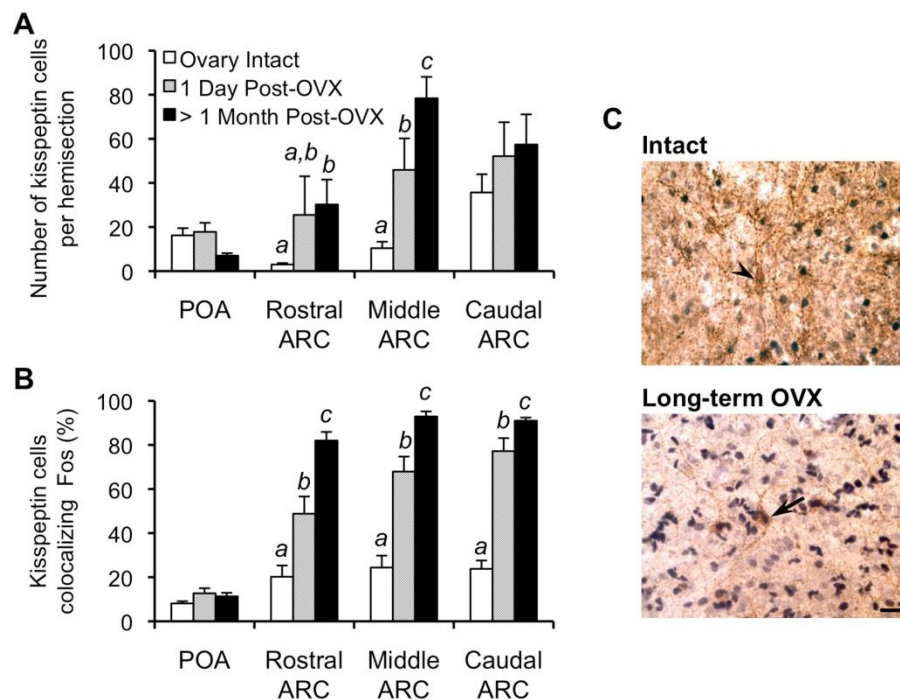


Figure 3.5. Ovariectomy increases activation of kisspeptin cells in the ARC, but not the POA. Kisspeptin cell number (A) and percent colocalization with Fos (B) in ovary-intact, 1 d post-OVX, and more than 30 days post-OVX ewes in the POA and rostral, middle, and caudal sections of the ARC. Letters (a, b, and c) represent significant differences from each other within the same area, $P < 0.05$. C, Sections immunostained for kisspeptin and Fos through the middle ARC in gonadal-intact and long-term (> 30 d) OVX ewes. Arrowhead and arrows indicate examples of single- and dual-labeled cells, respectively. *Scale bar*, 10 μm .

3.4. Discussion

Using a well-defined surge model, we observed that kisspeptin neurons of both the ARC and POA are activated after the onset of the preovulatory surge, indicating a role for both populations in E2-positive feedback in the ewe. In contrast, we observed that ARC, but not POA, kisspeptin neurons are activated following the withdrawal of E2, suggesting that only ARC kisspeptin neurons are likely important mediators of E2-negative feedback.

Several lines of evidence support the involvement of both ARC and POA kisspeptin neurons in E2-positive feedback in the ewe. First, in the late follicular phase, *Kiss1* mRNA has been shown to increase in the middle and caudal ARC, as well as in the POA (10, 36). Second, there was also an increase in kisspeptin protein in the ARC during this time (10). Third, E2 treatment of OVX ewes increased kisspeptin mRNA and protein expression in the POA (35). Leading up to the surge, we observed an increase in kisspeptin cell number and fiber intensity in the middle ARC, which may reflect an increased sequestration of the peptide in the cell body as a result of the up-regulated *Kiss1* mRNA (10, 36).

Although the aforementioned data show that *Kiss1* mRNA and kisspeptin protein are up-regulated prior to the surge, our data show that soon after the onset of the preovulatory surge, kisspeptin cells are transcriptionally activated in both the ARC and POA. Interestingly, Smith *et al.* (10) failed to show an increase in Fos expression within POA kisspeptin cells 1 hr after a surge-inducing injection of E2 to OVX ewes. In their paper, they propose that the activation of these neurons may occur outside of the time window they chose to study (10), which could be consistent with our results. In contrast to the findings of Smith *et al.* (10), a recent study using ovary-intact ewes observed Fos expression in POA, but not ARC, kisspeptin neurons during the preovulatory LH surge (31). These authors suggested that the contrasting findings to previous work (10) might be due to different animal models (ovary-intact vs. OVX). Our data confirm the activation of POA kisspeptin at the time of the LH surge reported by Hoffman *et al.* (31), but we also observed increased Fos expression in the ARC population. One possible explanation for this discrepancy is that the chronic guide tubes traversing the ARC (used to monitor GnRH in the median eminence) in the animals used by Hoffman *et al.* (31)

may have disrupted activation of ARC kisspeptin neurons. Another possible explanation is that different antibodies for detection of Fos were used in these two studies, suggesting the possibility that differences in observed Fos may be due to a different antibody sensitivity (Smith *et al.* used the same antibody we did). Despite this discrepancy, most mRNA studies in the ewe are consistent with the idea that both ARC (KNDy) and POA kisspeptin neuronal populations participate in mediating E2 positive feedback in this species (10, 36).

In the second experiment, we observed a significant increase in both numbers of kisspeptin-positive cells and their activation (as measured by Fos) in the ARC of long-term OVX ewes. Kisspeptin cell numbers and activation were also elevated, coincident with increased LH release, as early as 24 h post-OVX, demonstrating the rapid response of ARC kisspeptin neurons to the removal of E2. No changes were observed in the POA kisspeptin neurons at either time point. Thus we suggest that ARC, not POA, kisspeptin neurons mediate E2 negative feedback and therefore are likely key regulators of episodic LH secretion. These data confirm previous studies which found that chronic removal of E2 by OVX increased (22), and chronic E2-treatment decreased, both Kiss1 mRNA expression (10, 22) and number of kisspeptin-ir cells in the ovine ARC (10) during the breeding season. Our data extend these observations by demonstrating an early increase in both kisspeptin cell number and Fos colocalization following OVX. Similar to the work in sheep, studies in both humans and rodents also point to ARC kisspeptin neurons as mediators of E2 negative feedback. Postmenopausal women show both hypertrophy of kisspeptin neurons and increased *kisspeptin* gene expression in the infundibular nucleus (37), suggesting that kisspeptin in this area is normally held in check by steroid negative feedback. In rodents, OVX increases, and E2-treatment decreases, Kiss1 expression in the ARC (26), but there is no evidence that OVX increases Fos expression in these neurons (38).

In comparing kisspeptin populations involved in the positive and negative feedback actions of E2 among species in which this question has been addressed, two consistent patterns emerge: 1) the more rostral population (POA in sheep, AVPV and associated areas in rodents) is dedicated to the initiation of the LH surge, and 2) the ARC kisspeptin population participates in the negative feedback control of pulsatile LH

secretion. In sheep, between 40 and 60% of POA kisspeptin neurons are activated during the surge (this study and (31)). This roughly corresponds with the ~50% of POA kisspeptin-ir neurons that express ER α (7), but whether this population overlaps with the activated kisspeptin cells observed here remains to be determined. These neurons may be stimulated directly by E2, but this seems unlikely because in the ewe the mediobasal hypothalamus, not the POA is the site at which E2 acts to induce an LH surge (39). Alternatively, these cells may be indirectly regulated, and subsequently activated, by steroid-sensitive afferents. In rodents, E2 increases both kisspeptin expression (26) and Fos-colocalization with kisspeptin in the AVPV (24, 38, 40). Interestingly, the percentage (ranging from 30-60%) of rostral kisspeptin neurons activated at the time of the surge in rodents (24, 38, 40) is similar to that in sheep. Almost all of these neurons in rodents contain ER α (26) and evidence suggests that E2 positive feedback in rodents occurs directly on these kisspeptin neurons (41). Studies that examined the more rostral kisspeptin population observed relatively low levels of kisspeptin/Fos colocalization in the absence of an LH surge in both sheep (10, 31) and rodents (24, 38, 40), again consistent with this population being dedicated to the LH surge.

It is also generally accepted that the ARC kisspeptin (KNDy) neurons in rodents and sheep mediate the negative feedback actions of E2. It has also been proposed that they drive episodic GnRH secretion (42-44), but there may be some species differences in their activation. As already noted, OVX increased Fos expression in these neurons in sheep (this study), but apparently not in rodents (38). Moreover, in both current experiments, we observed that approximately 20% of KNDy neurons contain Fos during the follicular phase or during anestrus, even though LH secretion is at a basal level. This finding is apparently inconsistent with that of Hoffman et al. (31), who reported only about 5% of ARC kisspeptin neurons are activated in early follicular phase ewes (31). As discussed above, this may reflect differences in the antibodies used to detect Fos immunoreactivity or that Hoffman *et al.* (31) used tissue from ewes with chronic guide tubes traversing the ARC. Smith *et al.* (10) observed approximately 15% ARC kisspeptin/Fos colocalization during the luteal phase of ewes, which increased to about 35% during the late follicular phase. The relatively high level of Fos expression in ovine KNDy neurons, compared with POA kisspeptin neurons, during basal secretion of LH is

consistent with the hypothesis that KNDy neurons are important in tonic secretion of GnRH (42-44). In contrast, colocalization of kisspeptin and Fos in the ARC of rats is less than 5% on diestrus 2 of the cycle (24).

The one major difference between KNDy neurons in sheep and rodents is that they appear to participate in the LH surge in the former, but not the latter. Two of three studies observed high levels of Fos expression in KNDy neurons associated with the surge in sheep, whereas, with one exception (23), this has not been observed in rodents (24). As noted above, this difference is consistent with evidence that the positive feedback action of E2 occurs in the mediobasal hypothalamus in the sheep, and in the region of the AVPV in rodents (41). Interestingly, while almost all ovine KNDy neurons express ER α in the ewe (7), only 45-60% are activated during the surge. What remains unclear from these data is if this activation is due to heterogeneity in the ARC population, so that different subsets of neurons govern positive and negative feedback, or alternatively, if the same neurons govern positive and negative feedback, but are not activated at the same time. Moenter *et al.* (45), showed that approximately 40% of GnRH neurons were activated during the surge, and postulated that different neurons may be activated over time throughout the GnRH surge in order to maintain high levels of GnRH release. The high level of Fos expression in KNDy neurons of OVX ewes (70-90%, Figure 3.4) suggests that the same neurons are involved in both E2-positive and negative feedback. Reciprocal connections between KNDy neurons, that are postulated to play an important role in episodic GnRH secretion (42-44), may be responsible for the high percentage of activated KNDy neurons after OVX. If this is the case, the lower percentage of KNDy neurons containing Fos during the LH surge, may suggest a less important role for this reciprocal innervation. This proposal is consistent with the changes in multi-unit electrical activity associated with LH pulses at the time of the LH surge in monkeys, rats and goats (46-48). Fos induction in most neurons is thought to be a consequence of glutamate release and its postsynaptic action, and it is interesting in this regard that a majority of KNDy neurons in the sheep colocalize glutamatergic markers (49), including expression within terminals that form reciprocal KNDy-KNDy contacts (50). Thus, release of glutamate from KNDy presynaptic terminals may be partly responsible for the Fos induction seen during increased pulsatile activity.

In summary, these data demonstrate that both the ARC (KNDy) and POA kisspeptin neurons are activated during, and therefore likely play an important role in, the GnRH/LH surge in ewes. In contrast, only ARC kisspeptin neurons had increased kisspeptin expression and activation following E2 removal, indicating the key role of ARC kisspeptin in the negative feedback regulation of episodic GnRH secretion by E2. Whether the same or different subsets of ARC kisspeptin neurons are activated during surge and tonic GnRH secretion remains to be determined.

3.6. References

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CHAPTER 4

**KNDy (Kisspeptin/Neurokinin B/Dynorphin) neurons provide direct
input to GnRH neurons in the sheep**

4.1. Introduction

Recent studies that have focused on a subset of neurons in the arcuate nucleus (ARC) containing three neuropeptides important to reproduction, the ‘KNDy’ (Kisspeptin/Neurokinin B/Dynorphin) neurons, have yielded important insights into the control of gonadotropin-releasing hormone (GnRH) release. First identified in sheep (1), this neuronal population appears to be well conserved among mammals and has now been identified in a number of other species (1-6). The co-expression of KNDy peptides is unique to the ARC, as neurons containing one of the KNDy peptides elsewhere in the hypothalamus and preoptic area (POA) do not express either of the other two peptides (1, 5, 6).

Each of the KNDy peptides has been shown to play important roles in reproduction. Kisspeptin, encoded by the *Kiss1* gene (7), was cast to the forefront of reproductive neuroendocrine studies in 2003, with the finding that inactivating mutations of the kisspeptin receptor (GPR54 or Kiss1R) produced hypogonadotropic hypogonadism and a failure to enter puberty (8, 9). Since that discovery, evidence from a variety of species has shown that kisspeptin peptide and protein are under steroid hormone regulation (10), that kisspeptin is an extremely potent stimulator of GnRH secretion (11-14), and that kisspeptin acts directly on GnRH neurons, almost all of which express Kiss1R (11, 15-17). A fairly well-conserved population of kisspeptin neurons outside of the ARC is seen in the POA of sheep (18) and primates (2, 19, 20), and, in rodents, this cell group extends as a continuum within the rostral periventricular area of the third ventricle (RP3V) (21). This population appears to mediate the positive feedback influence of E2 onto GnRH neurons (22-24). The tachykinin, neurokinin B (NKB), has gained much recent attention because of genetic studies that identified mutations in the gene encoding the peptide (TAC3) or its receptor (TACR3 or NK3R) in humans with hypogonadotropic hypogonadism (25, 26), a similar phenotype to that seen for mutations in *Kiss1* and its receptor. KNDy neurons in sheep and rodents (5, 6, 27) express NK3R; however only a subset of GnRH neurons in rats express NK3R (28, 29), while GnRH neurons in sheep do not appear to express NK3R (27). Dynorphin, an endogenous opioid peptide (EOP), appears to play an important role in mediating progesterone negative feedback on GnRH/LH secretion (3, 30, 31). The receptor for dynorphin, the κ -

EOP receptor (KOR), is expressed in KNDy neurons of the mouse (6), and appears to mediate the suppression of LH pulse frequency in OVX goats (3).

The KNDy population forms a reciprocally interconnected network (5, 32, 33) which may be important for synchronization of this neuronal network for pulse generation (6, 34). Axon terminals and fibers containing individual KNDy peptides (kisspeptin, NKB, or dynorphin) have also been shown to directly contact GnRH cell bodies and terminals, at both the light, and electron microscopic (EM) level in a variety of mammalian species (31, 34-40). However it is not known whether all or some of these inputs derive specifically from the KNDy population. In addition, GnRH neurons in the sheep are located in a broad distribution, which includes the preoptic region (POA), the anterior hypothalamic area (AHA) and the mediobasal hypothalamus (MBH), and it is not known whether there are regional differences among GnRH cells in KNDy inputs. Supporting this possibility are the existence of regional differences among ovine GnRH neurons in single-labeled dynorphin inputs, with more inputs per cell in the MBH than the POA (31). However, it is not known whether this regional difference is due to inputs from KNDy cells or single-labeled dynorphin populations elsewhere.

In recent years, dual immunostaining for two of three KNDy peptides has been used as an approach to determine the projections of this population throughout the brain, and to GnRH cell bodies and terminals. Preliminary observations in sheep suggested that KNDy neurons project directly to GnRH cell bodies in the POA and MBH (34), and in humans, a small percentage of kisspeptin and NKB afferents to GnRH coexpress both peptides and thus likely arise from the KNDy population (41). In addition, observations in these and other species suggests that KNDy-GnRH connections may also exist at the level of the median eminence where axo-axonic contacts have been observed (2, 4, 17, 33, 42). However detailed quantification of KNDy terminals in apposition to GnRH cell bodies has not been carried out, nor is it known whether there are regional differences among GnRH cells in this input. Thus, in this study, we used triple-label immunofluorescence, to identify both KNDy inputs (dual kisspeptin/dynorphin) and single-labeled kisspeptin and dynorphin inputs to GnRH neurons and determine: 1) whether KNDy terminals directly contact GnRH cell bodies in the POA, anterior

hypothalamic area (AHA) and MBH, and 2) whether there are regional differences in KNDy inputs, and single-labeled kisspeptin and dynorphin inputs, to GnRH neurons.

4.2. Materials and Methods

4.2.1. Animals

Adult Suffolk ewes with regular estrous cycles were maintained under normal conditions in an open barn with free access to food and water during the breeding season (September through February). Ewes were moved to an indoor facility 3-5 days before any experimental procedures. In this facility, they were exposed to photoperiod simulating natural outdoor day length, had free access to water and a mineral lick, and were fed a pelleted maintenance diet daily. Tissue was collected from luteal phase ewes (n=3) on days 9-10 of an estrous cycle that had been synchronized using PGF2 α (43); stage of cycle was confirmed by examination of the ovaries and progesterone concentrations was with an RIA. All experimental procedures involving animals were approved by the West Virginia Animal Care and Use Committee, and followed the National Institutes of Health guidelines for animal research.

4.2.2. Tissue collection

Tissue collection and processing was performed as previously described (1). Ewes were euthanized via an iv overdose of sodium pentobarbital (~2g in 7ml saline; Sigma, St. Louis, MO) after two iv injections of heparin (25,000 U), 10 min and immediately prior to pentobarbital. The heads were removed and perfused bilaterally through the carotid arteries with 6 liters of fixative (4% paraformaldehyde containing 10 U/ml heparin and sodium nitrite). After perfusion, brains were removed and POA/hypothalamic tissue was dissected out. The tissue was then infiltrated with 30% sucrose, and 6 parallel series of coronal sections (45 μ m thick) were cut on a freezing microtome and stored at -20 C in cryoprotectant for later processing.

4.2.3. Immunocytochemistry

All immunocytochemistry was carried out on free-floating sections at room temperature (RT), and washed with 0.1 M phosphate-buffered saline (PBS) following

each incubation. For all experiments, sections were incubated in 10% hydrogen peroxide (10 min in PBS; EMD Chemicals, Inc., Gibbstown, NJ) to occupy endogenous peroxidase activity, followed by incubation in a solution containing 20% normal goat serum (NGS; Jackson Immunoresearch Laboratories, Inc., Gibbstown, NJ) in PBS containing 0.4% Triton X-100 (Fisher Scientific, Pittsburgh, PA) for 1 h to minimize nonspecific binding.

Kisspeptin, GnRH, Dynorphin

To examine KNDy (kisspeptin/dynorphin) inputs to GnRH cell bodies, tissue sections from the POA, AHA and MBH were processed. First, to visualize kisspeptin, tissue sections were incubated for 17h in polyclonal rabbit anti- kisspeptin-10 serum (1:200,000; No. 564, A. Caraty, Université Tours, Nouzilly, France (1, 40). Next, sections were incubated in biotinylated goat anti- rabbit IgG (1:500 in PBS containing 0.4% Triton X-100 and 4% NGS; 1 h; Jackson Immunoresearch Laboratories, Inc.), followed by ABC-elite (1:500 in PBS; 1h; Vector Laboratories, Burlingame, CA). Sections were incubated with TSA (1:250 in PBS containing 3% hydrogen peroxide/mL; 10 min; Perkin Elmer LAS, Inc., Boston, MA) followed by 30 minutes in Cy5-Streptavidin (1:100 in PBS; 30 min; Cat No. 016-170-084, Jackson Immunoresearch Laboratories, Inc.). Subsequently, sections were co- incubated with monoclonal mouse anti-GnRH serum (1:400; SMI41R, Sternberger Monoclonals, Inc., Princeton, NJ) and rabbit anti- Dynorphin A serum (1:1,000; Cat No. H-021-03, Phoenix Pharmaceuticals, Inc., Burlingame, CA) for 17 h, which have been previously validated in sheep tissue. Next, sections were incubated sequentially for 30 minutes with Alexa 488-goat anti-mouse IgG (1:100; Cat No. A11001, Invitrogen, Carlsbad, CA) and Alexa555-goat anti-rabbit IgG (1:100; Cat No. A21428, Invitrogen). Sections were mounted onto Superfrost slides, dried and coverslipped with gelvatol (32). All three primary antibodies have been validated for use in sheep (1, 32, 40). Additional controls for this experiment included the omission of one or more of the primary antibodies, which resulted in a lack of staining for the corresponding antigen at the appropriate wavelength, and demonstrating lack of cross-reactivity of secondary antibodies.

4.2.4. Confocal Analyses

All confocal analyses were completed using an LSM510 laser- scanning confocal microscope (Zeiss, Thornwood, NY). Alexa 488 was visualized with a 505 nm emission filter and Argon laser, while Alexa 555 and Cy5 were imaged with HeNe lasers and a 560 and 650 nm emission filters, respectively. Neurons in which complete cell bodies were visible were selected for analysis and images were taken in 1 μm intervals along the z-plane. For each animal, close contacts onto GnRH cells were defined as an immunolabeled terminal in close apposition (no intervening pixels) with a cell body in a single, 1 μm thick optical section. When analyzing through the entire z-stack, markers were placed on putative terminals, so that terminals flanking optical sections were not counted twice. Furthermore, orthogonal views confirmed contacts so that only markers contacting the neuron in all planes were accepted as close contacts. Minor brightness and level adjustments were made to the image using Adobe Photoshop (San Jose, CA).

The percentage of GnRH neurons receiving one or more close contact was determined from a randomly-selected sample of GnRH neurons (10 neurons/ewe) in each area. GnRH neurons from the same sample were used to quantify the number of single and dual labeled inputs per neuron. To quantify the number of single-labeled kisspeptin inputs per neuron, GnRH neurons with one or more single-labeled kisspeptin input were analyzed. Likewise, to quantify the number of single-labeled dynorphin inputs per GnRH neuron, GnRH neurons with one or more single-labeled dynorphin input were analyzed. To exclude the possibility that comparisons were confounded by cell size, optical thickness measurements were taken of each cell in this analysis by counting the number of 1 μm optical sections in each z-stack, and computing an average for each animal and group. We found no significant differences in optical thickness between GnRH neurons in the POA, AHA and MBH.

4.2.5. Statistical Analysis

For all of the confocal contact analyses (including optical thickness measurements), one-way ANOVA tests were used to make comparisons between regions (POA, AHA, MBH) in luteal phase animals. Post hoc analyses of all pairwise

comparisons were computed using Fisher LSD method. All results are reported as mean \pm SEM, and statistical significance was considered as $P < 0.05$.

4.3. Results

4.3.1. Direct inputs from KNDy (Kisspeptin/Dynorphin) neurons to GnRH neurons.

In all regions examined, GnRH neurons received KNDy (dual-labeled kisspeptin/dynorphin) inputs which were seen as varicosities in close appositions to their cell bodies (Figure 4.1). A majority of GnRH neurons in the MBH ($78 \pm 6\%$) and AHA ($67 \pm 7\%$) received at least one KNDy input. GnRH neurons with KNDy inputs was significantly higher in the MBH compared to the POA ($P = 0.02$; Figure 4.2A), and there was a nonsignificant trend towards significantly more GnRH neurons in AHA with KNDy inputs compared to the POA ($P = 0.06$). Of those cells that received at least one input, GnRH neurons in the AHA possessed a greater number of KNDy inputs than those in the POA ($P = 0.04$; Figure 4.2D), and there was a non-significant trend towards a greater number of KNDy inputs contacting MBH compared with POA GnRH neurons ($P = 0.07$). Numbers of KNDy inputs onto AHA and MBH GnRH neurons were not significantly different.

In the median eminence, we found many kisspeptin-containing fibers and varicosities that co-localized dynorphin in the external zone of the median eminence adjacent to the pars tuberalis of the anterior pituitary, where they were frequently seen in close apposition to GnRH fibers and terminals (Figure 4.1B). Some single-labeled fibers containing kisspeptin or dynorphin exclusively were observed in the external zone as well.

Single-labeled kisspeptin and dynorphin inputs to GnRH cell bodies were also examined. In all three regions, 37-53% of GnRH neurons receive at least one single-labeled kisspeptin input (Figure 4.2B), and there were no significant differences in either the percentage of GnRH cells with at least one contact (Figure 4.2B), or in the number of inputs per GnRH cell for those receiving at least one contact (Figure 4.2E). By contrast,

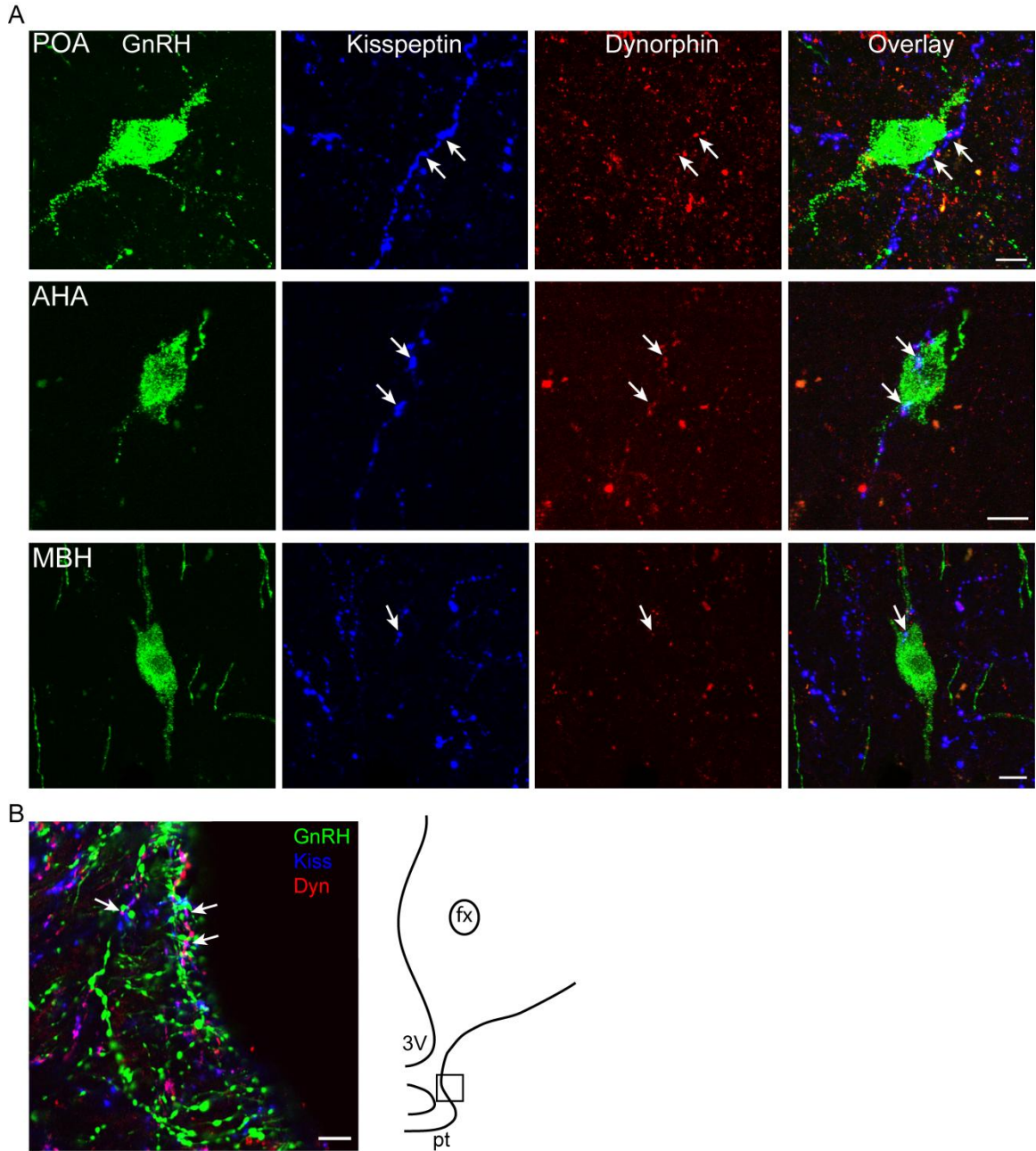


Figure 4.1. Dual-labeled kisspeptin/dynorphin inputs to GnRH cell bodies and terminals. A, Confocal image (1 μm optical thick section) of GnRH neurons (*green*) in the POA (*top*), AHA (*middle*) and MBH (*bottom*) contacted by kisspeptin (*blue*) and dynorphin (*red*) fibers and varicosities. Dual-labeled kisspeptin/dynorphin terminals in contact with GnRH neurons are indicated by white arrows. B, Confocal image (1 μm thick optical section) of the external zone of the median eminence immunolabeled for GnRH (*green*),

kisspeptin (*blue*), and dynorphin (*red*), showing close associations between KNDy fibers (dual-labeled kisspeptin/dynorphin) and GnRH fibers. *Scale bar*, 10 μm .

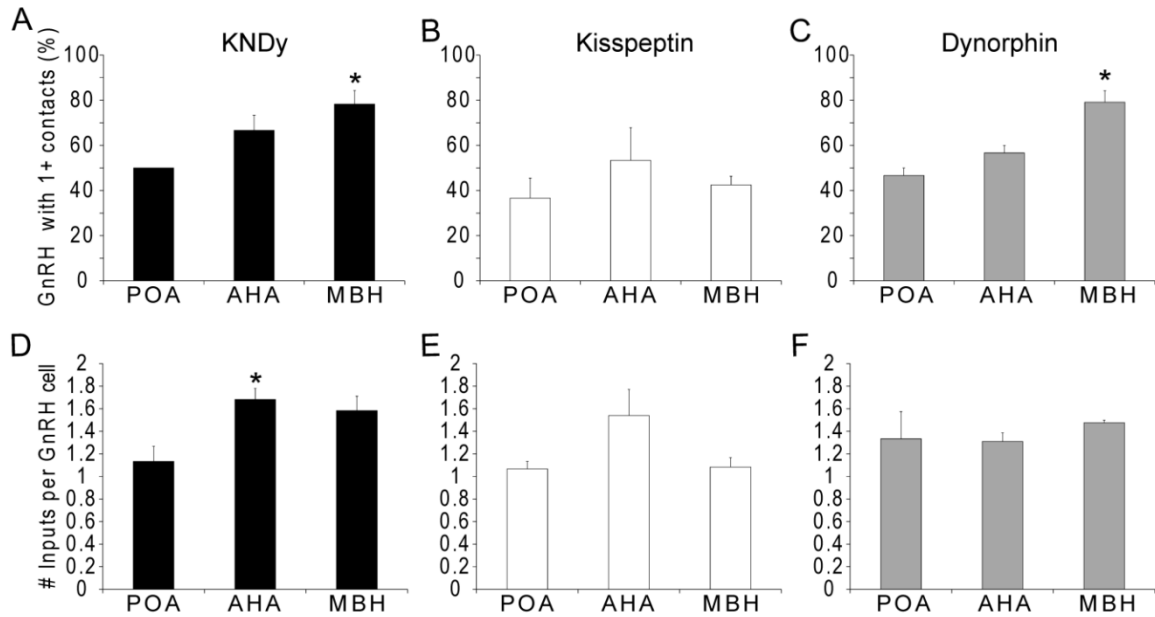


Figure 4.2. Inputs to GnRH neurons in the POA, AHA and MBH. A-C, The percentage (mean \pm SEM) of GnRH neurons showing one or more dual-labeled KNDy (kisspeptin/dynorphin) (A), single-labeled kisspeptin (B) or dynorphin (C) input. D-F, The number (mean \pm SEM) of inputs per neuron of those GnRH cells with at least one input. *, $P < 0.05$.

the percentage of GnRH neurons with single-labeled dynorphin inputs was significantly higher in the MBH ($79 \pm 5\%$) than in the POA ($47 \pm 3\%$; $P < 0.01$) or AHA ($57 \pm 3\%$; $P = 0.017$) (Figure 4.2C). However, the number of single-labeled dynorphin inputs/cell did not show a significant regional difference (Figure 4.2F).

Within each region, GnRH cells received relatively similar proportions of each different type of input (KNDy, single-labeled kisspeptin, single-labeled dynorphin) examined (Figure 4.3). KNDy inputs represented a majority of kisspeptin input (both single-labeled and dual-labeled inputs; to GnRH cells in each region (58-68%). In addition, KNDy inputs represented approximately half (45-57%) of all dynorphin input to GnRH neurons (both single-labeled and dual-labeled inputs) in each region. In the MBH, the proportion of single-labeled kisspeptin input was significantly less than that of either KNDy or single-labeled dynorphin inputs; no significant differences were seen between input type in the POA or AHA.

4.4. Discussion

The results of this study provide strong anatomical evidence that KNDy neurons project directly to a majority of GnRH neurons in the ovine POA, AHA and MBH. Projections from KNDy neurons represent the majority of kisspeptin inputs, and half of all dynorphin inputs onto GnRH neurons in the hypothalamus. The presence of single-labeled kisspeptin and dynorphin terminals onto GnRH cell bodies suggests that kisspeptin and dynorphin populations outside of the ARC also project to GnRH neurons, and indicate that these different populations likely contribute to the control of GnRH secretion.

Results from this study reveal interesting regional differences in the innervation of GnRH by KNDy (kisspeptin/dynorphin) terminals and non-KNDy dynorphin terminals: a greater percentage of GnRH neurons in the MBH receive single-labeled dynorphin and KNDy inputs than GnRH neurons in the POA, and AHA. In addition, MBH GnRH neurons receive a greater number of KNDy inputs per neuron than GnRH cells in the other two regions. The existence of functional subpopulations of GnRH neurons has not been fully elucidated. Although there does not appear to be a distinct

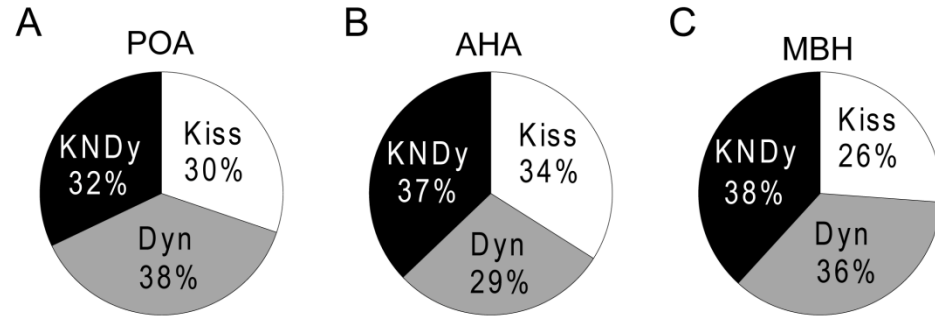


Figure 4.3. Circle charts representing the relative proportion of the mean number of kisspeptin, dynorphin and KNDy inputs onto GnRH neurons in the POA (A), AHA (B) and MBH (C).

anatomical subgroup of GnRH cells that are involved in the surge (44), subpopulations of GnRH neurons may be preferentially involved in pulsatile secretion of LH, and negative feedback of progesterone (31, 45). Previous work in sheep suggests that dynorphin inputs to MBH GnRH cells may mediate progesterone inhibition of LH pulse frequency (31), and that arcuate dynorphin cells (KNDy cells) specifically, are critical to this action, as severed connections between the POA and MBH do not affect EOP-mediated progesterone negative feedback (46) and most ARC dynorphin contain progesterone receptors (47). In addition, studies using Fos as a marker in sheep have demonstrated that GnRH neurons in the MBH but not the POA or AHA, are activated during an acute increase in GnRH/LH pulse frequency (45). Thus, taken together, these results suggest that KNDy-MBH GnRH connections in sheep may be a critical component for the pulsatile secretion of GnRH consistent with the proposed role of the KNDy subpopulation as the “GnRH pulse generator” (34).

This study is the first to distinguish between kisspeptin and dynorphin inputs to GnRH from those derived specifically from the KNDy neurons. A previous study by Goodman et al. (2004) investigated dynorphin inputs to GnRH neurons in various hypothalamic regions; however, the source of these dynorphin inputs was not identified, and at the time, the co-expression of dynorphin, kisspeptin, and NKB in the arcuate nucleus had not been identified (1). Their results showed the percentage of GnRH neurons with dynorphin inputs (and the mean number of inputs per cell) was twice as high in the MBH as in the POA and other regions (diagonal band of Broca, AHA, lateral hypothalamic area) (31). Importantly, they also demonstrated that close contacts between dynorphin terminals and GnRH cell bodies observed at a light microscopic level were bona fide synaptic inputs when viewed under the electron microscope (31). Consistent with those findings, we found that a greater percentage of GnRH neurons receive dynorphin inputs (non-KNDy) in the MBH than the POA and AHA, although there were no regional difference in the number of single-labeled dynorphin inputs per cell. However, since these data (single-labelled dynorphin) do not take into account dynorphin-positive contacts arising KNDy cells, we analyzed total dynorphinergic inputs (both single- and dual-labeled inputs) and found that the number of total dynorphin inputs onto GnRH cells in the MBH was greater than that onto POA GnRH cells. One slight

difference between our results and that of the earlier study of dynorphin inputs to GnRH cells in the sheep was that the total number of inputs onto GnRH cells in the MBH was lower in the current study. This may be due to the methodological differences: light microscopy was used for analysis of immunoperoxidase-labeled sections in Goodman et al. (2004), while the present study used fluorescent-labeling and confocal microscopy. Overall, however, our findings are consistent with the previous work showing that GnRH neurons in the ovine MBH receive a greater number of dynorphin inputs, and extend that finding by showing that approximately half of these are derived from the ARC KNDy population.

Although our results show that the majority of kisspeptin inputs to GnRH arise from KNDy cells, GnRH neurons are also contacted by single-labeled kisspeptin inputs, and these are not restricted to a particular anatomical subgroup of GnRH cells. The single-labeled kisspeptin inputs observed in this study may be derived from the POA kisspeptin population, which has been recently implicated as having a role in the preovulatory GnRH/LH surge in sheep (16, 23, 48). Interestingly, the percentage of GnRH neurons with single kisspeptin inputs in the POA, AHA and MBH (~44%) reported in this study is strikingly similar to the percentage of GnRH neurons that express cFos during the estradiol-induced surge in sheep (~41%), reported two decades ago (44). Based on the convincing evidence for the role of POA kisspeptin neurons in the preovulatory surge in sheep (16, 23, 48), it is tempting to speculate that they may be projecting to the same GnRH neurons that are activated during that time, although this remains to be demonstrated. Although approximately half of all POA kisspeptin neurons in sheep express ER α (49), the site of estrogen action in the ewe to induce the preovulatory GnRH surge is in the MBH (50). It is unclear whether the kisspeptin neurons that contain ER α are the ones activated during the surge or the ones that project to GnRH neurons.

Conventional tract tracing studies in rodents and sheep (33, 51-53) have identified projections from ARC neurons to the POA, in the vicinity of GnRH cell bodies. However, studies using dual- immunostaining to identify KNDy projections (kisspeptin/NKB) in the POA, report only a small number of fibers (2, 17, 41, 42, 54), but inputs to GnRH neurons were not analyzed in most of these studies. The limited number

of KNDy fibers identified in these studies, suggests that the kisspeptin inputs onto GnRH neurons in various species (see review for summary (10)), arise from either the well-conserved POA/AVPV kisspeptin population, or possibly other small kisspeptin populations elsewhere (10). A recent study in human by Molnar et al. (2012) showed dual kisspeptin/NKB inputs comprise a very small percentage of the kisspeptin and NKB inputs to GnRH cell bodies in the human infundibular nucleus (41). This is in contrast with the present findings in sheep which show that a majority of the kisspeptin inputs onto GnRH neurons derive from the KNDy neurons. One possibility for this discrepancy may be species differences, but another may be sex: the present study used tissue from female sheep, while Molnar et al. used tissue from human males. Expression of kisspeptin and NKB, as well as kisspeptin inputs onto GnRH neurons, are sexually dimorphic in humans, with females showing greater numbers of kisspeptin- and NKB-immunoreactive cells (55), and increased numbers of kisspeptin inputs onto GnRH neurons (2). A sexual dimorphism in KNDy peptides is also present in sheep, with rams having less than half the number of kisspeptin, NKB and dynorphin cells in the arcuate nucleus than ewes (56). Therefore, it is possible that a sex difference in the innervation of GnRH by KNDy neurons exists in humans, as well as sheep, and may account for the fewer KNDy inputs to GnRH observed in human males.

A number of studies suggest that the majority of the interaction between KNDy and GnRH neurons is thought to take place at the level of the median eminence (2-4, 42), although localization of receptors for KNDy peptides within GnRH terminals remains to be shown. In sheep, light microscopic and electron microscopic evidence for contacts between dynorphin terminals and GnRH terminals in the median eminence has been reported (34), and preliminary results from retrograde tracing ratifies the source of these dynorphin terminals being the KNDy cells (34). In addition, anterograde tracing from the ARC in sheep has identified dual BDA/kisspeptin fibers in the ME (17). Kisspeptin is able to stimulate GnRH release in the median eminence *in vitro* (17, 57), but has also been shown to stimulate GnRH at the cell bodies located in the POA (58, 59). Our current data provides further evidence that KNDy neurons project to the median eminence, in which their fibers are intermingled with GnRH fibers in the external zone. Therefore, kisspeptin appears to act at various levels of the GnRH neuron (cell body, dendrite,

terminal) to influence GnRH secretion. In addition to a direct action at GnRH neurons and terminals, kisspeptin may act on other interneurons to indirectly regulate GnRH secretion.

In summary, these findings demonstrate that KNDy neurons in the ovine ARC project directly to GnRH cells throughout the POA and hypothalamus, contacting GnRH neurons both at the level of their cell bodies and at their terminals in the median eminence. The KNDy-GnRH circuitry likely plays a key role in pulsatile secretion of GnRH across the estrous cycle, and the MBH GnRH neurons specifically may be critical for this.

4.5. References

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CHAPTER 5

**Plasticity of glutamatergic inputs to kisspeptin and GnRH neurons
during the preovulatory LH surge in the ewe**

5.1. Introduction

Kisspeptin, coded for by the *Kiss1* gene, is a member of the RFamide-related peptide family that has been established as a key neuronal afferent to GnRH cells, and is suitably poised to convey gonadal steroid feedback to GnRH neurons (1, 2). Most kisspeptin neurons contain estrogen receptor- α (ER α) (3, 4) and kisspeptin is a potent stimulator of gonadotropin (and GnRH) release in various mammals, including mice (5, 6), rats (7-10), sheep (11, 12), monkeys (13) and humans (14). Kisspeptin antagonists have revealed important roles for kisspeptin in both tonic GnRH/LH secretion and the preovulatory surge in females. Kisspeptin antagonists block pulsatile episodic GnRH secretion in pubertal monkeys as well as ovariectomized (OVX) ewes (15). Moreover, kisspeptin antisera in rats (16) and kisspeptin receptor antagonist in sheep (17, 18) were shown to attenuate the preovulatory surge.

In female rodents, kisspeptin neurons of the arcuate nucleus (Arc) appear to mediate E2 negative feedback, as OVX increases *Kiss1* mRNA expression, with E2 replacement reversing this effect (7, 19, 20). In contrast, kisspeptin neurons of the rodent anteroventral periventricular nucleus (AVPV) play a role in mediating E2 positive feedback, as OVX down regulates and E2 replacement increases the expression of the *Kiss1* mRNA (20). AVPV kisspeptin neurons also show extensive colocalization with the transcriptional activation marker, c-Fos, during the LH surge (3). These data are consistent with earlier evidence that the AVPV is the site where E2 acts to induce the LH surge in rodents (21).

In contrast to rodents, ovarian steroids appear to exert both negative and positive feedback effects onto kisspeptin neurons at the level of the ARC in the sheep. Their role in negative feedback is supported by evidence that OVX increases *Kiss1* mRNA and kisspeptin protein expression in the ARC, reversed with E2 replacement (19, 22), and that OVX increases (23) and E2 treatment inhibits (24) cFos expression in these neurons. There are also several lines of evidence to suggest that ARC kisspeptin neurons mediate E2 positive feedback: *Kiss1* mRNA has been shown to be upregulated in the ARC in late follicular phase or the preovulatory period (24, 25), ARC kisspeptin neurons express cFos in response to a surge-inducing dose of E2 (25) and cFos expression is elevated at the

time of the LH surge (23). Furthermore, E2 implants in the MBH (in proximity to the ARC) but not in the POA are able to induce an LH surge (26).

A distinguishing feature of ARC kisspeptin neurons is their extensive colocalization with the endogenous opioid peptide, dynorphin, and the tachykinin, neurokinin B (27). This subpopulation, collectively referred to as KNDy cells, is critical for the steroid feedback regulation of GnRH neurons (28-30). KNDy cells appear to be conserved across multiple mammalian species in addition to sheep, including mice (31), rats (32, 33), goats (34) and humans (35). Anatomically, another distinguishing feature of the KNDy population is that they are reciprocally interconnected, based on the presence of KNDy peptide-containing terminals in synaptic contact with KNDy cell bodies in sheep and rodents (32, 36, 37). In addition to containing the neuropeptides, kisspeptin, neurokinin B and dynorphin, there is also evidence that KNDy cells in rodents contain other neuropeptides and transmitters (38, 39). Notably, KNDy cells in mice express the mRNA for the vesicular glutamate transporter, vGlut2, which is generally accepted as a marker for glutamatergic neurons (40, 41), but this has not yet been demonstrated in other species.

Glutamate has long been implicated in control of GnRH secretion, with several lines of evidence suggesting changes in glutamatergic tone and synaptic inputs to GnRH neurons across the estrous cycle. Glutamate mediates the activation of GnRH neurons at the time of the surge (42, 43) and glutamate receptor antagonists block the surge in rodents (42). Immunohistochemical studies in sheep, rodents and humans have shown that GnRH neurons receive close appositions from glutamatergic and GABAergic terminals (44), with increased glutamatergic inputs during breeding season in sheep (45, 46), and during proestrus in rodents (47, 48). Finally, GnRH neurons in rodents have been shown to express glutamate receptors (49, 50), and this expression of specific receptor subunits have been shown to increase at the time of the preovulatory surge (49, 51). One potential source for glutamatergic input to GnRH neurons is the KNDy population, as KNDy cells have direct projections to GnRH cell bodies (30, 33, 52, 53).

In this study, we investigated potential neuroplasticity in inputs to kisspeptin and GnRH neurons across the ovine estrous cycle. We hypothesized that an increase in excitatory synaptic inputs would be present at the time of the GnRH/LH surge, and that

changes in the presence of these inputs across the estrous cycle may be important for their role in positive feedback. In our first objective, we sought to determine whether terminals from ovine kisspeptin neurons contained glutamate, using vGlut1 and vGlut2 as markers, and if some or all of these were derived from the KNDy population. In our second objective, we examined changes across the estrous cycle in synaptic inputs (including those containing vGlut2 and kisspeptin) to kisspeptin neurons in both the ARC and the POA. Finally, our third objective was to investigate changes across the estrous cycle in kisspeptin and vGlut2 inputs to GnRH cell bodies in both the POA and MBH.

5.2. Materials and Methods

5.2.1. Animals

Adult Suffolk ewes with regular estrous cycles were maintained under normal conditions in an open barn with free access to food and water during the breeding season (September through February). Ewes were moved to an indoor facility 3-5 days before any experimental procedures. In this facility, they were exposed to photoperiod simulating natural outdoor day length, had free access to water and a mineral lick, and were fed a pelleted maintenance diet daily. Blood samples (3-5 ml) were taken by jugular venipuncture and placed into heparinized tubes, and plasma was collected and stored at -20 C. All experimental procedures involving animals were approved by the West Virginia Animal Care and Use Committee, and in accordance to the National Institutes of Health guidelines for animal research.

5.2.2. Animal Protocol

To collect tissue from ewes at specific times of the estrous cycles, these cycles were synchronized with injections of prostaglandin $F_{2\alpha}$ (10 mg; i.m.; PGF_{2 α} ; Lutalyse; Upjohn, Kalamazoo, MI) to induce regression of the corpus luteum. Once cycles had been synchronized, on d9-d10 of the luteal phase, brain tissue was collected or PGF_{2 α} was again injected and two Controlled Internal Drug Releasing (CIDR) devices were inserted (54). The CIDRs were removed 10 d later, and blood samples were collected every 4 h starting 12 hr after CIDR removal until perfusion. Tissue was collected 4 hrs after onset of estrous behavior and, based on LH concentrations, animals were classified

into surge and pre-surge groups; only tissues from animals showing a LH surge were used in this study. Mean LH levels at perfusion were: 2.43 ± 1.02 and 18.52 ± 6.28 ng/ml for luteal and surge groups, respectively.

5.2.3. Tissue Collection

Tissue collection and processing was performed as previously described (27). Ewes were euthanized via an iv overdose of sodium pentobarbital (~2g in 7ml saline; Sigma, St. Louis, MO) after two iv injections of heparin (25,000 U), 10 and 0 min prior to pentobarbital. The heads were perfused through both carotid arteries with 6 liters of fixative (4% paraformaldehyde containing 10 U/ml heparin and sodium nitrite). After perfusion, brains were removed and POA/hypothalamic tissue was dissected out. The tissue was then infiltrated with 30% sucrose, and 6 parallel series of coronal sections (45 μ m thick) were cut on a freezing microtome and stored at -20 C in cryoprotectant for later processing.

5.2.4. Assays

LH samples were measured in duplicate aliquots of 50-200 μ l, using a previously validated RIA (55), and expressed in terms of NIH-LH-S12. The minimal detectable concentration of LH in these assays average 0.077 ng/tube; inter- and intraassay coefficients of variation were 3.8% and 1.7%, respectively. Circulating progesterone was measured in duplicate aliquots of 150 μ l plasma using a commercially available solid-phase RIA kit (Coat-A-Count P₄, Diagnostic Products, Corp., Los Angeles, CA), which has been validated in sheep (56) to confirm stage of cycle.

5.2.5. Immunocytochemistry

All immunocytochemistry was carried out on free-floating sections at room temperature (RT), and washed with 0.1 M PBS between incubations. For all experiments, sections were incubated in 10% hydrogen peroxide (10 min in PBS; EMD Chemicals, Inc., Gibbstown, NJ) to occupy endogenous peroxidase activity, followed by incubation in a solution containing 20% normal goat serum (NGS; Jackson Immunoresearch

Laboratories, Inc., West Grove, PA) in PBS containing 0.4% Triton X-100 (Fisher Scientific, Pittsburgh, PA) for 1 h to minimize nonspecific binding.

Kiss/vGlut1/Synaptophysin

To examine the expression of vGlut1 within kisspeptin terminals and presence of inputs to kisspeptin cell bodies, triple- label immunofluorescence was conducted on a series of every sixth POA and ARC section from luteal (n=3) and surge (n=3) animals for kisspeptin, vGlut1 and synaptophysin. First, sections were incubated for 17 h in polyclonal rabbit anti- kisspeptin10 serum (1:200,000; No. 564, A. Caraty, Université Tours, Nouzilly, France), which has been previously characterized as specific for kisspeptin10 in sheep tissue (22, 27). Next, sections were incubated with biotinylated goat anti- rabbit IgG (1:500 in PBS containing 0.4% Triton X-100 (Fisher Scientific) and 4% NGS; 1 hour, Jackson Immunoresearch Laboratories, Inc.), ABC-elite (1:500 in PBS; 1 hour, Vector Laboratories, Burlingame, CA), TSA (1:250 in PBS containing 3% hydrogen peroxide/mL; 10 min, Perkin Elmer LAS, Inc., Boston, MA) and Alexa 488- Streptavidin (1:100 in PBS; 30 minutes; S32354, Invitrogen, Carlsbad, CA). Subsequently, sections were co- incubated in polyclonal rabbit anti-vGlut1 serum (1:1,000; Synaptic Systems, 135002, Goettingen, Germany) and monoclonal mouse anti- synaptophysin serum (1:200; Sigma, S5768) for 17 h. Next, sections were incubated sequentially for 30 minutes with Alexa 555 goat anti-rabbit (1:100; A21428, Invitrogen) and Cy5- donkey anti- mouse (1:100; 715-175-151, Jackson Immunoresearch Laboratories, Inc.). Sections were mounted onto Superfrost slides, dried and coverslipped with gelvatol (36).

Kisspeptin, vGlut2, Synaptophysin

Triple label immunofluorescence for kisspeptin/synaptophysin, and vGlut2 was conducted on luteal (n=4) and surge (n=4) tissue from a series of every sixth POA and ARC section using the identical protocol as described above, but with the substitution of rabbit anti- vGlut2 serum (1:2,000; Synaptic Systems, 135403).

Kisspeptin, vGlut2, Dynorphin

KNDy neurons and glutamate terminals in ARC sections of luteal and surge animals (n=3) were detected using the identical protocol as above using rabbit anti-kisspeptin10 serum (1:200,000; no. 564, A. Caraty), monoclonal mouse anti- vGlut2 (1:500; MAB5504, Chemicon, Billerica, MA), and rabbit anti- Dynorphin A (1:1,000; H-021-03, Phoenix Pharmaceuticals, Inc, Burlingame, CA). The kisspeptin signal was detected using TSA and Cy5- Streptavidin (1:100; 016-170-084, Jackson Immunoresearch Laboratories, Inc.), while vGlut2 and dynorphin were visualized using Alexa 488 goat anti- mouse IgG (1:100; Invitrogen) and Alexa555- goat anti-rabbit IgG (1:100; Invitrogen), respectively.

Kisspeptin, vGlut2, GnRH

To examine changes in kisspeptin and glutamatergic inputs to GnRH neurons across the estrous cycle, POA and MBH tissue sections of luteal (n=4) and surge (n=4) animals were processed for triple-label immunodetection of kisspeptin, vGlut2 and GnRH, using the same protocol as above. Kisspeptin was visualized using TSA and CY5-streptavidin. GnRH neurons were detected using monoclonal mouse anti-GnRH (1:400; SMI41R, Sternberger Monoclonals, Inc., Princeton, NJ), and Alexa 488-goat anti-mouse IgG (1:100; A11001, Invitrogen). vGlut2 was visualized using Alexa555-goat anti-rabbit IgG (1:100; A21428, Invitrogen).

5.2.6. ICC Controls

Specificity for kisspeptin and dynorphin antibodies has previously been determined (27, 36). Preabsorption of the rabbit vGlut2 antibody (1:2,000, Synaptic Systems) and vGlut2 peptide (1, 10, 25 and 50 µg/ml; Synaptic Systems, 135-4P) resulted in a complete abolishment of all vGlut2 staining in hypothalamic sections and western blot analysis showed a single band using sheep hypothalamic protein. To control for specificity of the Mouse anti-vGlut 2 antibody (Chemicon), dual- label immunofluorescence experiment was performed with mouse anti- vGlut2 (Chemicon) and rabbit anti-vGlut2 (Synaptic Systems). Confocal microscopy analysis confirmed that both vGlut2 antibodies exhibited complete overlap of the same terminals. To confirm that the rabbit vGlut2 antibody (Synaptic Systems) does not crossreact with kisspeptin, rabbit

vGlut2 antiserum (1:2,000; Synaptic Systems) was preincubated with kisspeptin peptide (10, 50 and 100 ug/ml; kisspeptin-10/Metastatin 45-54 amide; human, 048-56, Phoenix Pharmaceuticals, Inc). Preabsorption of the vGlut2 antibody with kisspeptin peptide did not interfere with vGlut2 immunostaining or co-expression of kisspeptin and vGlut2 in synaptic terminals for any peptide concentration. Dual-label immunofluorescence was carried out to confirm whether vGlut1 and vGlut2 are co-expressed in the same varicosities in the sheep MBH. The distribution and expression patterns of vGlut1 and vGlut2 are primarily complementary, present in different brain regions (57-59), although some terminals in the rat have been shown to express both (60). Although expression profiles differ, these vGluts retain very similar functional properties (58, 61). Tissue sections were stained for both vGlut1 (1:80,000; Synaptic Systems) and vGlut2 (1:2,000; Synaptic Systems) using the amplification protocol described above (vGlut1 amplified with TSA), as both antibodies are raised in rabbit. Confocal microscopy revealed no instances of colocalization of vGlut1 and vGlut2. Finally, for each triple- and dual-label experiment, omission of one or more of the primary antibodies resulted in a lack of staining for the corresponding antigen, demonstrating lack of cross-reactivity of secondary antibodies.

5.2.7. Confocal Analyses

All confocal analyses were conducted with an LSM510 laser- scanning confocal microscope (Zeiss, Thornwood, NY). Alexa 488 was visualized with a 505 nm emission filter and Argon laser, while Alexa 555 and Cy5 were imaged with HeNe lasers and a 560 and 650 nm emission filters, respectively. Neurons in which complete cell bodies were visible were selected for analysis and images were taken in 1 μ m intervals along the z-plane. For each animal, close contacts onto kisspeptin or GnRH cells were defined as an immunolabeled terminal in close apposition (no intervening pixels) to a cell body or proximal dendrite. When analyzing through the entire image z-stack, markers were placed on putative terminals, so that terminals flanking optical sections were not counted twice. Furthermore, orthogonal views confirmed contacts so that only markers contacting the neuron in all planes were accepted as close contacts. Minor brightness and level adjustments were made to the image using Adobe Photoshop (San Jose, CA).

For all analyses, the percentage of either kisspeptin or GnRH neurons receiving one or more specific close contacts was determined on a large sub-population (~20-30 neurons/ewe). A subset of the neurons which received at least one immunoreactive close contact (7-9) were then selected to quantify the number and type of synaptic close contacts in tissue from luteal phase and preovulatory surge groups. To ensure comparisons were not confounded by cell size, optical thickness measurements were taken of each cell in this analysis by counting the number of 1 μm optical sections in each z-stack. No significant differences were identified in optical thickness between luteal and surge groups, or within groups between POA and ARC.

To investigate whether dual-labeled kisspeptin+vGlut2 terminals expressed dynorphin, all putative kisspeptin boutons in a 1 μm optical section within the ARC and POA were counted and analyzed for the presence of vGlut2 and/or dynorphin, independent of whether they contact a labeled neuron. The percentage of kisspeptin/vGlut2 dual-labelled varicosities that also expressed dynorphin, and the percentage of dual-labelled kiss/dynorphin varicosities that expressed vGlut2 were calculated.

5.2.8. Statistical Analysis

Statistical significance for all confocal analyses was determined using two-way ANOVAs, with group and region as factors. All pairwise comparisons were done using the Holm-Sidak method with a 95% confidence level. All results are reported as mean \pm SEM, and statistical significance was considered as $P < 0.05$.

5.3. Results

5.3.1. vGlut2 as a marker for KNDy neurons and their terminals

Because of evidence in rodents that a majority of KNDy cells are glutamatergic (40), we first examined KNDy cells and fibers in the sheep ARC for colocalization of vGlut1 and vGlut2. KNDy neurons and their projections were identified by colocalized dynorphin and kisspeptin in individual cells, fibers and boutons. There was no colocalization of kisspeptin and vGlut1 in cell bodies or fibers in luteal and surge animals, and the majority (>95%) of kisspeptin cell bodies in the POA and ARC showed

at least one apposition by dual- labelled vGlut1/synaptophysin presynaptic terminals. By contrast, extensive colocalization of vGlut2 with kisspeptin and dynorphin was noted in fibers and terminals (Figure 5.1). Therefore, subsequent analyses focused on vGlut2 colocalization in KNDy cells and their projections.

In the ARC and POA, a large majority (>90%) of dual-labelled kisspeptin/dynorphin terminals colocalized vGlut2 (Figure 5.1). Conversely, only few instances of kisspeptin/vGlut2 dual-labeled terminals that did not colocalize dynorphin were noted (<2% and <8% in ARC and POA, respectively). The high degree of colocalization of vGlut2 with multiple KNDy peptides thus suggests that vGlut2 is an additional marker for fibers originating from kisspeptin cells in the ARC in the sheep. As expected, KNDY terminals in the ARC were frequently observed in direct contact with KNDy cell bodies and dendrites. Almost all (98%) of these KNDy-KNDy contacts also colocalized vGlut2 (arrows in Figure 5.1), indicating that these interconnections, like other KNDy projections, are glutamatergic.

5.3.2. Changes in inputs onto kisspeptin cells across the estrous cycle

To unambiguously identify close contacts between kisspeptin/vGlut2 terminals and kisspeptin cells as presynaptic, synaptophysin was used as a marker for synaptic terminals (22)(Figure 5.2), and compared the total number of synaptophysin-positive contacts onto kisspeptin cells of the ARC (KNDy cells) and POA, as well as the number of triple-labeled kisspeptin/vGlut2/synaptophysin inputs between luteal and surge groups.

Kisspeptin neurons of the POA and ARC, in both luteal and surge animals, received triple-labeled kisspeptin/vGlut2/synaptophysin-positive (Kiss + vGlut2 + Syn) inputs, as well as dual-labeled vGlut2/synaptophysin (vGlut2 + Syn), kisspeptin/synaptophysin (Kiss + Syn), and single-labeled synaptophysin (Syn only) inputs to their cell bodies (Table 5.1). Statistical analysis showed significant main effects of group for the total number of synaptic inputs (all synaptophysin-positive inputs; $F_{1,12}= 5.378$; $P = 0.039$), vGlut2 + Syn ($F_{1,12}= 5.189$; $P = 0.042$), and Syn only ($F_{1,12}= 5.694$; $P = 0.034$) inputs. Post hoc analyses revealed these effects of group on numbers of synaptic inputs were restricted to the ARC. The total number of synaptic inputs (all synaptophysin-positive contacts) onto ARC kisspeptin neurons was significantly higher

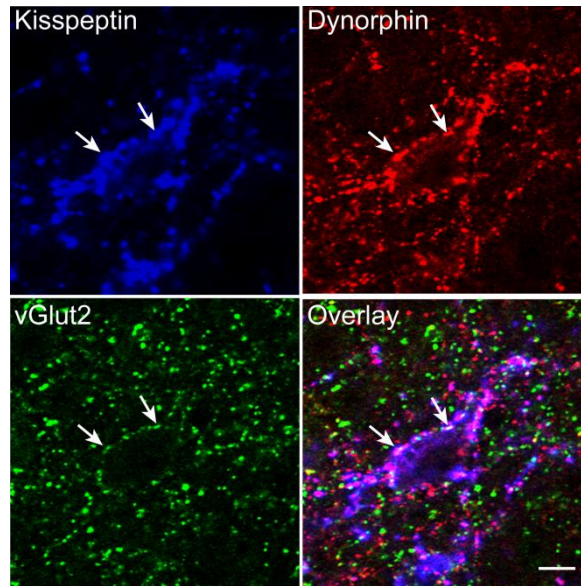


Figure 5.1. Confocal images (1 μm thickness; 63x) of triple-labeling for kisspeptin (*blue*), dynorphin (*red*) and vGlut2 (*green*) in the ARC. White arrows indicate examples of triple labelled kisspeptin/vGlut2/dynorphin terminals in close apposition to a dual-labeled kisspeptin/dynorphin cell body. *Scale bar*, 10 μm .

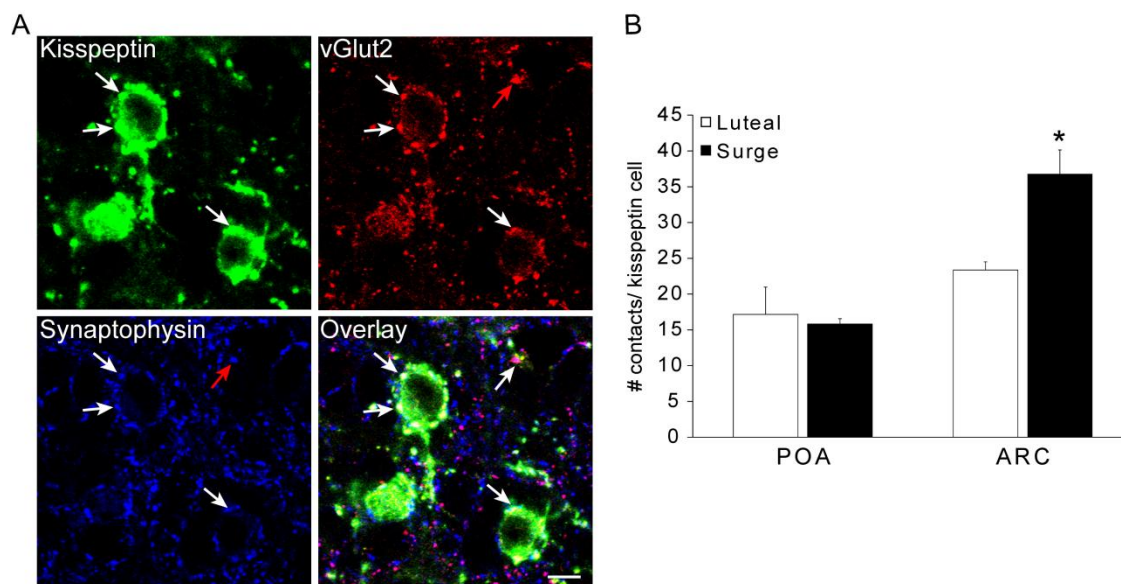


Figure 5.2. Synaptic inputs to kisspeptin neurons in the ARC and POA. A, Confocal images (1 μ m thickness; 63x) of triple-labeling for kisspeptin (*green*), vGlut2 (*red*) and synaptophysin (*blue*) in the ARC of a luteal phase ewe. *White arrows* indicate examples of a triple labelled kisspeptin/vGlut2/synaptophysin terminal in close contact with a kisspeptin soma, and *red arrow* indicates an example of a dual-labeled vGlut2/synaptophysin terminal. *Scale bar*, 10 μ m. B, Total number (mean \pm SEM) of synaptophysin-positive contacts onto kisspeptin neurons in luteal (n=4) and surge (n=4) animals. No changes were seen in synaptic inputs to POA kisspeptin neurons, but ARC kisspeptin neurons received significantly more synaptic inputs during the surge. *, $P < 0.05$.

POA	Luteal (n=4)	Surge (n=4)
Kiss + vGlut2 + Syn	1.94 ± 0.29	1.77 ± 0.18
vGlut2 + Syn	7.52 ± 1.53	6.99 ± 0.40
Kiss + Syn	0.03 ± 0.033	0 ± 0
Syn Only	7.67 ± 2.19	7.08 ± 0.25
ARC	Luteal (n=4)	Surge (n=4)
Kiss + vGlut2 + Syn	7.33 ± 1.31 [#]	7.12 ± 0.59 [#]
vGlut2 + Syn	7.30 ± 0.40	13.21 ± 1.73 ^{*#}
Kiss + Syn	0.03 ± 0.03	0.13 ± 0.09
Syn Only	8.71 ± 0.93	16.35 ± 1.74 ^{*#}

Table 5.1. Contacts onto kisspeptin neurons (mean ± SEM).

*Significant difference between luteal and surge, within brain region;

[#]Significant difference between ARC and POA, within luteal or surge groups.

during the surge compared to the luteal phase ($P < 0.01$), while no differences were seen between groups in contacts onto POA kisspeptin neurons (Figure 5.2). This difference in inputs onto ARC kisspeptin was not due to changes number of Kiss + vGlut2 + Syn inputs, which did not change across the estrous cycle, but rather to a near doubling in the numbers of vGlut2 + Syn ($P < 0.01$) and Syn-only contacts ($P < 0.01$) onto ARC kisspeptin neurons in surge animals compared to luteal phase (Table 5.1). By contrast, Kiss + Syn inputs were rarely seen in close apposition to kisspeptin neurons in either the POA or ARC (Table 5.1), and did not differ between luteal and surge animals.

Statistical analyses detected a significant main effect of region for total inputs, ($F_{1,12} = 27.030$; $P < 0.01$), vGlut2 + Syn ($F_{1,12} = 6.437$; $P = 0.026$), kiss + vGlut2 + Syn ($F_{1,12} = 53.053$; $P < 0.01$) and syn-only ($F_{1,12} = 12.174$; $P < 0.01$) inputs. Post hoc analyses revealed that during the surge, ARC kisspeptin cells receive significantly more total inputs ($P < 0.01$), vGlut2 + Syn ($P < 0.01$), and Syn only ($P < 0.01$) inputs than POA kisspeptin cells, whereas no regional differences were detected during luteal phase for these inputs types. In addition, ARC kisspeptin cells receive significantly more triple kiss + vGlut2 + Syn inputs than POA kisspeptin cells ($P < 0.01$), regardless of phase. Interestingly, the proportion of kiss + vGlut2 + Syn inputs as a function of total inputs showed a significant main effect for both group ($F_{1,12} = 4.79$; $P = 0.049$) and region ($F_{1,12} = 26.001$; $P < 0.01$). Post hoc analyses revealed that these triple labeled inputs represent a greater proportion of total synaptic contacts onto ARC kisspeptin cells compared with POA kisspeptin neurons, in both luteal ($31.07 \pm 4.36\%$ ARC vs. $11.93 \pm 1.47\%$ POA) and surge ($19.88 \pm 2.76\%$ ARC vs. $11.17 \pm 1.05\%$ POA) animals ($P < 0.01$).

5.3.3. Changes in inputs to GnRH cells across the estrous cycle

GnRH neurons in both the POA and MBH were contacted by dual-labelled kisspeptin/vGlut2 (Kiss + vGlut2) terminals, as well as by single-labeled vGlut2 and kisspeptin terminals (Figure 5.3; Table 5.2). Almost all (>95%) GnRH neurons in the POA and MBH received at least one single-labelled vGlut2 input to their cell body, and between 45% and 60% of GnRH neurons in the POA and MBH, respectively, received at least one dual-labeled Kiss + vGlut2 input. In contrast to kisspeptin cells in the ARC and

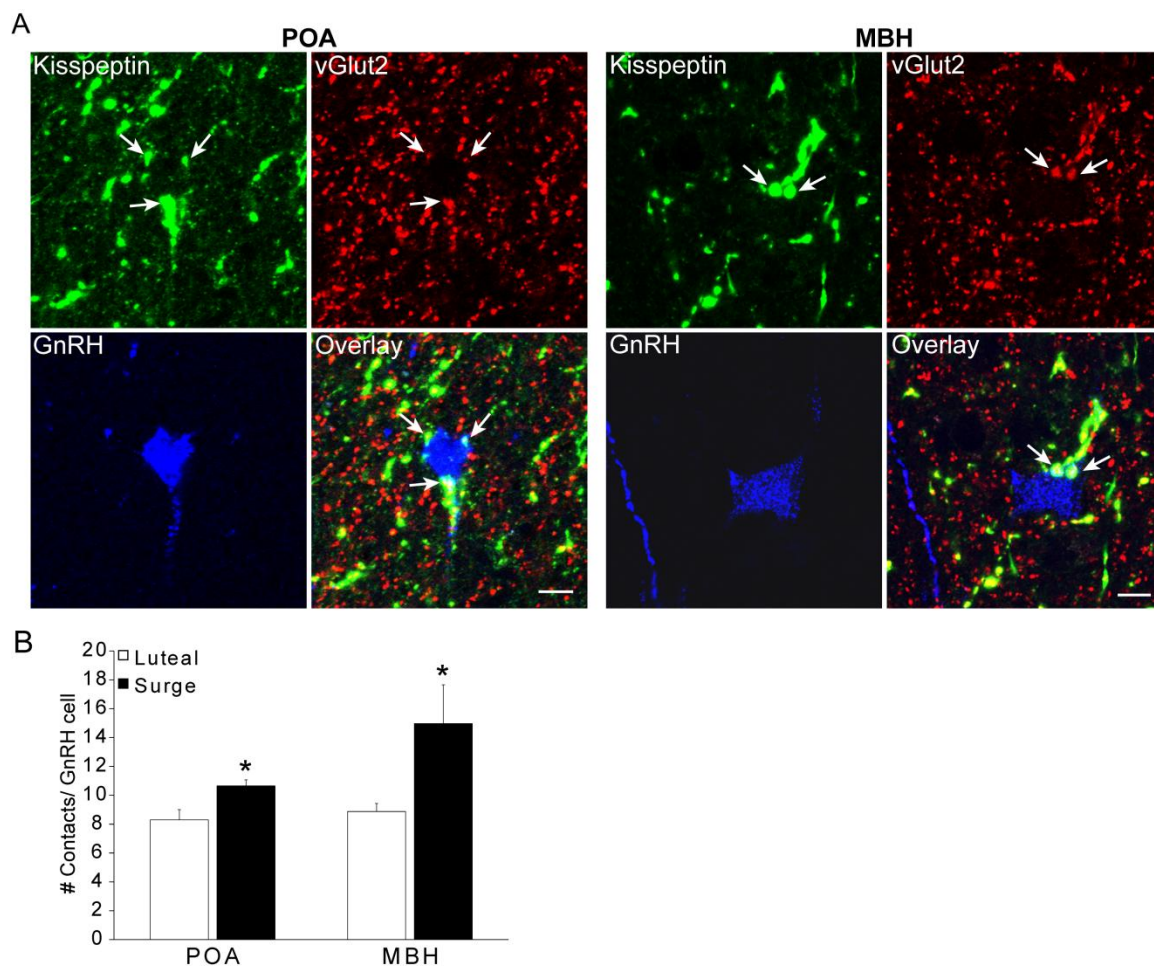


Figure 5.3. Synaptic inputs onto GnRH neurons in the POA and MBH. A, Confocal images (1 μm thickness; 63x) triple-labelled for kisspeptin (*green*), vGlut2 (*red*) and GnRH (*blue*) in the POA and MBH. White arrows indicate examples of dual labelled kisspeptin/vGlut2 terminal in close contact with a GnRH soma. *Scale bar*, 10 μm . B, Total number of contacts onto GnRH neurons from kisspeptin and vGlut2 labelled terminals (single and dual-labelled). GnRH neurons in the POA and MBH receive significantly more of these inputs during the surge than during the luteal phase. *, $P < 0.05$.

POA	Luteal (n=4)	Surge (n=4)
Kiss + vGlut2	1.57 ± 0.15	1.91 ± 0.16
vGlut2	4.67 ± 0.48	6.63 ± 0.60
Kiss	2.06 ± 0.30 [#]	2.13 ± 0.39 [#]
MBH	Luteal (n=4)	Surge (n=4)
Kiss + vGlut2	2.05 ± 0.28	4.85 ± 0.53 ^{*#}
vGlut2	5.65 ± 0.80	8.67 ± 2.46
Kiss	1.18 ± 0.16	1.45 ± 0.44

Table 5.2. Contacts onto GnRH neurons (mean ± SEM).

*Significant difference between luteal and surge groups, within brain region;

[#]Significant difference between POA and MBH, within luteal or surge groups.

POA which received almost no single-labeled kisspeptin inputs, 55-65% of GnRH neurons in the POA and MBH were contacted by at least one single-labeled kisspeptin terminal in both luteal and surge groups. Interestingly, 76-80% of MBH GnRH neurons received at least one input that was single or dual-labeled for kisspeptin in luteal and surge groups, while 20-23% showed no kisspeptin inputs from single or dual labeled terminals. In the POA, 61-70% of luteal phase and surge animals received at least one kisspeptin input (from single or dual-labeled terminals), and likewise, 30-39% did not receive any kisspeptin inputs from single or dual-labeled terminals.

Two-way ANOVA revealed significant main effects of group on total number of kisspeptin and vGlut2 inputs (both single- and dual-labeled; $F_{1,12} = 8.683$; $P = 0.012$), and dual kiss + vGlut2 inputs ($F_{1,12} = 24.636$; $P < 0.01$). Post hoc analysis revealed that the total number of kisspeptin and vGlut2 inputs (both single- and dual-labelled) per GnRH neuron was increased during the surge, in both the POA and MBH ($P = 0.012$) (Figure 5.3). In the MBH, this increase appears to be due to an increase in the number of Kiss + vGlut2 inputs ($P < 0.01$), as single vGlut2 and single kiss inputs did not differ (Table 5.2).

Statistical analysis also showed a main effect of region for single kiss ($F_{1,12} = 5.399$; $P = 0.039$) and kiss + vGlut2 ($F_{1,12} = 29.179$; $P < 0.01$) inputs. Post hoc comparisons revealed that POA GnRH neurons receive significantly more single kisspeptin inputs than MBH GnRH cells, regardless of phase ($P = 0.039$; Table 5.2), and that MBH GnRH neurons receive significantly more kiss + vGlut2 inputs during the surge ($P < 0.01$), but not luteal phase (Table 5.2).

In addition to dual-labeled Kiss + vGlut2 inputs onto GnRH cell bodies, we also observed kiss + vGlut2 fibers within the internal and external zones of the median eminence (Figure 5.4). Dual-labeled Kiss + vGlut2 fibers were often seen in close proximity to GnRH fibers and terminals (Figure 5.4). Single-labeled vGlut2-positive terminals were also frequently seen in the median eminence, nearby GnRH fibers and terminals.

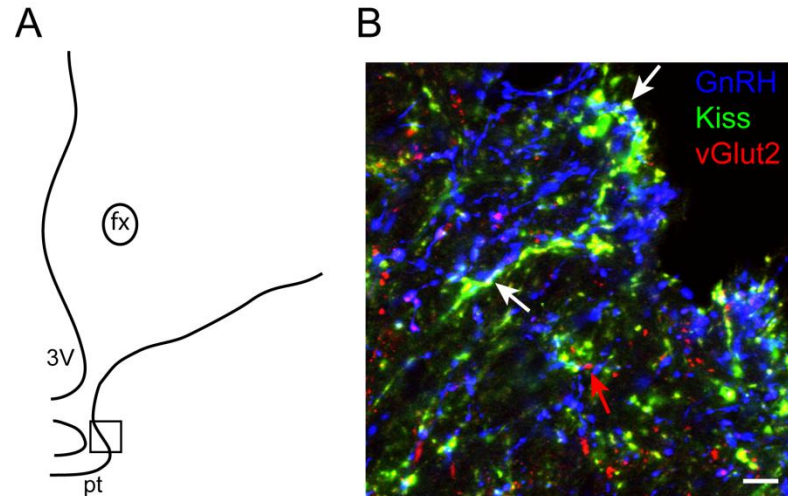


Figure 5.4. Kisspeptin and vGlut2 associations with GnRH fibers in the median eminence. A, Drawing showing a section through the ovine median eminence and the location of the image (boxed area) shown in B. B, Confocal image (1 μm thickness; 63x) of a section through the ovine median eminence triple-labelled for kisspeptin (*green*), vGlut2 (*red*) and GnRH (*blue*). White arrow indicates examples of dual labelled (*yellow*) kisspeptin/vGlut2 fibers and terminals adjacent to GnRH fibers (*blue*), and the red arrow indicates a single labelled vGlut2 terminal. fx = fornix; pt= pars tuberalis; 3V= third ventricle. *Scale bar*, 10 μm .

5.4. Discussion

The results of this study provide strong evidence for neuroplasticity of synaptic inputs to KNDy cells and GnRH neurons across the estrous cycle in sheep. Specifically, we found that KNDy neurons examined in animals perfused at the onset of the LH surge had nearly twice the number of total (synaptophysin-positive) inputs per cell as did KNDy cells in luteal phase animals. Similarly, there were more kisspeptin/vGlut2-containing contacts onto GnRH cell bodies at the time of the surge in the MBH. As evidence for estrous cycle-related neuroplasticity, our results complement and extend earlier observations in rodents and reinforce the long held notion that the ARC is a focal region for neuroplastic changes that accompany female reproductive cyclicity (62-64).

In addition, the results provide evidence that, in addition to the three neuropeptides, KNDy cells in the ewe also contain the excitatory neurotransmitter, glutamate (based on the presence of vGlut2). Our conclusion that KNDy cells are glutamatergic was based on the high percentage (>90%) of dual-labelled kisspeptin/dynorphin terminals that colocalized vGlut2. Two other lines of evidence also support this contention. First, vGlut2 and ER α are co-expressed in the same neurons in the sheep ARC, as shown using immunohistochemistry in the ARC (65). Given that KNDy neurons possess the highest degree of co-localization of ER α of any neuropeptide cell group of the ARC (1), it is likely that many of these vGlut2/ ER α cells were KNDy neurons. Second, studies in rodents have shown that ARC NKB neurons express vGlut2 protein (41), and ARC kisspeptin cells express vGlut2 mRNA (40), indicating that at least in rodents, these neurons are glutamatergic. One interesting corollary of the observation that KNDy neurons contain vGlut2 is that kisspeptin terminals that do not contain vGlut2 must originate from other kisspeptin neurons. One likely source is the POA kisspeptin neurons, although small populations of kisspeptin neurons have also been described in the dorsomedial and ventromedial hypothalamic nuclei of sheep (1, 4, 24). It is also possible that a subset of KNDy neurons are not glutamatergic, and is supported by our finding that a small number of KNDy (kisspeptin + dynorphin) terminals in the ARC and POA were not labelled with vGlut2. Ultimately, confirmation of the cellular origin of vGlut2/kisspeptin terminals in sheep awaits the analysis of *Kiss1* mRNA and vGlut2 mRNA co-expression in the same neurons.

A collection of evidence supports a direct effect of kisspeptin on GnRH neurons: kisspeptin stimulates GnRH electrophysiological activity (6, 66), GnRH neurons express the Kiss1 receptor (Kiss1R) (6, 7, 18, 67), and confocal studies in various mammals show kisspeptin contacts onto GnRH cell bodies (1). An intriguing finding from our work is that POA and MBH GnRH neurons receive single-labeled kisspeptin inputs as well as dual-labeled vGlut2/kisspeptin inputs. In the POA, single-labeled kisspeptin terminals make up a larger proportion of the total inputs to these neurons, while in the MBH, glutamatergic kisspeptin inputs comprise a larger proportion of the total inputs. This regional difference suggests that kisspeptin inputs to MBH GnRH neurons arise primarily from nearby KNDy cells, whereas the kisspeptin contacts onto POA GnRH neurons are most likely to originate from POA kisspeptin neurons. Interestingly, in rodents, where GnRH neurons are found in the POA but not in the MBH, the primary source of kisspeptin inputs to GnRH neurons is similarly from the rostral kisspeptin population in the AVPV (68).

While the majority of kisspeptin input to GnRH cells in the POA appeared as single-labeled terminals, there were a considerable number that colocalized vGlut2 and kisspeptin, suggesting that ARC KNDy cells do provide some input to POA GnRH cells. Moreover, we have shown using markers of KNDy terminals that KNDy neurons provide direct inputs to GnRH neurons in the POA, as well as in the AHA and MBH (69). This is supported by evidence by tract tracing in the sheep showing retrogradely-labeled glutamatergic cells in the ARC after Fluoro-Gold injection into the ventral POA (65). However, injections of anterograde tracers in the sheep ARC have revealed only very few fibers projecting to the POA (70) and contacting GnRH neurons (64). This discrepancy may be due to the limitations of the tracing techniques, since anterograde tracer injections only label a small number of cell bodies in comparison to retrograde tracers that are taken up by terminals arising from many cell bodies. It is likely that our use of dual immunocytochemical markers for KNDy cells is also a more sensitive way of labelling projections than anterograde tracer injections. However, studies in rodents using dual-immunolabeling to identify KNDy projections also show only a small number of fibers in the POA (18, 33, 35, 52, 71) but in most of these studies contacts onto GnRH neurons were not analyzed. Our data also support a projection from KNDy neurons to GnRH

terminals in the median eminence because GnRH-immunoreactive axons in the external zone of the median eminence are frequently juxtaposed by glutamatergic KNDy axonal fibers. These observations are consistent with other data in sheep (18) and support a proposed stimulatory action of kisspeptin (and KNDy) cells on GnRH nerve terminals located in the neurosecretory zone of the median eminence in a variety of mammals (1).

Previous electron microscopic (EM) and confocal studies demonstrated that vGlut2-containing terminals provide synaptic inputs onto POA GnRH cells in both rodents and sheep (45-48, 72) and that these inputs increase during the breeding season in the ewe (46). However, plasticity across the estrous cycle in kisspeptin and glutamatergic inputs to GnRH neurons had not yet been explored in any species. Our data extends previous findings of seasonal plasticity in glutamatergic inputs in sheep to show that the number of glutamatergic inputs to MBH GnRH neurons, and specifically that of dual-labeled glutamatergic/kisspeptin inputs, increases during the preovulatory surge. This change is consistent with evidence in rats and mice implicating glutamate in the preovulatory LH surge: glutamate neurotransmission to GnRH is increased during positive feedback (73, 74) and the expression and activation of glutamate receptors on GnRH neurons are increased in the presence of E2 and during the preovulatory surge (49, 51).

The current study also provides key anatomical evidence for glutamatergic action proximal to kisspeptin neurons. Recently, d'Anglemont de Tassigny and colleagues (2010) showed that peripheral NMDA is sufficient to induce Fos activation within mice kisspeptin neurons (75). This data, along with our finding of increased glutamatergic inputs to kisspeptin (KNDy) neurons at the time of the surge, suggests an action of glutamate proximal to kisspeptin cells that may play a crucial role in their activation at the onset of the surge (76, 77). This would be consistent with the increase in Fos expression that occurs both in kisspeptin neurons (23, 25) and GnRH neurons (78, 79) at the time of the LH surge. Moreover, an increase in the number of glutamate-kisspeptin contacts onto GnRH neurons in the MBH during the surge is consistent with the hypothesis that KNDy neurons play a key role in the activation of GnRH neurons at the time of positive feedback (23, 80).

Our findings extend previous observations of neurochemical (27) and morphological (81) differences between ARC and POA kisspeptin neurons to include differences in synaptic input. In luteal phase animals, ARC kisspeptin (KNDy) cells received more vGlut2/kisspeptin (KNDy) inputs than POA kisspeptin cells (Table 5.2). Moreover, synaptic inputs to ARC kisspeptin cells, but not those in the POA, increased during the preovulatory surge. Interestingly, this increase in total synaptic inputs reflected an increase in glutamatergic terminals (and other unidentified synaptic terminals), but not kisspeptin-containing synapses. In rodents, synaptic plasticity occurs across the estrous cycle, with an increase in axosomatic synapses onto AVPV neurons during proestrus (82, 83) that is estrogen-dependent (63); however the phenotype of the neurons onto which this plasticity occurs has not been described. In rodents the AVPV is the site of E2 positive feedback (21), while in sheep, this site lies within the MBH (26). These anatomical and temporal correlations in both rodents and sheep raise the possibility that this increase in the number of glutamatergic synaptic inputs may be an important mechanism in the control of GnRH and gonadotropin secretion leading to the generation of the preovulatory surge.

Our observation of colocalized kisspeptin and glutamate in terminals contacting GnRH neurons is consistent with the hypothesis that these two stimulatory neurotransmitters act synergistically. Studies in middle aged female rats reveal that delayed and attenuated LH surges are correlated with both a decrease in glutamatergic neurotransmission and *Kiss1* mRNA, and that infusion of kisspeptin restores surge amplitude and glutamate levels in these rats (74, 84, 85). Interestingly, blockade of NMDA receptor activation can prevent this restoration of surge amplitude by kisspeptin, while still maintaining heightened glutamate levels (84). These data do not eliminate the possibility that glutamate is acting at least in part, in an autoregulatory fashion upon the same kisspeptin neurons from which it is released, but also suggests that kisspeptin neurons may provide afferent input to other glutamatergic neurons. Electrophysiological studies demonstrate an indirect effect of kisspeptin on GnRH, suggesting that glutamate release may be occurring downstream of kisspeptin neurons (66, 84). Thus, the action of glutamate is most likely occurring at various levels of the system (i.e. on kisspeptin and

KNDy neurons, within the KNDy-KNDy circuitry, and downstream of kisspeptin), and the physiological role at each level remains to be determined.

In summary, our results suggest that KNDy neurons in the female sheep are glutamatergic, and that glutamatergic markers are present within terminals that constitute reciprocal circuitry among KNDy neurons, as well as in their inputs to GnRH cells. In addition, we found that inputs to both KNDy and GnRH neurons are increased during the preovulatory surge, suggesting a role for the ARC KNDy neurons and glutamatergic synaptic inputs in positive feedback regulation of GnRH. This work provides new and exciting evidence for neuroplasticity within the kisspeptin and GnRH system across the estrous cycle, and highlights the complexity of this network in the feedback regulation of reproduction.

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CHAPTER 6

Estrogenic regulation of presynaptic inputs to Kiss1 neurons in the mouse is independent of estrogen receptor- alpha ($ER\alpha$) in Kiss1 neurons.

6.1. Introduction

Kisspeptin, coded for by the *Kiss1* gene (1), is now widely recognized as an important player in the control of GnRH secretion and reproductive axis (2). In 2003, simultaneous reports identified mutations in the kisspeptin receptor (Kiss1R) that produced hypogonadotropic hypogonadism (3, 4), thereby casting kisspeptin into the reproductive neuroendocrine spotlight. Since then, kisspeptin neurons have been shown to be potent stimulators of GnRH/LH secretion in a variety of species (5-10), and provide direct input to GnRH cell bodies, terminals, and dendrites (11-17).

Kisspeptin neurons are present in a wide variety of species in two well-conserved populations: in the arcuate nucleus (ARC), and the rostral periventricular area of the 3rd ventricle (RP3V), including the anteroventral periventricular nucleus (AVPV) and the periventricular preoptic nucleus (PVpo) (18). ARC and AVPV kisspeptin neurons appear to mediate E2 feedback and both populations colocalize the estrogen receptor- α (ER α) (19, 20). E2 exerts negative and positive feedback actions on *Kiss1* mRNA in the ARC and AVPV, respectively (21), and ER α is critical for these actions in mice, as ER α knockout mice are not responsive to OVX (19). More specifically, ER α within kisspeptin neurons has recently been shown to regulate these feedback actions of E2 (22), supporting a direct action of E2 on kisspeptin cells to mediate these effects.

Kisspeptin neurons located in the ARC coexpress kisspeptin, neurokinin B (NKB), and dynorphin (Dyn; known as the 'KNDy' cells), and form a reciprocally interconnected network (23-27). It is hypothesized that the stimulatory actions of NKB and kisspeptin, and the inhibitory actions of Dyn within the KNDy network regulate GnRH/LH pulses (28). Moreover, recent studies in mice and sheep have shown that KNDy cells (29) and their reciprocal connections (12), coexpress vGlut2. Therefore, at least some of the glutamatergic input to KNDy cells arises from the ARC. In this study, we sought to investigate whether changing levels of E2 alter glutamatergic and KNDy inputs to kisspeptin cells, and whether ER α signaling in kisspeptin neurons is critical for any E2-mediated changes.

Recent electrophysiological data has shown that E2 influences inhibitory synaptic activity on ARC and AVPV kisspeptin cells, and that these actions of E2 are mediated via ER α action within kisspeptin neurons (22). In addition, prepubertal kisspeptin neurons

show increased inhibitory tone compared with adult animals (22), suggesting developmental changes in the inhibition of kisspeptin cells. Thus, in the current study, we also extended these findings to investigate E2-mediated changes in GABAergic and other synaptic inputs and ask whether they are dependent on ER α signaling in kisspeptin neurons. Finally, based on findings of increased inhibitory tone in prepubertal animals, we examined developmental changes in presynaptic GABAergic inputs to Kiss1 cells.

6.2. Materials and Methods

6.2.1. Animals

Female (8-10 weeks old) Kiss1-Cre/GFP mice expressing enhanced green fluorescent protein (eGFP) under the transcriptional control of Cre-recombinase were used (29). Estrous cycles were monitored and Kiss1-Cre/GFP females were divided into four groups: diestrous (ovary intact; estrous cycle measured by vaginal cytology), ovariectomized (OVX, 7-10 days before perfusion), ovariectomized and implanted with SILASTIC capsule (Dow-Corning) containing 1.0 μ g of 17 β - estradiol (Sigma, company info) suspended in sesame oil (OVX+E2, 3-4 days before perfusion), and prepubertal (18-25 d old, showing no vaginal opening). To selectively delete estrogen receptor- α (ER α) from Kiss1 neurons, Kiss1-Cre/GFP mice were crossed with ER $\alpha^{flox/flox}$ (22, 30). All mice used in this study were housed at the University of Texas Southwestern Medical Center Animal Resource Center in a light (12 h on/12 h off) and temperature (21-23°C) controlled environment. Animals were given *ad libitum* access to water, and were on a standard chow diet (Harlan Teklad Global Diet, Harlan Laboratories). All experiments and procedures were carried out in accordance with the guidelines established by the National Institute of Health Guide for the Care and Use of Laboratory Animals, and with the University of Texas Institutional Animal Care and Use Committee.

6.2.2. Tissue Collection

Prior to perfusion, blood samples were collected directly from the heart, and hormone assays were performed by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core. Mice were deeply anesthetized (sodium pentobarbital; 270 mg/mL/kg; i.p.) and perfused intracardially with 50 mL of saline,

followed 10% formaline in 0.1 M phosphate buffer (PB). After perfusion, brains were removed and post-fixed in 10% buffered formalin, then stored at 4°C in 30% sucrose. Four parallel series of coronal sections (25 µm) were cut on a freezing microtome and stored at -20°C in cryoprotectant for later processing. Tissue sections were shipped to the University of Mississippi Medical Center at 4°C, where all immunocytochemistry was carried out.

6.2.3. ICC

All immunocytochemistry was carried out on free-floating sections at room temperature, and washed with 0.1 M PBS between incubations. For all experiments, sections were incubated in 1% hydrogen peroxide (10 min in PBS; EMD Chemicals, Inc., Gibbstown, NJ) to occupy endogenous peroxidase activity, followed by incubation in a PBS solution containing 0.1% bovine serum albumen (BSA) and 0.4% Triton X-100 (Fisher Scientific, Pittsburgh, PA) (PBS+) for 1 h to minimize nonspecific binding.

GFP/NKB/vGlut2

To investigate changes in glutamatergic inputs to AVPV Kiss1 and ARC KNDy neurons, sections from OVX (n=7), OVX+E (n=7) and ER $\alpha^{flox/flox}$ (n=7) mice were processed for GFP, vGlut2 and NKB. First, sections were incubated for 17 h in polyclonal chicken anti-GFP (1:1000 in PBS+; GFP-1010, Aves Labs Inc., Tigard, OR, USA) and polyclonal guinea pig anti- vGLUT2 (1:3000 in PBS+; AB2251, Millipore, Billerica, MA, USA). Next, sections were incubated in Goat anti-chicken Alexa 488 IgG (1:100 in PBS; A11039, Molecular Probes, Carlsbad, CA, USA) followed by incubation in goat anti- guinea pig 647 IgG (1:100 in PBS; A21450, Molecular Probes) for 30 minutes each. Subsequently, sections were incubated for 17 h in polyclonal rabbit anti-Neurokinin B (1:3000; NB300-201, Novus Biologicals, Littleton, CO, USA). Next, sections were incubated with biotinylated goat anti- rabbit IgG (1:500 in PBS+; Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) for 1 hour, followed by incubation in ABC-elite (1:1000 in PBS; 1 hour, Vector Laboratories, Burlingame, CA), TSA (1:250 in PBS containing 3% hydrogen peroxide/mL; 10 min, Perkin Elmer LAS, Inc., Boston, MA) and Alexa 555-Streptavidin (1:100 in PBS for 30 minutes; S32355,

Molecular Probes). Finally, sections were mounted onto Superfrost slides, dried and coverslipped with gelvatol.

NKB/Kisspeptin

To visualize KNDy projections to KNDy neurons in the ARC, and to AVPV Kiss1 neurons, tissue from OVX (n=6), OVX+E (n=5) and ER $\alpha^{flox/flox}$ (n=6) mice, dual-label immunofluorescence was conducted to process tissue for NKB and kisspeptin. In this experiment, sections were not processed for GFP immunoreactivity, but were sheltered from light to preserve endogenous GFP, which was used to identify GFP-labeled neurons in this experiment. The same protocol for detection of NKB was used as described above except the substitution of Cy5- Streptavidin (1:100 in PBS; 016-170-084, Jackson Immunoresearch Laboratories, Inc.) for Alexa 555-Streptavidin. To visualize kisspeptin, tissue sections were incubated for 17 h in polyclonal rabbit anti-kisspeptin10 serum (1:1000 in PBS+; AC 564, A. Caraty, Université Tours, Nouzilly, France), which has been previously characterized as specific for kisspeptin10 in mouse tissue (14), followed by incubation in goat anti-rabbit Alexa 555 IgG (1:100 in PBS; 30 minutes; A21428, Molecular Probes). Finally, sections were mounted onto Superfrost slides, dried and coverslipped using gelvatol.

GFP/GAD65&67/Synapsin

To examine GABAergic inputs and other presynaptic inputs to Kiss1 cells in the AVPV and ARC, triple label immunofluorescence was conducted on tissue sections from OVX (n=6), OVX+E (n=5) and ER $\alpha^{flox/flox}$ (n=6) (Experiment 3) and diestrus (n=6) and prepubertal (n=5) mice (Experiment 4) for GFP, GAD65&67 (marker of presynaptic GABAergic terminals) and synapsin (marker of presynaptic terminals). Sections were incubated for 17 h in goat anti- Synapsin (1:100 in PBS+; sc-8295, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) followed by incubation in donkey anti- goat Cy3 IgG (1:100 in PBS; 705-165-147, Jackson Immunoresearch Laboratories, Inc.) for 30 minutes. Next, sections were incubated for 17 h in polyclonal chicken anti-GFP (1:1000 in PBS+; GFP-1010, Aves Labs Inc.) and rabbit anti- GAD65&67 (1:500 in PBS+; AB1511, Millipore). Subsequently, sections were incubated in Goat anti-chicken Alexa

488 IgG (1:100 in PBS; A11039, Molecular Probes, Carlsbad, CA, USA) followed by donkey anti-rabbit Cy5 (1:100 in PBS; 711-175-152, Jackson ImmunoResearch Laboratories, Inc.) for 30 minutes each. Finally, sections were mounted onto Superfrost slides, dried and coverslipped using gelvatol.

6.2.4. ICC Controls

The rabbit anti-Neurokinin B antibody (1:3000; Novus Biologicals) was preabsorbed with Neurokinin B control peptide (1, 10, 25 $\mu\text{g}/\text{mL}$; NB300-201PEP, Novus Biologicals), and resulted in complete abolishment of NKB staining at all peptide concentrations and full staining with the positive control (no peptide). Preabsorption of the guinea pig vGlut2 antibody (1:3000; Millipore) with vGlut2 control peptide (10, 25 $\mu\text{g}/\text{mL}$; AG209, Millipore) completely abolished all vGlut2 staining. In addition, goat anti-Synapsin antibody (1:100; Santa Cruz) was preabsorbed with synapsin control peptide (10, 25 $\mu\text{g}/\text{mL}$; sc-8295P, Santa Cruz), and rabbit anti-GAD65 & 67 antibody (1:500; Millipore) was preabsorbed with GAD65&67 peptide (10, 25 $\mu\text{g}/\text{mL}$; AG252, Millipore), and resulted in complete abolishment of immunostaining. In additional control experiments, primary antibodies were omitted and resulted in complete absence of immunostaining.

6.2.5. Confocal Analysis

All confocal analyses were conducted with a Leica TCS SP2 laser scanning confocal microscope (Leica Microsystems, Buffalo Grove, IL, USA). Alexa 488 was visualized with a 458/476/488/514 nm emission filter and Argon laser, while Alexa 555 and Cy5 were imaged with HeNe lasers and a 543 and 633 nm emission filters, respectively. Neurons in which complete cell bodies (through the entire nucleus) were visible were selected for analysis and images were taken in 1 μm intervals along the z-plane. All images were analyzed using LAS AF Lite program, and presynaptic inputs were defined as immunolabeled terminals in close apposition (no intervening pixels) to a cell body. Soma perimeter measurements were taken in three or four 1 μm sections through the middle of each neuron, and presynaptic inputs were counted only in those 1 μm sections. Markers were placed on putative terminals to ensure that terminals present

in two adjacent 1 μm sections were not counted twice. Minor brightness and level adjustments were made to the image using Adobe Photoshop (San Jose, CA).

6.2.5.1. Experiment 1: Glutamatergic inputs to AVPV Kiss1 and ARC KNDy neurons

To examine glutamatergic inputs onto Kiss1 neurons in the mouse, ten GFP-labeled Kiss1 cells in the AVPV and Kiss1 of OVX (n=7), OVX+E (n=7) and ER α ^{flox/flox} (n=7) mice were selected. The number of close appositions by single-labeled NKB, single-labeled vGlut2, and dual-labeled NKB/vGlut2 were counted. These, as well as total vGlut2 (single-vGlut2 + dual vGlut2/NKB) and total NKB (single NKB + dual vGlut2/NKB) inputs to Kiss1 (GFP-labeled) cells were expressed as inputs per 100 μm soma perimeter.

6.2.5.2. Experiment 2: KNDy (Kiss/NKB) inputs to AVPV Kiss1 and ARC KNDy neurons

To identify E2 induced changes in KNDy inputs to KNDy cells or AVPV Kiss1 cells, and the role of ER α , kisspeptin and NKB (single and dual-labeled inputs) were counted in apposition to GFP-labeled Kiss1 cells in the ARC (KNDy cells) and AVPV (10 cells/animal/region). Although GFP was not immunolabeled, endogenous GFP was still present in both ARC and AVPV neurons, and those neurons were selected for analysis. Numbers of single-labeled kisspeptin, single-labeled NKB, and dual-labeled Kisspeptin/NKB inputs were counted and expressed as inputs per 100 μm soma perimeter.

6.2.5.3. Experiment 3: GABAergic and other synaptic inputs onto AVPV Kiss1 and ARC KNDy neurons

(a) E2-Induced Changes in Adult Mice

GFP-labeled cells (10/animal/region) were selected in the ARC and AVPV of OVX (n=6), OVX+E (n=5) and ER α ^{flox/flox} (n=6) mice. All GAD65&67 (GAD) terminals in contact with GFP (Kiss1) cells were co-labeled with the synaptic vesicle protein, synapsin. Therefore all dual-labeled GAD65&67/synapsin as well as single-labeled

synapsin terminals were counted and expressed as number of GAD65&67/Synapsin, Synapsin, and Total Synapsin (dual GAD65&67/synapsin + single synapsin) inputs per 100 μm soma perimeter.

(b) Developmental Changes in GABAergic and other synaptic Inputs

GFP-labeled cells (10/animal/region) were selected in the ARC and AVPV of diestrus (n=6) and prepubertal (n=5) mice. All analyses on this tissue are previously described in 3a.

6.2.6. Statistical Analyses

For each experiment, numbers of inputs per 100 μm soma perimeter was calculated. Average soma perimeter and inputs/100 μm soma perimeter were averaged for all cells within each animal, and an average of all animals per group was calculated. One-way ANOVAs were conducted for vGLut2, NKB, NKB/vGlut2, total vGlut2 and total NKB inputs. All pairwise comparisons were conducted using Tukey test with a 95% confidence interval. Results are reported as mean \pm SEM, and statistical significance was considered as $P < 0.05$.

6.3. Results

6.3.1. Experiment 1: E2-induced changes in glutamatergic input to Kiss1 cells do not require ER α signaling in Kiss1 cells

E2 treatment in OVX mice significantly decreased the number of single-labeled vGlut2 inputs onto Kiss1 cells in both the ARC ($P = 0.02$; Figure 6.1A) and AVPV ($P < 0.01$; Figure 6.1). Deletion of ER α from Kiss1 cells did not reverse the effects of E2 to downregulate vGlut2 inputs in either area, suggesting that this effect is regulated by ER α signaling in non-kisspeptin cells. Very few single-labeled NKB inputs were found in close contact with Kiss1 cells in the ARC and AVPV, and E2 did not appear to have an effect on numbers of inputs.

Very few single-labeled NKB inputs were present onto Kiss1 cells in the ARC and AVPV, and these did not show changes with E2 treatment. Dual-labeled vGlut2/NKB inputs were present in contact with ARC Kiss1 cells, but did not show any E2-mediated changes. Variability in the inputs to ER $\alpha^{flox/flox}$ animals did not allow for

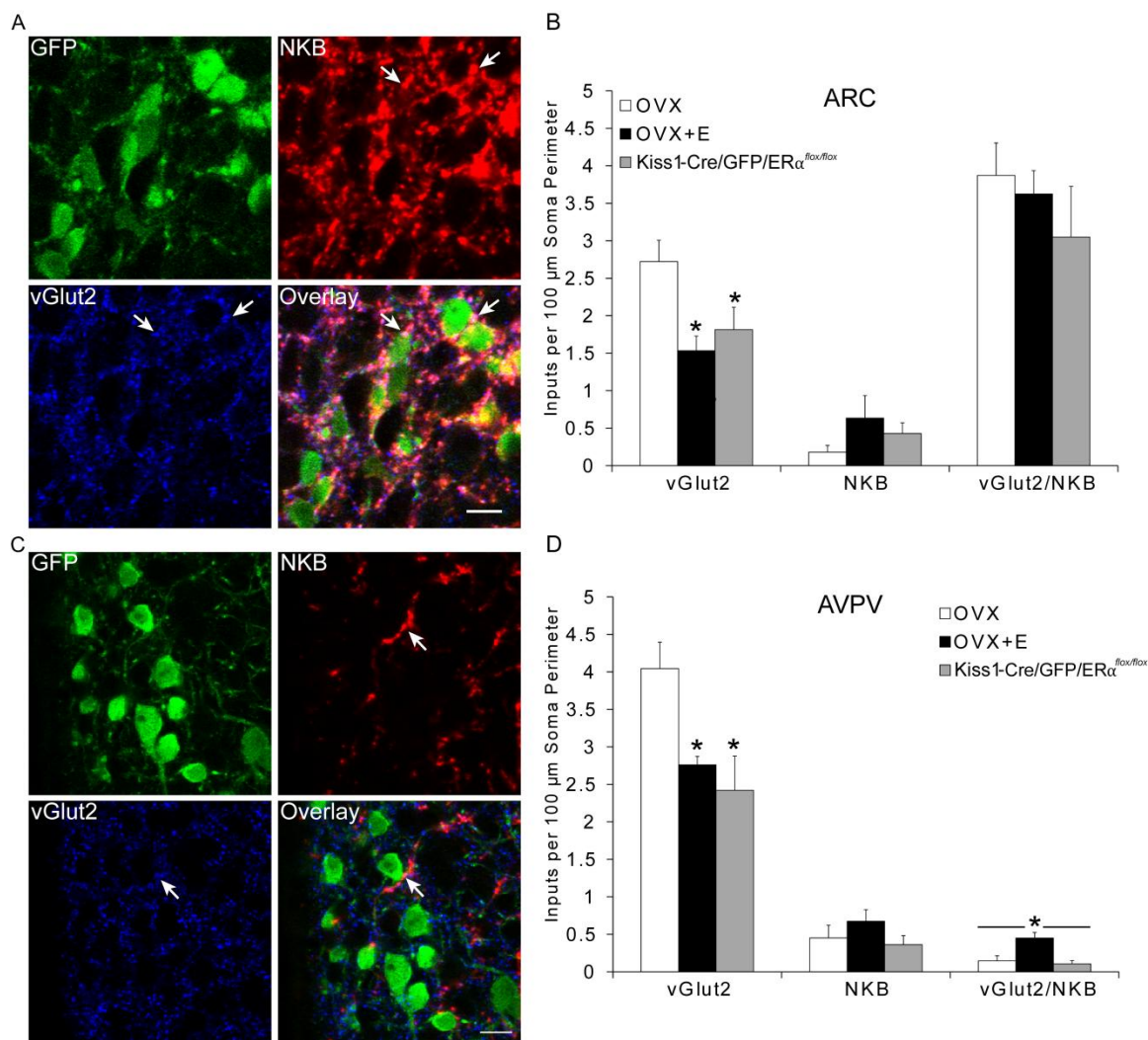


Figure 6.1. Glutamatergic and NKB Inputs to ARC and AVPV Kiss1 cells. A, Confocal image (1 μm thick optical section) showing ARC Kiss1 cells (*green*) in an OVX mouse with NKB (*red*) and vGlut2 (*blue*) inputs. Arrows indicate dual-labeled vGlut2/NKB fibers in apposition to Kiss1 (GFP-labeled) cells. B, Number of single and dual-labeled vGlut2 and NKB inputs onto ARC Kiss1 cells. C, Confocal image (1 μm thick optical section) of AVPV Kiss1 cells in an OVX+E mouse with vGlut2 and NKB input. White arrow indicates dual-labeled terminal. D, Number of single and dual labeled vGlut2 and NKB inputs onto AVPV Kiss1 cells. *Scale bar*, 10 μm . Data are presented as means \pm SEM. *, $P < 0.05$.

significant differences from OVX or OVX+E2 mice. In the AVPV, although vGlut2/NKB inputs to Kiss1 neurons were infrequent, there was a significant increase in the number of vGlut2/NKB inputs in OVX+E2 mice ($P < 0.01$; Figure 6.1B). Interestingly, deletion of ER α from Kiss1 cells reversed this effect of E2 on vGlut2/NKB inputs ($P < 0.01$; Figure 6.1B).

The total number of vGlut2 inputs, which included single-labeled vGlut2 and dual-labeled vGlut2/NKB, were decreased in OVX+E2 compared with OVX mice in both the ARC ($P = 0.01$) and AVPV ($P = 0.02$; data not shown). This decrease was due, in large part, to the significant decrease in single-labeled vGlut2 inputs in OVX+E2 mice (Figure 6.1). Deletion of ER α in Kiss1 cells did not reverse the effects of E2 on total vGlut2 inputs in the ARC or AVPV, where Kiss1-Cre/GFP/ER $\alpha^{flox/flox}$ mice were significantly different from the OVX (ARC, $P = 0.05$; AVPV, $P = 0.01$) but not the OVX+E group. Although results showed no effect of E2 on the total number of NKB inputs (single NKB and dual vGlut2/NKB) in the ARC or AVPV, in the AVPV, Kiss1 cells in ER $\alpha^{flox/flox}$ animals showed significantly fewer total NKB compared with OVX+E2 mice ($P = 0.05$). Because total NKB is the sum of both single NKB and dual vGlut2/NKB, this difference is likely attributed to the robust difference and low variability in the numbers of vGlut2/NKB inputs.

6.3.2. Experiment 2: E2 decreases Kiss inputs and increases Kiss/NKB (KNDy) inputs to AVPV Kiss1 cells

To identify KNDy inputs onto ARC Kiss1 cells (i.e. reciprocal connections) as well as KNDy inputs onto AVPV Kiss1 cells, dual-labeled kisspeptin/NKB inputs were analyzed. In the ARC, single-labeled kisspeptin and single-labeled NKB inputs to KNDy cells were exceptionally rare, and virtually all kisspeptin inputs co-expressed NKB and vice versa; because of this only the dual-labeled kiss/NKB data is shown (Figure 6.2A,B). E2 replacement in OVX animals did not significantly change the number of KNDy inputs onto ARC KNDy cells. Despite a lack of an effect of E2, there were significantly fewer KNDy inputs to ARC Kiss1 cells in Kiss1-Cre/GFP/ER $\alpha^{flox/flox}$ mice compared to OVX ($P = 0.01$) or OVX+E2 ($P = 0.02$) mice.

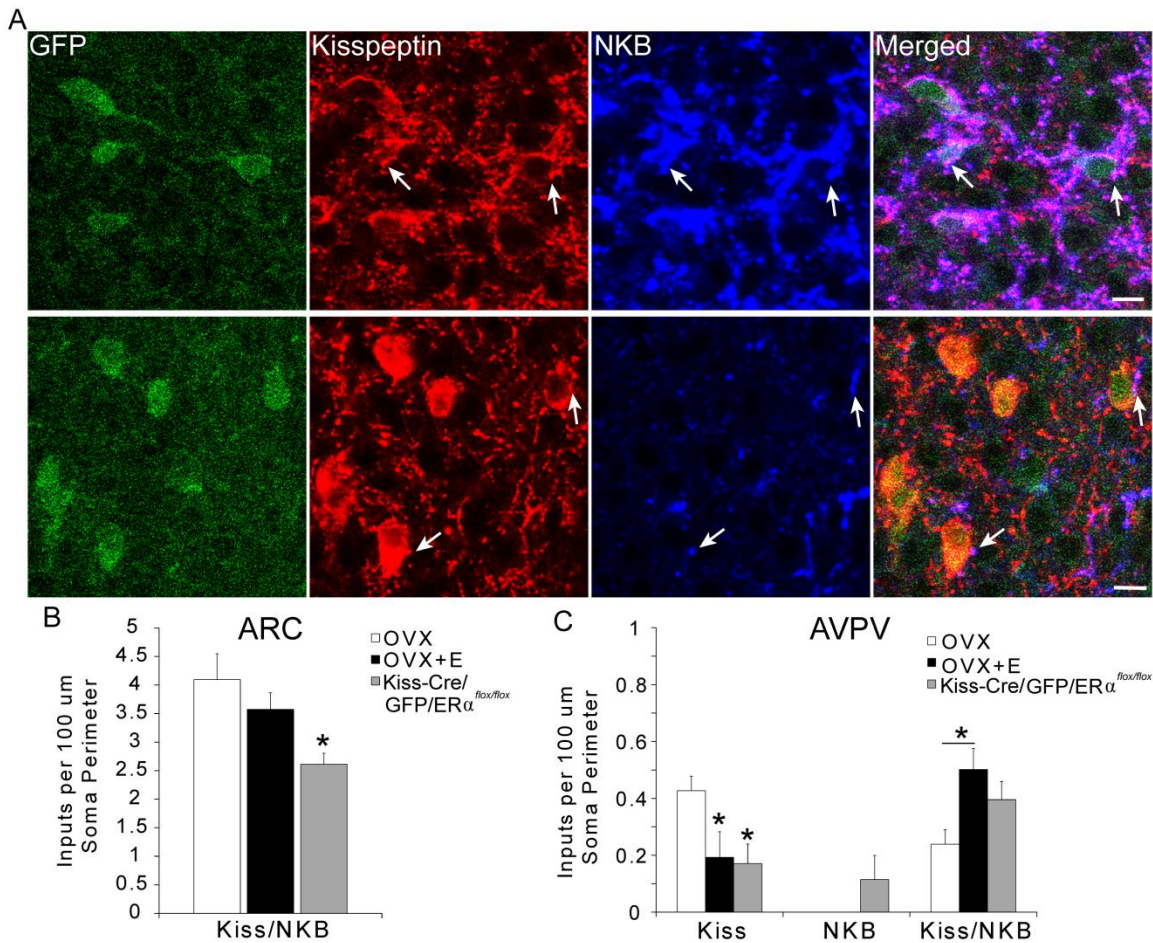


Figure 6.2. Kisspeptin and NKB Inputs to ARC and AVPV Kiss1 cells. A. Confocal images (1 μm optical thickness) of the ARC (A, *top panels*) and AVPV (A, *bottom panels*) in tissue from an OVX+E mouse showing Kiss1 cells (endogenous GFP; *green*), with kisspeptin (*red*) and NKB cells (*blue*). Arrows indicate dual-labeled KNDy (kisspeptin/NKB) inputs. *Scale bar*, 10. B. Number of KNDy inputs onto ARC Kiss1 cells are not regulated by E2. C. Numbers of single and dual-labeled kisspeptin and NKB inputs to AVPV Kiss1 cells. Data presented as means \pm SEM. *, significant ($P < 0.05$) difference from OVX.

Like ARC kisspeptin cells, AVPV kisspeptin cells received dual-labeled kisspeptin/NKB (KNDy) inputs (Fig. 6.2A,C). In addition, AVPV Kiss1 cells received kisspeptin inputs that did not coexpress NKB (Figure 6.2C). These single-labeled kisspeptin inputs were significantly decreased in OVX+E2 mice ($P = 0.05$), and this effect was not reversed in Kiss1-Cre/GFP/ER $\alpha^{flox/flox}$ mice ($P = 0.04$; Figure 2C). KNDy inputs to AVPV Kiss1 cells were upregulated in OVX+E2 mice compared with OVX ($P = 0.03$). However, it was not clear whether this effect was specifically due to ER α signaling within kisspeptin neurons, as the ER $\alpha^{flox/flox}$ group was not significantly different from either OVX or OVX+E animals.

6.3.3. Experiment 3a: E2-induced decrease in synaptic inputs to ARC Kiss1 cells is not dependent on ER α signaling in Kiss1 cells.

To address whether E2 mediates changes in inhibitory inputs in the present study, GABAergic terminals were immunolabeled with GAD65&67 (GAD), and contacts with Kiss1 cells in the ARC and AVPV were counted. Synapsin (syn), a synaptic vesicle marker, was used alongside GAD, to ensure GAD was labeling bona fide synaptic terminals, but was also analyzed as a single-label to identify all synaptic inputs onto Kiss1 cells, regardless of phenotype.

E2 treatment did not produce any changes in GAD/syn inputs to Kiss1 cells in either the ARC or AVPV (Figure 6.3). GAD/syn inputs to AVPV Kiss1 cells were decreased in Kiss1-Cre/GFP/ER $\alpha^{flox/flox}$ mice was present in the AVPV ($P = 0.02$; Figure 6.3B) despite the lack of effect of E2.

Single-syn inputs showed a significant decrease in the ARC with E2 treatment ($P = 0.04$; Figure 6.3A), and this effect was not reversed in the Kiss1-Cre/GFP/ER $\alpha^{flox/flox}$ mice, which like OVX+E mice were significantly different from OVX mice ($P < 0.01$; Figure 6.3A). In the AVPV, there were no significant differences in number of single-syn inputs to Kiss1 cells. E2-induced changes in total syn inputs (including dual-labeled GAD/syn and single-syn) were in the same direction as the single syn inputs, with a decrease in the ARC with E2 replacement ($P = 0.06$), and in Kiss1-Cre/GFP/ER $\alpha^{flox/flox}$ mice ($P < 0.01$; Figure 6.3A). There were no E2 induced changes in total syn inputs in

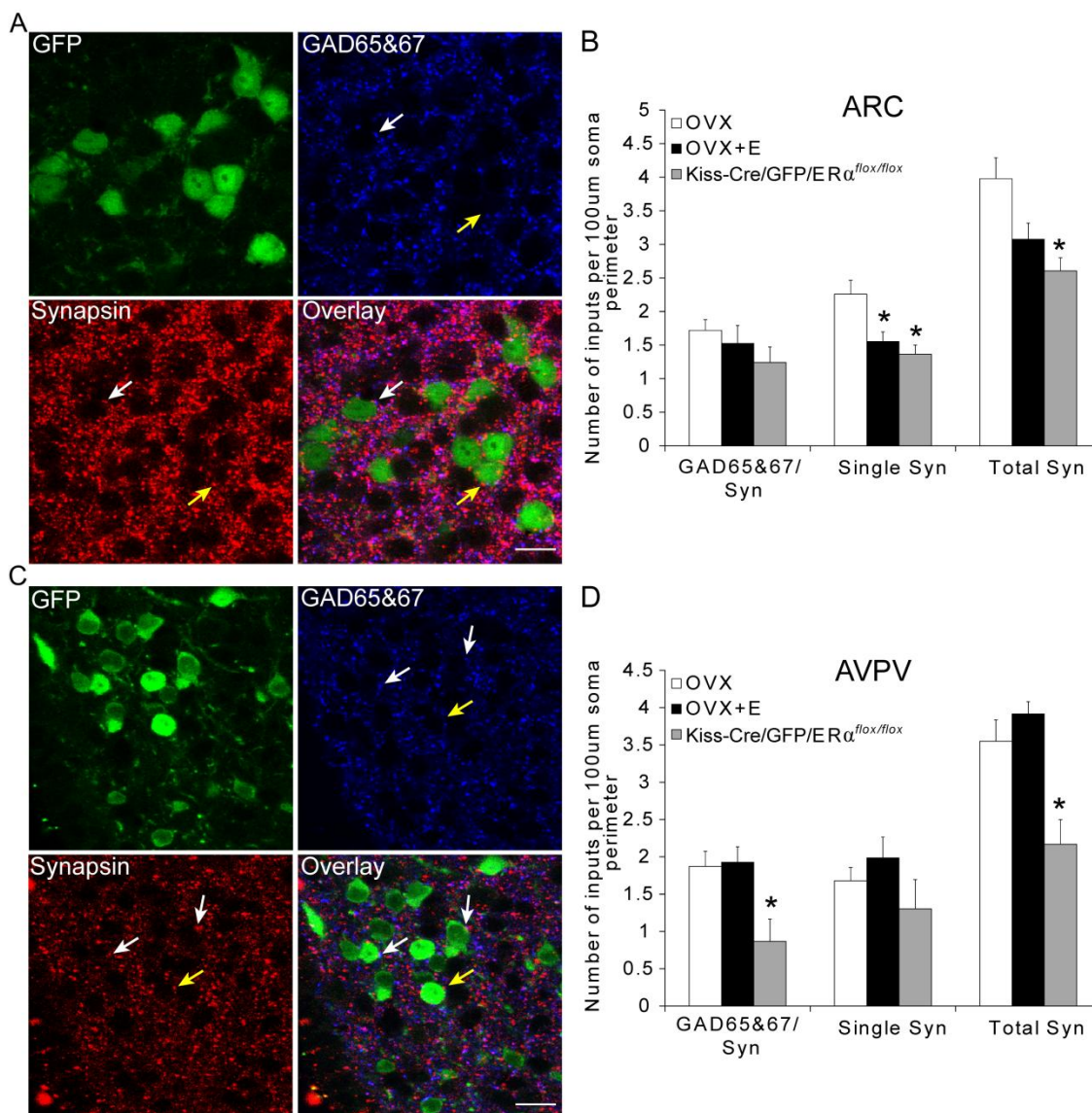


Figure 6.3. GABAergic and Synaptophysin-labeled inputs to ARC and AVPV Kiss1 cells. A,C. Confocal images (1 μm optical thickness) showing ARC (A) and AVPV (C) Kiss1 cells (green) in OVX mice with GABAergic (GAD65&67; red) and non-GABAergic (Single syn; blue) presynaptic inputs. White arrows indicate dual GAD65&67/syn inputs and yellow arrows indicate single-syn terminals on Kiss1 (GFP) cells. Scale bars, 10 μm . B,D. Numbers of single and dual-inputs to ARC (B) and AVPV (D) Kiss1 cells. Data presented as means \pm SEM. *, $P < 0.05$.

the AVPV, although a significantly decreased number of total syn inputs was seen in Kiss1-Cre/GFP/ER $\alpha^{flox/flox}$ mice (Figure 6.3B).

6.3.4. Experiment 3b: GABAergic inputs to Kiss1 cells do not differ between prepubertal and adult diestrus female mice.

To investigate whether there are changes in presynaptic inhibitory inputs to Kiss1 cells from the prepubertal stage to adulthood, GAD and syn immunolabeled terminals were analyzed in apposition to ARC and AVPV kisspeptin cells as in Exp. 3a (Section 6.3.3). There were no significant differences in numbers of GAD/syn, single syn or total syn inputs to Kiss1 cells in the ARC or AVPV between prepubertal and diestrus animals (Figure 6.4). There was a trend towards a decrease in single syn inputs to ARC Kiss1 cells in prepubertal animals ($P = 0.08$), although this failed to reach significance.

6.4. Discussion

To investigate whether changing circulating levels of E2 modulate presynaptic inputs to Kiss1 cells, we used a Kiss1-Cre/GFP mouse model that has been previously described (29). Our data demonstrate that changing levels of E2 alter presynaptic inputs to both ARC (KNDy cells) and AVPV Kiss1 cells. In the ARC, the presence of E2 decreased the number of glutamatergic and total presynaptic inputs to Kiss1 cells, while on AVPV Kiss1 cells, mediated both a decrease in glutamatergic inputs and an increase in KNDy (dual-labeled kiss/NKB) and dual-labeled glutamate/NKB inputs. To determine whether E2-induced changes were dependent on ER α signaling specifically within kisspeptin neurons, mice with selective deletion of ER α in Kiss1 cells were used (22, 30). Our results show that E2-mediated changes in glutamatergic and presynaptic inputs are not dependent on ER α in Kiss1 neurons, but that changes in KNDy and glutamate/NKB inputs onto AVPV Kiss1 cells are dependent upon ER α in Kiss1 cells.

6.4.1. E2 negatively regulates glutamatergic inputs to ARC and AVPV Kiss1 cells and does not depend on ER α signaling in Kiss1 cells

In mice, E2 elicits negative and positive feedback effects on Kiss1 mRNA in the ARC and AVPV, respectively, and these effects are not only dependent on ER α (19, 31),

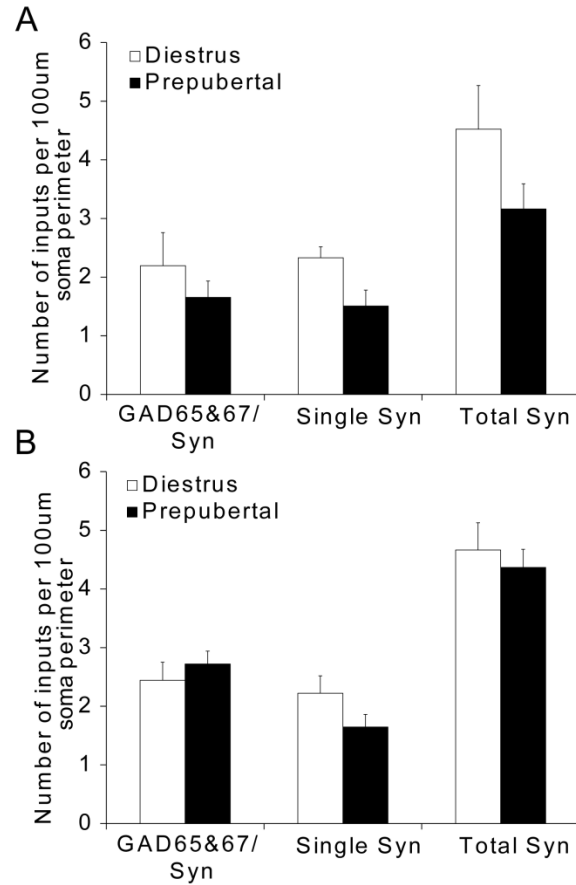


Figure 6.4. GABAergic and Presynaptic Inputs to ARC and AVPV Kiss1 cells in diestrus and prepubertal female mice. A, B. Numbers of single and dual-labeled GAD and syn inputs to ARC (A) and AVPV (B) Kiss1 cells. No significant differences between groups were detected. Data presented as means \pm SEM.

but are mediated directly on Kiss1 cells (22). Recently, using the same Kiss1-Cre/GFP mouse model that was used in the present study, Cravo et al. (2011) demonstrated that the majority of ARC Kiss1 (KNDy) cells are glutamatergic (29). Based on the documented presence of reciprocal circuits in the KNDy network of rodents and other species (25, 26), a source of glutamatergic input to ARC KNDy cells must arise from ARC KNDy cells themselves. Recent sheep studies have identified glutamatergic inputs to ARC KNDy cells that arise from both KNDy (vGlut2 terminals coexpressing kisspeptin) and non-KNDy glutamatergic sources (non-kisspeptin vGlut2), and change in density across the estrous cycle (12) (Chapter 5). In the current study, we sought to investigate E2-mediated changes in glutamatergic inputs and whether any changes depend on ER α signaling in Kiss1 cells. Our results show that E2 decreases glutamatergic inputs to ARC and AVPV Kiss1 cells. Because the effects of E2 were not altered in the mice with deletion of ER α in Kiss1, these effects appear to be mediated via steroid-sensitive neurons upstream in which ER α signaling is intact. Furthermore, the same population of upstream neurons may project to Kiss1 cells in both regions, as E2 regulates glutamatergic inputs in the same direction in both areas.

In the same experiment, NKB terminals were analyzed alongside vGlut2. Numerous dual-labeled terminals in the ARC and in contact with ARC Kiss1 cells were identified, with very few single-labeled NKB inputs apparent in the ARC. Our data demonstrate that these glutamatergic NKB terminals are not altered with E2. It is tempting to speculate that the dual vGlut2/NKB inputs represent KNDy terminals because of the high coexpression of NKB (24) and vGlut2 (29) within Kiss1 cells of the ARC of mice. However, a small population of NKB cells exists in the ARC that does not coexpress Kiss1 (24), and it is not known whether or not those NKB cells are glutamatergic. Therefore, although some glutamate/NKB terminals likely arise from KNDy neurons, we cannot say for certain whether they do until this question is addressed by *in situ* experiments or additional ICC experiments to detect a second KNDy peptide.

In the AVPV, very few NKB and vGlut2/NKB terminals were present, and although only rarely identified, still provided input to AVPV Kiss1 cells. Interestingly, E2 increased the number of glutamate/NKB inputs onto Kiss1 cells, and ER α deletion within Kiss1 cells reversed this effect. This supports the idea that these terminals arise

from Kiss1 cells, since ER α within kisspeptin is regulating the E2 effect. However, it also suggests that inputs from glutamatergic NKB neurons projecting to the AVPV Kiss1 cells are positively regulated by E2. If these are part of the KNDy population, then they may only represent neurons that are regulated differently by E2 than the majority of the population, which is primarily under E2 negative feedback regulation (19). In the sheep, KNDy neurons are activated during conditions of both positive and negative feedback (32), and it may be that subsets of mouse KNDy neurons are similarly responsive to either positive or negative feedback effects of E2.

6.4.2. Changes in KNDy inputs to AVPV Kiss1 cells require ER α

To identify E2 induced changes in reciprocal KNDy circuitry in the ARC, and in KNDy inputs to AVPV Kiss1 cells, we immunostained for two KNDy peptides, kisspeptin and NKB. Because their coexpression is unique to the ARC (23, 24), presynaptic terminals dual-labeled with both kisspeptin and NKB would identify a KNDy source. The current findings demonstrate that virtually all kisspeptin and NKB inputs to ARC Kiss1 cells were in terminals that contained both peptides, with very few single-labeled inputs present, and these were not altered with E2. Therefore, despite a small population of ARC NKB cells in the mouse that are not part of the KNDy population (24), these single-labeled NKB neurons do not appear to provide substantial input to KNDy cell bodies. These data are also consistent with the idea that the glutamatergic NKB terminals (described in previous experiment above), and these KNDy terminals represent the same inputs. First, similar to in this experiment, they are not altered with E2. Second, virtually all NKB terminals expressed vGlut2 or kisspeptin.

By the use of the unique combination of peptides present in KNDy cells, we have shown for the first time in rodents that KNDy neurons project to kisspeptin cells in the AVPV, and that these inputs are positively regulated by E2. KNDy inputs to preoptic kisspeptin cells have been identified in the sheep (12), and there is evidence in rodents and other species that KNDy (kisspeptin/NKB) fibers are present in the AVPV/POA (13, 15, 33-35), although they have not yet been described in contact with the kisspeptin cells in that area. Notably, the KNDy inputs observed in the current study represent a very small number of inputs to AVPV Kiss1 cells, because not all cells show KNDy inputs.

However, our data demonstrates that there is a significant increase in KNDy inputs to AVPV Kiss1 cells with E2. Unfortunately, it was not clear whether or not this effect was partially reversed by ER α deletion in Kiss1 cells because of high variability in data from that group. Nonetheless, the E2 effects on KNDy inputs to AVPV Kiss1 cells are particularly interesting. E2 exerts negative feedback effects on KNDy (kiss/NKB) cells in the mouse (24), however we have described KNDy inputs to AVPV Kiss1 cells that are upregulated with E2. Although it has not been investigated, it is possible that this discrepancy is due to a small sub-population of KNDy cells that provide rostral projections and are positively regulated by E2 in rodents.

Kiss1 cells in the AVPV received single-labeled kisspeptin inputs, in addition to KNDy inputs, and these are reduced in OVX+E2 mice. The presence of single-labeled kisspeptin inputs is unique to the AVPV, as ARC KNDy cells receive few (if any) kisspeptin inputs that do not coexpress NKB (present data). The presence of kisspeptin inputs to AVPV Kiss1 cells that are devoid of NKB- immunoreactivity suggests that these are not coming from the ARC. It is possible that these terminals are still KNDy terminals but the levels of NKB peptide are below detectability using ICC methods; however, kisspeptin and NKB are both negatively regulated by E2 in the ARC (19, 24, 36), and so the presence of E2 would likely decrease the levels of both peptides in the terminals to a similar extent. Therefore, it seems more likely that these single-labeled kisspeptin inputs are arising from regions other than the ARC. The largest kisspeptin population outside of the ARC is in the rostral periventricular area of the 3rd ventricle (RP3V; includes the AVPV and periventricular preoptic nucleus (PVpo)) in rodents (14, 37), and the preoptic area (POA) in sheep, monkeys and humans (38-40), while smaller populations have been reported in the dorsomedial hypothalamus (DMH), medial amygdala (MeA) and bed nucleus of the stria terminalis (BNST) (18). If the single kisspeptin inputs reported here originate from the RP3V, then the downregulation of these inputs with E2 is not consistent with the action of E2 to increase kisspeptin in these cells (19). Until another immunohistochemical marker of the AVPV kisspeptin population is identified, the source of these kisspeptin inputs remains elusive. Nevertheless, the reduction in single- kisspeptin inputs onto AVPV Kiss1 cells with E2 was not reversed by ER α deletion in Kiss1 cells, which strongly suggests that this effect

of E2 is relayed via an estrogen-sensitive interneuron, and not via direct E2 action on kisspeptin cells.

6.4.3. E2 decreases the total number of presynaptic inputs onto ARC Kiss1 cells

Recent electrophysiological findings using the same mouse model have shown E2 modulates a decrease and increase in inhibitory tone of Kiss1 cells in the ARC and AVPV, respectively (22). This data supports the notion that GABAergic neurons lie upstream of the Kiss1 neurons, and are modulated by E2. In addition, in the ARC of female monkeys and rats, ultrastructural studies have revealed an E2-induced decrease in the number of GABAergic/inhibitory terminals (41, 42) although the postsynaptic cell type was not identified in these studies. In the current study, we did not detect any E2-mediated differences in the number of GABA inputs in contact with Kiss1 cells in the ARC or AVPV. It may be that E2 alters GABAergic inputs to non-kisspeptin cells in the ARC without affecting inhibitory inputs to kisspeptin cells in the same region. In contrast to the ARC, our findings in the AVPV, are consistent with previous observations by Polston et al. (2004) showing that E2 replacement did not affect GAD65 inputs to AVPV cells in peripubertal rats (43). In a recent EM study, E2 increased GABAergic inputs onto cells expressing both ER α and ER β , but did not affect GABAergic inputs to ER α -expressing AVPV neurons (44). Because virtually all Kiss1 expressing cells in the mouse AVPV express ER α mRNA (19), and only 30% coexpress ER β mRNA (19), the kisspeptin neurons expressing both estrogen receptors only represent one third of the kisspeptin population in the AVPV (19), and, taken together, thereby suggests that GABAergic inputs to a majority of AVPV Kiss1 cells are not regulated by E2.

Using a marker of all synaptic terminals, we investigated changes in total presynaptic inputs to ARC and AVPV Kiss1 cells with E2, and whether ER α in Kiss1 cells is critical for any E2-mediated changes. In the ARC, E2 decreased total synaptic inputs to Kiss1 cells, and this change was not dependent on ER α signaling in Kiss1 cells, suggesting E2 action on upstream neurons to modulate changes in presynaptic inputs. In rodents, estrogen induces dynamic synaptic remodeling in synapses onto ARC neurons across the estrous cycle, with preovulatory levels of E2 decreasing the number of inputs (45). In rats, the subpopulation of ARC neurons that project to the median eminence,

respond to E2 with a decrease in axosomatic synapses, while synapses onto ARC neurons that do not project to the ME are unaffected by changes in E2 (46). These ME-projecting neurons may in part, be the ARC KNDy neurons, as a number of studies have shown that ARC KNDy neurons project to the ME (13, 15, 17, 34, 35, 47, 48). Our data shows that E2 decreases presynaptic inputs to ARC Kiss1 cells. By contrast, in the AVPV, E2 did not modulate the number of presynaptic inputs, including non-GABAergic synaptic inputs and total synaptic (including GABAergic and non-GABAergic) inputs. This data confirms earlier observations by Kurunczi et al. (2009), who reported a lack of influence of E2 on non-GABAergic synapses with E2 replacement in rats (44). Even though total presynaptic inputs do not appear to be mediated by E2, concomitant increases and decreases in different input types may participate in achieving this balance. Taken together, all of our present findings in the AVPV are supportive of a lack of E2-mediated changes in total presynaptic inputs: E2 decreases glutamatergic inputs while increasing glutamatergic NKB inputs and KNDy inputs to AVPV Kiss1 cells.

Data from primates have demonstrated that kisspeptin neurons relay GABA signals to GnRH neurons, serving as the substrate for GABAergic inhibition of GnRH prior to puberty (49). This data strongly suggests that GABAergic neurons are present upstream of kisspeptin neurons before pubertal onset. Electrophysiological data in rodents supports this finding, showing Kiss1 neurons in pubertal mice are under higher inhibitory presynaptic influence than adult Kiss1 neurons (22). Although we have shown that GABAergic terminals do provide input to ARC and AVPV Kiss1 neurons in prepubertal female mice, our data did not reveal any differences in the number of inputs from adult females. A caveat to this study was that tissue from prepubertal and adult mice was used, but not from any time points in between. In future experiments, tissue from time points across the pubertal transition (i.e. prepubertal, pubertal and post-pubertal) may reveal a more specific timeline for changes in inhibition of kisspeptin cells that might have been missed in the current study.

In summary, the present results provide evidence for E2 modulation of presynaptic inputs to ARC KNDy and AVPV Kiss1 neurons in the mouse. Direct effects of E2 on KNDy neurons through ER α , mediate KNDy projections to AVPV Kiss1

neurons, while ER α signaling in kisspeptin neurons is not required for the action of E2 on other presynaptic (including glutamatergic) neurons upstream of the KNDy and AVPV Kiss1 populations. Changes in these inputs to kisspeptin cells may play an important role in the relay of steroid feedback and other signals to the reproductive system and GnRH neurons.

6.5. References

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CHAPTER 7

NK3R internalization in KNDy and other neurons of the arcuate nucleus does not vary across the ovine estrous cycle

7.1. Introduction

The hypothalamic mechanisms by which ovarian steroids exert their feedback effects to modulate gonadotropin-releasing hormone (GnRH) secretion during the estrous cycle are not well understood. In recent years, kisspeptin/neurokinin B/dynorphin (KNDy) neurons of the arcuate nucleus (ARC) have received attention as a potential key mediator of steroid hormone feedback in the neuroendocrine control of reproduction (1, 2). Initially, observations showing that mutations in the kisspeptin receptor produced hypogonadotropic hypogonadism in humans (3, 4) and mice (4), cast kisspeptin into the forefront of reproductive neuroendocrine studies, and kisspeptin is now established as a key mediator of GnRH secretion and steroid feedback (5, 6). In the ARC but not other regions, kisspeptin is co-expressed with the tachykinin, neurokinin B (NKB), and the endogenous opioid peptide, dynorphin (Dyn) in a number of mammalian species (7-11). This single population (termed KNDy cells) is steroid-sensitive and virtually all express estrogen receptor- α (ER α ; (12-15)), as well as progesterone receptors (PR; (16)). An interesting feature of the KNDy population is the presence of axon terminals labeled with one or more KNDy peptide in frequent apposition to KNDy cell bodies (7, 16). These KNDy-KNDy connections, which may play a role in the coordination of the KNDy network, may involve NKB as an autoregulatory system, as NK3R, the high affinity receptor for NKB, is localized within KNDy neurons (7, 10, 17). Although KNDy neurons contact GnRH cells (9, 18-23), the lack of NK3R in GnRH neurons (17, 24), suggests that the effects of NKB on GnRH and LH secretion (see below) are not due to direct actions upon GnRH neurons.

While the stimulatory effects of kisspeptin have been well documented (6, 25-28), the effects of NKB on GnRH and luteinizing hormone (LH) release are not as clear. Senktide, an agonist for NK3R, has been shown to suppress LH secretion in rats and mice, although these effects are present under specific hormonal conditions in which E2 levels are low (10, 29, 30). In ovary intact proestrus rats, or rats with E2 replacement that mimics proestrus levels, senktide increases LH (31, 32). Stimulatory effects of senktide are also seen in male mice (33), prepubertal male monkeys (34), goats (11), and sheep (35). In sheep, senktide increases LH in follicular phase, but not luteal phase (35), suggesting that an increase in NKB release or sensitivity during follicular phase may play

a role in synchronizing the KNDy neurons leading up to the preovulatory surge. In addition, KNDy neurons in sheep show synaptic plasticity across the estrous cycle, and are activated during the preovulatory surge (19, 36). Based on these findings, we sought to investigate whether endogenous NKB release onto KNDy and other ARC neurons may change across the estrous cycle. Upon ligand binding, NK3R undergoes endocytosis and is internalized, and confocal detection of internalized G-protein coupled receptors, including NK3R (37-39), has been used as an indicator of endogenous release of peptides in behaving animals (38-40). Therefore cellular internalization of immunoreactive NK3R was used in this study as a marker for endogenous NKB release during the estrous cycle.

7.2. Materials and Methods

7.2.1. Animals

Adult ewes were maintained in an open barn with free access to food and water. All experiments were conducted during the breeding season (September to February) with cycling ewes, and estrous behavior was monitored using vasectomized rams. Ewes that had been observed in estrous 5 to 12 days earlier were treated with two injections of prostaglandin F₂ α (PGF₂ α ; (41)) 10 hours apart to induce regression of the corpus luteum. A cohort of ewes was euthanized 24 h after the first injection of PGF₂ α and were classified into a follicular phase group (N=6). Another group of ewes was observed for signs of estrous every 3 h by exposing them to a vasectomized ram beginning 32 h after the first injection of PGF₂ α . Ewes that were euthanized 1 h after the onset of behavioral estrous were classified into the surge onset group (N=5), and those euthanized 7 days after onset of detected estrous were classified into the luteal phase group (N=5). Blood samples were collected from all ewes immediately before euthanasia and serum was harvested by centrifugation. Mean concentrations of luteinizing hormone (LH) at the time of perfusion were: 1.7, 4.96, and 0.74 ng/mL, for follicular phase, surge onset, and luteal phase groups, respectively. Mean concentrations of progesterone (P₄) at perfusion were: 0.91, 0.42, 4.96 ng/mL for follicular, surge, and luteal phase groups, respectively. Animal procedures were conducted at Texas A&M University- College Station, TX, USA, in collaboration with Dr. Marcel Amstalden, and were approved by the Institutional Agricultural Animal Care and Use Committee of the Texas A&M University System.

7.2.2. Tissue Collection

Ewes were euthanized with an i.v. overdose of sodium pentobarbital (Sigma, St. Louis, MO) following two i.v. injections of 25000 U of heparin (Abraxiz Pharmaceutical Products, Schaumburg, IL, USA). Heads were removed and perfused through both carotid arteries with 6 liters of fixative (4% paraformaldehyde containing 10 U/mL heparin and 0.1% sodium nitrite). After perfusion, brains were removed and post-fixed for 24 h in 4% paraformaldehyde. Brains were infiltrated with 30% sucrose and stored at 4° C. A block of tissue containing the preoptic area (POA) and hypothalamus was dissected out and cut into 4 parallel series of coronal sections (50 µm thick) using a freezing microtome. Tissue sections were stored at -20° C in cryopreservative until further processing.

7.2.3. Assays

Concentrations of LH were determined by RIA using rabbit anti-ovine LH (AFP-192279; NHPP) and ¹²⁵I-labeled ovine LH (AFP-8614) as tracer. Concentrations of progesterone were determined using a commercially-available RIA kit (Siemens Healthcare Diagnostics Inc., Newark, DE, USA).

7.2.4. Double-label Immunofluorescence for NK3R and Kisspeptin

Tissue sections in the arcuate nucleus were processed for immunofluorescent detection of NK3R, kisspeptin and NISSL. Preliminary studies in our lab have shown that only a very small percentage (less than 5%) of POA kisspeptin cells express NK3R (Lehman, unpublished observations), and therefore in this study, NK3R internalization in POA kisspeptin cells was not examined. All immunocytochemistry was conducted on free floating sections at room temperature (RT) from follicular phase (N=6), surge onset (N=5), and luteal phase (N=5) animals. Between all incubations, sections were washed extensively with phosphate-buffered saline (PBS; pH=7.35). First, tissue sections were incubated for 10 min in 10% hydrogen peroxide (H₂O₂; EMD Chemicals, Inc., Gibbstown, NJ) diluted in PBS, to quench endogenous peroxidase activity, followed by incubation in a blocking solution containing 20% normal goat serum (NGS; Jackson Immunoresearch Laboratories, Inc., West Grove, PA) in PBS containing 0.4% Triton X-

100 (TX-100; Fisher Scientific, Pittsburgh, PA). Next, sections were incubated in polyclonal rabbit anti- NK3R (1:10,000 in PBS containing 4% NGS and 0.4% TX-100; NB300-102, Novus Biologicals, Littleton, CO, USA) for 17 h, followed by incubations in biotinylated goat anti- rabbit IgG (1:500 in PBS containing 4% NGS and 0.4% TX-100; Jackson Immunoresearch Laboratories, Inc.) for 1 h, ABC-elite (1:500 in PBS; Vector Laboratories, Burlingame, CA) for 1 h, TSA (1:250 in PBS containing 3% H₂O₂ per mL; Perkin Elmer LAS, Inc., Boston, MA) for 10 min and Alexa 488- Streptavidin (1:100 in PBS; S32354, Invitrogen, Carlsbad, CA) for 30 min. Subsequently, tissue sections were incubated in polyclonal rabbit anti- kisspeptin10 serum (1:10,000 in PBS containing 4% NGS and 0.4% TX-100; No. 564, A. Caraty, Université Tours, Nouzilly, France) for 17 h followed by incubation in Alexa 555 goat anti-rabbit (1:100 in PBS; A21428, Invitrogen) for 30 min. Finally, sections were incubated in Fluor-NISSL (1:100 in PBS; N21483, Invitrogen) to aid in the demarcation of cytoplasmic and nuclear boundaries. Sections were mounted onto Superfrost slides, dried and coverslipped with gelvatol.

Both the NK3R and kisspeptin antibodies have been previously validated for use in sheep (17) and (18), using preabsorption controls in which the antibody is preabsorbed with the corresponding peptide, and yield a complete lack of immunostaining. Additional controls included the omission of each primary antibody from the immunostaining protocol, which eliminated staining for the corresponding antigen.

7.2.5. Confocal Imaging and Data Analysis

All confocal analyses were conducted with a Leica TCS SP2 laser scanning confocal microscope (Leica Microsystems, Buffalo Grove, IL, USA). Alexa 488 was visualized with a 458/476/488/514 nm emission filter and Argon laser, while Alexa 555 and Cy5 were imaged with HeNe lasers and a 543 and 633 nm emission filters, respectively. Images were taken at 1 μ m intervals on the z plane, and analyzed using the LAS AF Lite program (Leica Microsystems). A total of 687 kisspeptin cells (42.9 ± 4.4 per animal) were analyzed for the presence of NK3R-immunoreactivity (ir), and the mean percentages of kisspeptin cells with NK3R in each animal were calculated. For analysis of NK3R internalization, 20 cells (10 kisspeptin/NK3R and 10 NK3R) were randomly selected in two middle ARC sections but were required to meet the following criteria:

the entire cell had to be present in the z-stack, and a Nissl-stained nucleolus had to be present in three consecutive z-sections. Three consecutive optical sections (1 μm thick) in the middle of each neuron (identified by the presence of a Nissl-stained nucleolus) were used for analysis, and internalized particles were defined as immunolabeled cytosolic particles clearly dissociated from the membrane. Numbers of internalized particles clearly not associated with the cell membrane were counted and presented as total in the 3 optical sections. Neurons with three or more cytosolic particles were considered to be *internalized* (17, 40). Neurons with less than three internalized particles were considered to not be internalized cells, and NK3R in those cells was considered to be primarily membrane bound. The percentage of NK3R-ir kisspeptin neurons, mean number of particles per NK3R cell, percentage of internalized NK3R cells, and mean number of particles per internalized NK3R cell (3 optical sections) were averaged for each group, and compared using one-way ANOVAs with Fishers LSD post-hoc comparisons. Statistical significance was considered as $P < 0.05$.

7.3. Results

In agreement with a previous study in sheep, NK3R-ir in the ARC was predominant localized to cell surface, outlining cell bodies (17) (Figure 7.1 and Figure 7.2). In addition, numerous NK3R-ir fibers were visualized in the ARC, where they made frequent appositions onto NK3R, kisspeptin, and kisspeptin/NK3R cell bodies. Double-labeled NK3R/kisspeptin varicosities were identified, in addition to NK3R varicosities that did not coexpress kisspeptin (Figure 7.1).

Of the 687 kisspeptin cells analyzed in the ARC, 35-36% expressed NK3R in luteal, follicular and surge animals (Table 7.1). This percent colocalization was not significantly different across the estrous cycle. We also observed no significant difference between groups for: number of immunoreactive particles per NK3R cell, percentage of NK3R cells that were internalized (showed ≥ 3 internalized particles), or the mean number of immunoreactive particles in internalized neurons for both kisspeptin/NK3R (Table 7.2) and non-kisspeptin/NK3R (Table 7.3) cells in the ARC.

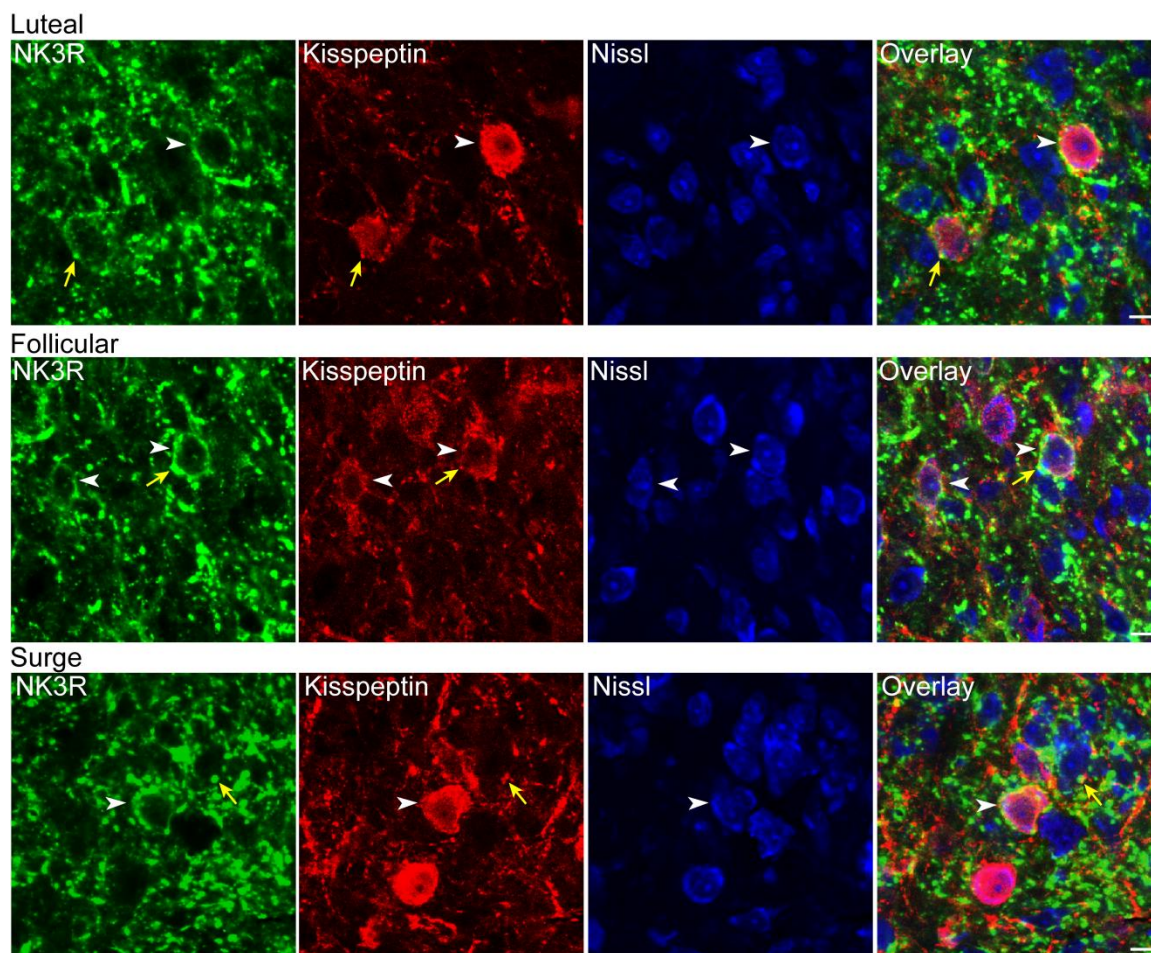


Figure 7.1. Confocal images (1 μm optical thick sections) of NK3R-ir (*green*), kisspeptin (*red*), and Nissl-stained (*blue*) neurons in the middle ARC of luteal (*top panels*), follicular phase (*middle panels*) and surge (*bottom panels*) ewes. White arrowheads indicate dual kisspeptin/NK3R cells, and yellow arrows indicate dual-labeled kisspeptin/NK3R appositions onto both kisspeptin and non-kisspeptin NK3R cells. *Scale bar*, 10 μm .

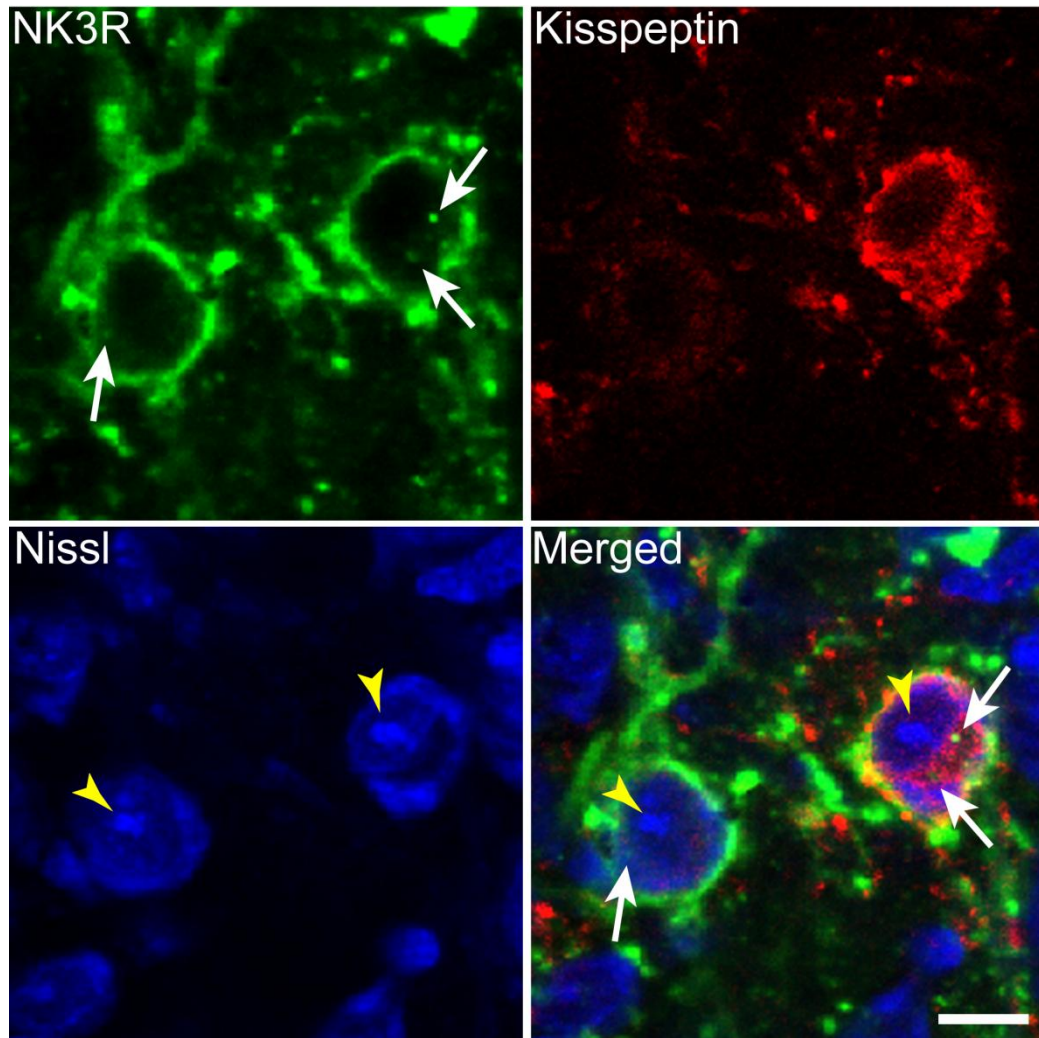


Figure 7.2. Confocal image (1 μm optical thick sections) showing internalization of NK3R-ir (*green*) in kisspeptin (*red*), and non-kisspeptin Nissl (*blue*) stained cells in the ARC of a luteal phase ewe. Yellow arrowheads indicate Nissl-stained nucleoli, and white arrows indicate NK3R particles dissociated from the cell membrane. *Scale bar*, 10 μm .

	# Kisspeptin cells per animal	Kisspeptin cells co-expressing NK3R per animal (%)
Luteal phase	34.6 ± 4.76	35.02 ± 4.46
Follicular phase	47.67 ± 8.65	34.81 ± 2.1
Surge onset	45.6 ± 8.18	36.13 ± 2.26

Table 7.1. Co-expression of kisspeptin and NK3R in the ARC. No significant differences were observed in the percentage of kisspeptin cells expressing NK3R. Data are presented as mean ± SEM.

	# NK3R-ir particles per kisspeptin cell	Internalized kiss/NK3R cells (%)	# NK3R-ir particles per internalized kiss/NK3R cell
Luteal phase	2.40 ± 0.56	43.56 ± 14.50	4.01 ± 0.43
Follicular phase	2.31 ± 0.32	40 ± 6.83	3.86 ± 0.25
Surge onset	2.48 ± 0.40	52 ± 10.68	3.74 ± 0.23

Table 7.2. NK3R internalization in ARC kisspeptin cells across the estrous cycle. No significant differences were observed between groups for numbers of NK3R particles per kisspeptin/NK3R cell, the percentage of internalized kisspeptin/NK3R cells, or numbers of NK3R particles per internalized kisspeptin/NK3R cell. Data are presented as mean ± SEM.

	# NK3R-ir particles per cell	Internalized NK3R cells (%)	# NK3R-ir particles per internalized NK3R cell
Luteal phase	1.12 ± 0.29	10 ± 6.32	3.75 ± 0.16
Follicular phase	1.46 ± 0.21	20 ± 6.83	3.4 ± 0.22
Surge onset	1.24 ± 0.09	10 ± 4.47	3.0 ± 0

Table 7.3. NK3R internalization in ARC non-kisspeptin cells across the estrous cycle. No significant differences were observed between groups for numbers of NK3R particles per NK3R cell, the percentage of internalized NK3R cells, or numbers of NK3R particles per internalized NK3R cell. Data are presented as mean ± SEM.

7.4. Discussion

To determine whether endogenous release of NKB onto ARC kisspeptin and other ARC neurons, changes across the estrous cycle, NK3R internalization was characterized in the ARC of ewes during the luteal, early follicular and late follicular (surge) phases. Results indicated that NK3R internalization, at least as seen in kisspeptin and other cells of the ARC, does not change across the estrous cycle.

NKB is a tachykinin with the highest binding affinity to the neurokinin receptor, NK3R (42). Binding of tachykinins or tachykinin agonists to their receptors induces internalization, and this internalization can be used as a marker of endogenous neurokinin release (37-39, 43, 44). A previous study in sheep investigating NK3R expression in ARC neurons identified a pattern of labeling in luteal phase animals that showed NK3R primarily associated with the cell membrane (17). Thus, based on the evidence for ligand binding endocytosis of NK3R (44), this would suggest that NKB is not being abundantly released (17), at least during the luteal phase. Other recent work in the sheep has demonstrated that senktide has a stimulatory effect on LH release in follicular, but not luteal phase (35). In addition, KNDy (NKB) cells of the ARC, along with rostral kisspeptin neurons in the preoptic area, are activated during the LH surge (36). These observations have led the hypothesis that increased NKB, released during the follicular phase, plays a role in the generation of the preovulatory GnRH/LH surge (35). In line with this hypothesis, preliminary findings showed an increased internalization of NK3R in ARC neurons in follicular phase ewes compared to luteal phase animals (45). In contrast to our expectations, we found no change in NK3R internalization between the luteal, follicular phase, and the surge in either KNDy neurons of the ARC, or their unidentified neighbors in the ARC. Besides the increased sample size, there are several important differences between the current study and the preliminary results of Amstalden et al. (45). Importantly, in the current study, both kisspeptin and non-kisspeptin NK3R cells were identified, while in the preliminary findings by Amstalden et al., phenotype of the NK3R neurons was not ascertained. Because NK3R-expressing KNDy cells only make up a third of all NK3R cells in the ARC (17), it may be that the cells sampled in the preliminary study represent a separate subpopulation. It is possible that the assay used, internalization of NK3R, is not sensitive enough to detect the magnitude of endogenous

release of NKB occurring at times examined. Alternatively, neurons in other brain regions, such as the RCh and POA (see below), may be more important functionally for the actions of NKB in the generation of the GnRH/LH surge. Furthermore, we do not know the precise time course of KNDy cell activation or NKB release during the estrous cycle, and it may be the critical times when NKB acts to stimulate the LH surge were not sampled in this study. In this regard, it should be noted that surge onset occurs 20 to 25 hours after a rise in estradiol in the sheep, so the window for actions leading to the surge is much longer than that in rodent species.

Although KNDy neurons in sheep and rodents express NK3R (7, 10, 17), GnRH neurons in sheep and mice do not (17, 24), with the exception of a small subset of GnRH cells in rats (46, 47). Therefore, the actions of NKB on LH secretion likely occur through other neurons (1, 2, 7, 48, 49), instead of at the level of the GnRH neurons. One possible candidate for relaying the effects of NKB on LH release are NK3R neurons of the retrochiasmatic area (RCh). Senktide-containing microimplants in the RCh stimulate a surge-like release of LH secretion during the follicular, but not luteal, phase (35). The RCh receives projections from the ventromedial hypothalamus (VMH) (50), which along with the ARC, is a potential site of positive feedback in the ewe (51). In addition to the RCh, the POA is another site of NK3R-containing cells where senktide microinjections are able to produce a marked and sustained elevation in LH (52). Preliminary findings indicate that senktide microimplants into the ARC are not as effective as those in either the RCh or POA in stimulating LH (52). Therefore, while the ARC and NK3R cells in that region may participate in the neural circuitry mediating the GnRH/LH surge, they may not be as important as the RCh and/or POA as targets for the actions of NKB released during the follicular phase. We observed many NK3R-positive fibers and varicosities in the ARC and these were frequently juxtaposed to KNDy cell bodies. A number of these NK3R fibers and varicosities expressed kisspeptin, suggesting that they likely arise from KNDy cells, since very few POA kisspeptin neurons in sheep express NK3R (Ahn & Lehman, unpublished observations). Interestingly, presynaptic NK3R terminals (co-expressed with synaptophysin) are present in close apposition to GnRH cell bodies in the POA and MBH of sheep (Ahn & Lehman, unpublished observations). Although the source of the presynaptic NK3R terminals onto GnRH neurons remains to

be identified, it is interesting to speculate that they play an autoregulatory role in controlling the secretion of KNDy peptides and other transmitters from presynaptic terminals. For example, NKB release from KNDy terminals in contact with GnRH might act via presynaptic NK3R to regulate the release of kisspeptin, dynorphin, or glutamate (19, 53, 54). Evidence in rodents and monkeys supports the contention that NKB actions are upstream of kisspeptin and Kiss1R (31-33, 55), and the hypothesis that NKB action on presynaptic NK3R terminals presents another alternative (in addition to actions on KNDy cell bodies) for which NKB/NK3R are poised to act upstream of kisspeptin to mediate its release. Future studies are needed to determine the precise site(s) of origin of NK3R-positive fibers and terminals, and more specifically, to investigate their possible functional role in controlling GnRH cells.

In summary, our findings suggest that the ARC may not be a major target for the actions of NKB during the estrous cycle, and instead lay the groundwork for future studies investigating the role of NKB/NK3R signaling in the RCh and POA. Senktide microinjections into the RCh and POA produce a surge-like secretion of LH (52) and it may be that NKB released from axons of KNDy neurons during follicular phase causes NK3R internalization in those regions. Regardless, the role of NKB in the preovulatory LH surge, the key event responsible for triggering ovulation, has to date received relatively little attention from reproductive neuroendocrinologists. Given the clear, detrimental effects of NKB and NK3R mutations on human reproduction, this is an area worth concerted study in the near future.

7.5. References

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CHAPTER 8:**Unanswered Questions and Future Directions**

8.1. Conclusions: Kisspeptin and KNDy Neurons in Reproduction

The emergence of kisspeptin and KNDy cells as key mediators of GnRH and LH secretion has catapulted reproductive neuroendocrine studies and revealed intricacies in this system beyond what was known prior. In particular, over the five years of my graduate work, there have been many significant advances in knowledge in this field. For example, in 2008, kisspeptin antagonists were first developed and have since been used to further characterize the physiological role of kisspeptin in control of gonadotropin secretion, as they are able to suppress GnRH and LH secretion in diverse animal species, using *in vivo* and *in vitro* approaches (1, 2). In addition, the recent identification of mutations encoding NKB (*TAC3* gene in humans) or its receptor (TAC3R in humans) that result in a hypogonadotropic hypogonadism, combined with data showing that NKB has stimulatory effects on reproduction in a variety of species (3-7), have cast a critical role for NKB in the reproductive spotlight. Another significant milestone in the last five years was the identification of the coexpression of kisspeptin, NKB and dynorphin in arcuate neurons, first identified in sheep (8), and soon afterward shown to also be present in other mammals including rodents, monkeys, goats and humans (6, 7, 9-11). This population of cells is now termed KNDy (Kisspeptin/Neurokinin B/Dynorphin) neurons (12), and have been shown to play an important role in control of the GnRH pulse generator (12, 13) through a variety of sophisticated anatomical (10, 14-16), electrophysiological (7, 17, 18) and functional (16, 19, 20) studies.

The work presented in this dissertation has added to this knowledge; our studies have clarified our understanding of the anatomical organization of the KNDy network, the plasticity of the KNDy neurons, their afferents, and projections to GnRH neurons, and have defined functional roles for the two major kisspeptin populations in the sheep. In 2008, the ARC had been established as a key site for steroid positive and negative feedback in the sheep (21, 22), however, this was primarily based on *in situ* data (21, 22), and the role of the preoptic area (POA) kisspeptin cells in the sheep remained elusive. Our work has identified a role for the POA kisspeptin neurons in positive feedback, and has also demonstrated that in the ARC, the same neurons likely mediate both positive and negative feedback ((16); Chapter 3). Another prevailing view based on rodent data was that rostral anteroventral periventricular (AVPV) or POA kisspeptin neurons provide

input to GnRH cell bodies while the ARC kisspeptin (KNDy) cells project to the median eminence to regulate GnRH release at the terminal level (23, 24). Our data has provided clear anatomical evidence that, at least in the sheep, KNDy and non-KNDy kisspeptin neurons project to GnRH cell bodies (Chapter 4). We have also shown that KNDy neurons project to POA kisspeptin cells, and that glutamate is a component transmitter of the ARC KNDy reciprocal circuitry (Chapter 5). Very recently, KNDy inputs to GnRH cell bodies have also been identified in the human (25), which corroborates our data and furthers the idea that kisspeptin is able to exert stimulatory actions on GnRH cell bodies. In addition, we have shown that the ARC KNDy neurons represent a population of cells that undergoes plasticity in synaptic afferents (glutamatergic, GABAergic, and others) across the estrous cycle, and with E2 in sheep and mice, respectively (Chapters 5 and 6).

8.2. Unanswered Questions and Future Directions

With the advances in our understanding of the anatomical and physiological role of kisspeptin and KNDy cells, as well as technical advances in our ability to study various aspects of the system, there are a number of significant issues and questions that are ripe for study in the coming years.

8.2.1. KNDy Cells in Steroid Feedback

The KNDy neurons have been hypothesized to constitute a key component of the GnRH pulse generator in various species (7, 10, 12, 13). With regards to steroid feedback, KNDy neurons in rodents and sheep mediate E2 negative feedback (22, 26, 27). In contrast to the rodent, in which E2 positive feedback appears to be restricted to the AVPV (26, 27), in sheep both the preoptic and arcuate kisspeptin (KNDy) populations appear to participate in positive feedback (16, 21, 28, 29). Because a majority of KNDy neurons are activated during both increased pulsatile activity (negative feedback) and the surge (positive feedback) (Chapter 3), it seems likely that individual KNDy neurons may respond to both the negative and positive feedback influence of E2 (16). This raises numerous questions such as: what are the mechanisms by which E2 can regulate both positive and negative feedback in the same cells, and do changes in E2 signaling pathways within these cells accompany changes in E2 feedback? The availability of

transgenic models (see below) in which individual KNDy cells can be identified and the electrophysiological properties studied should be an advantage in pursuing such questions at a cellular and molecular level. In addition, the question of whether KNDy neurons in mice might be activated during positive feedback has not been systematically studied and should be examined.

8.2.2. Projections from KNDy Neurons

Although inputs from single-labeled kisspeptin, neurokinin B or dynorphin terminals onto GnRH neurons have been described in a variety of species (9, 23, 24, 30-32), only recently have projections that arise specifically from KNDy neurons been examined using multiple-label immunohistochemistry. There are limitations of the immunohistochemical approach, for example, labeling depends on peptide detection which can vary depending on hormonal status, and increased/decreased detection may be due to a number of factors such as release of peptide, increased transcription/translation, and rate of trafficking to the terminals. The use of transgenic mouse models to completely label axonal projections from neurons in specific regions, will expand the ways in which mapping of projections can be carried out. For example, projections from KNDy neurons and their terminals can be labeled by use of a viral vector injected into the ARC of Kiss1-Cre mice that expresses a Cre-dependent fluorescent marker conjugated to synapsin, so that all synaptic terminals originating from ARC Kisspeptin (KNDy) neurons will be labeled accordingly (see below). In addition to the current work in Chapter 4, only one other study has used triple-label immunofluorescence to identify KNDy vs. single-labeled kisspeptin and NKB inputs onto GnRH neurons, conducted in the human (25). It is important to extend these findings to include more species in which KNDy peptides have individually have been shown to project to GnRH cells. Future studies will provide insight into comparative neuroanatomy of the reproductive neuroendocrine system, both in terms of projections throughout the brain and in interactions with GnRH neurons.

The documented existence of reciprocal interconnections among KNDy neurons is a unique feature of this kisspeptin population (15, 17, 33), and has been identified using various techniques including electron microscopy (EM)(34), conventional tract

tracing (17), and immunohistochemistry (35). An important unanswered question is whether these reciprocal connections arise from the same neurons, i.e., are recurrent synapses (autosynapses), or whether they represent synapses between different KNDy cells (36). This is a question that could potentially be addressed by dye-filling of individual, identified KNDy neurons in tissue slices, using Kiss1-Cre/GFP mice (Chapter 6, (37, 38)).

8.2.3. Projections to KNDy Neurons

Our data has demonstrated that there is significant morphological plasticity in afferent inputs to kisspeptin across the estrous cycle (Chapter 5 and 6). While these changes include inputs arising from glutamatergic neurons, the precise location of those glutamatergic cells is not known. In addition, there are also a large number of unidentified afferents to KNDy neurons that show plasticity across the estrous cycle in the sheep (Chapter 5), or are modulated by E2 in the mouse (Chapter 6). Thus, a major unresolved question is determining the origin and transmitter phenotype of inputs to KNDy cells in sheep, as well as in other species. Again, transgenic approaches may be useful in addressing this issue. For example, trans-synaptic retrograde tracers (e.g., WGA) could be delivered in a cell-specific manner into KNDy cells by viral vectors, and thereby could allow for identification of afferent neurons that specifically target KNDy cells. There is also evidence that other factors which modulate GnRH secretion, such as endogenous and environmental stressors, do so via kisspeptin cells (39, 40). Again the precise afferent responsible for conveying stress signals to KNDy neurons remain to be determined. Kisspeptin and KNDy cells are also regulated by metabolic signals, such as leptin and insulin, and their mediators, pro-opiomelanocortin (POMC) and agouti-related peptide (AGRP)/neuropeptide Y (NPY) neurons in the ARC (40). In the ARC of sheep, there are documented reciprocal innervations between kisspeptin neurons and both NPY and POMC cells (41). Whether metabolic signals such as leptin, act to regulate kisspeptin neurons by direct or indirect (i.e. via AgRP/NPY or POMC cells) mechanisms, remains to be determined. In summary, future exploration of the regulation of these and other systems that lie afferent to kisspeptin and KNDy cells, as well as the presence and

regulation of their cognate receptors, will be an important next step in uncovering the anatomical organization of the kisspeptin and KNDy neuronal networks.

8.2.4. Sites of Action of NKB and Dynorphin in the Control of GnRH/LH pulses

Our understanding of the sites of action of the KNDy peptides is in large part due to receptor localization in cells using immunohistochemistry to visualize protein, and in situ hybridization or RT-PCR to localize mRNA (10, 14, 42, 43). From these studies, NKB is hypothesized to act primarily on the KNDy neurons and not GnRH neurons to drive GnRH/LH pulsatility (12, 14, 44), although a study in the rat suggests that a subset of GnRH neurons may express NK3R (32, 45). There are various lines of evidence that support the idea that NKB acts upstream or on kisspeptin cells to influence GnRH (4, 5, 44, 46). First, desensitization of Kiss1R blocks the senktide-induced increase in GnRH, while desensitization of NK3R does not affect the GnRH response to kisspeptin in monkeys (46). Secondly, a GPR54 antagonist in rats blocked a senktide-induced increase in LH pulses (4). Third, senktide induces cFos in kisspeptin cells of rats and sheep (5, 47). Therefore, there are multiple routes by which stimulatory actions of NKB can alter GnRH secretion: via postsynaptic actions within KNDy-KNDy connections or on other NK3R-expressing neurons, as well as presynaptic actions on NKB/NK3R expressing terminals in contact with GnRH cells or other neurons. Preliminary studies have identified NK3R-immunoreactive synaptic terminals in contact with GnRH neurons (Ahn & Lehman, unpublished observations), which expands the role of NKB in the synchrony of the network to include presynaptic actions at the level of KNDy-GNRH contacts. Future studies that reveal the brain regions and precise anatomical sites by which NKB/NK3R act to stimulate GnRH release, will be important steps forward.

Future efforts will also help to decipher the precise sites of actions of dynorphin within the KNDy network and in the control of GnRH secretion. Although the ability of dynorphin to mediate P4 negative feedback has been established in the sheep (48), it is still not clear *where* dynorphin exerts its inhibitory actions to suppress GnRH and LH pulses. It may be via reciprocal connections in the KNDy circuitry, or an indirect mechanism. The high affinity opioid receptor for dynorphin, the kappa opioid receptor (KOR), is found on a small subset of KNDy cells in the male and female mice (20% in

females and less than 6% in males (10, 44). However, numerous other KOR mRNA-containing cells are present in the ARC, suggesting that dynorphin may be acting locally on other cells as well. Dynorphin is unlikely to act directly upon GnRH neurons since data from rats has shown that GnRH neurons are devoid of KOR (49, 50). Thus, in light of limited KOR in KNDy and GnRH cells (10, 44, 49, 50), these data suggest that dynorphin is acting on other neurons, in the ARC and elsewhere, that in turn may project to KNDy or GnRH cells to inhibit pulsatile GnRH/LH release (13). It is important to recognize that a majority of data regarding KOR localization is in rodents, and at this point it is not clear whether or not this reflects the site(s) of dynorphin action in other species, including the sheep. Thus, a key area for future research will be to clearly define, using either antibody or mRNA probes, the cellular sites of dynorphin action, and to determine which of these sites is critical for KNDy cells regulation of GnRH secretion.

8.2.5. Release of KNDy Peptides: simultaneous release or selective trafficking of receptors?

A fundamental unanswered question embedded in the current working hypothesis of how KNDy cells act as a pulse generator (Section 2.5), is what is responsible for the time lag between the release the stimulatory peptide (NKB) that triggers each pulse and the inhibitory peptide (dynorphin) that terminates it? The possibilities include both presynaptic and postsynaptic mechanisms. One presynaptic mechanism would be that NKB and dynorphin are localized in different presynaptic vesicles in the same terminals, and their release can thereby be independently regulated perhaps by presynaptic activity (51). Determining whether or not the KNDy peptides are localized within the same or different vesicles would be difficult since it would require dual-label EM immunolabeling, but would be the most straightforward way of examining this possibility. A postsynaptic mechanism to account for the time lag between the actions of NKB and dynorphin might be the localization and trafficking of NK3R and KOR on either the pre- or postsynaptic membrane. For example, if NK3R were rapidly internalized after the start of each pulse it might allow the inhibitory effect of dynorphin to surface. Alternatively, if KOR were rapidly brought to the cell surface following NKB stimulation, it might account for the termination of activity responsible for the end of

each individual pulse. In order to test these possibilities, reagents and/or tracers that allow for discrete localization of NK3R and KOR would be necessary, as well as a physiological model where the minute-by-minute dynamics of an individual GnRH pulse could be monitored. While difficult, these experiments are not outside the realm of possibility and would help move the current working hypothesis of the KNDy cell “pulse generator” into a more cellular and molecular framework.

8.2.6. Sites of Action Where NKB May Contribute to the GnRH Surge

In the sheep, NKB agonists are able to produce a surge-like LH secretion during follicular phase, perhaps reflecting a role for endogenous NKB in driving the GnRH/LH surge (3, 52). In Chapter 7, we examined the possibility, using receptor internalization of NK3R as a marker for endogenous NKB release, that the ARC and KNDy cells may be a site of action for NKB action related to the surge. However, we found no evidence that there were significant differences across the estrous cycle in internalization of NK3R that might reflect changes in endogenous release of NKB. In fact, this closely corresponds to recent data in follicular phase ewes showing that intra-ARC injection of NKB antagonist have only minor, modest effects on LH release (52). This modest effect is in striking contrast to that seen when NKB is microinjected into the retrochiasmatic area (RCh) or preoptic region (POA) of the ewe, suggesting that the latter two sites may be the relevant physiological targets for NKB action leading up to the preovulatory GnRH/LH surge (52). The sheep RCh and POA are both locations that contain NK3R-positive cells (14), as well as NKB fibers, but again the precise origin of those NKB fibers is not known. Some may indeed arise from KNDy cells, but since NKB cells are also found in other diencephalic and telencephalic regions (53-55), it may be that this input arise from multiple sites. In addition, little is known about the peptide/transmitter phenotype of NK3R-positive cells in the RCh or POA, or whether they project directly to GnRH and/or kisspeptin neurons. Thus, there is much to be learned about the anatomical organization of the NKB system outside of KNDy neurons, and it will be worthwhile in the future to study endogenous NKB release across the estrous cycle and its role in the control of the GnRH/LH surge.

8.2.7. Emergence of Transgenic Mouse Models of Kiss1 or KNDy Physiology

Recently, transgenic mouse models have been used to study the physiology of the kisspeptin system (18, 37, 56-59), and may revolutionize the way that this system is studied. Mouse models expressing markers of Kiss1 neurons (Kiss1-Cre/GFP) have emerged (37, 60), and are invaluable in electrophysiological experiments for targeting these specific neurons. In addition, as noted above, transgenic mice expressing kisspeptin-specific markers (i.e. farnesylated EGFP) will be important in fully delineating the efferent projections of KNDy and other kisspeptin cells, as well as defining their complete set of afferent inputs. These transgenic animals also allow for kisspeptin cell-specific manipulations, such as ablation of receptors (i.e. ER α (56, 57)), and the subsequent analysis of the reproductive physiology of such animals. However, in order to allow for selective manipulation of KNDy cells vs preoptic kisspeptin neurons (and kisspeptin cells expressed elsewhere), the use of techniques that permit conditional and/or regional expression of transgenes are required. One such approach, used by our colleague Martin Myers, is to combine the use of transgenic Kiss1-Cre mice with local delivery of Cre-dependent viral vectors. In this way, manipulations of the KNDy cell group in the ARC could be made independently of that of preoptic kisspeptin neurons, and the effects of KNDy-specific changes assessed. For example, injections in Kiss1-Cre mice of a vector encoding a Cre-dependent transgene expressing the fluorescent marker mCherry conjugated to synaptophysin is currently being used in the Myers lab to label all synaptic terminals arising from KNDy neurons in the mouse. With modifications, this same strategy could be employed in non-transgenic rats or sheep, so long as a transgene to confer KNDy cell specificity (e.g., kisspeptin promoter) is used.

8.3. Clinical Aspects in Reproductive Health

Since mutations in the gene coding for kisspeptin (*Kiss1*), NKB (*TAC3*) and the Kiss1R are associated with hypogonadotropic hypogonadism, delayed puberty, and an infertile phenotype (61-63), it is plausible that dysfunctions in this neuronal network are associated with other clinical disorders. One such disorder is polycystic ovarian syndrome (PCOS), which is characterized by both metabolic and reproductive deficits in women (64). In this disease, reduced sensitivity of the brain to feedback actions of

ovarian steroids are thought to play an important role in the reproductive deficits. Thus, in light of the evidence supporting the KNDy neurons as an important network in the steroid regulation of GnRH (12, 13), altered levels of KNDy peptides in PCOS might play a role in reduced sensitivity of the reproductive axis to steroid feedback. Recently, this hypothesis was tested using a well-established sheep model of PCOS (64), in which treatment with prenatal androgens during fetal life results in a PCOS-like phenotype (64), and increased LH pulse frequency (65). Interestingly, these researchers found that numbers of NKB and dynorphin neurons in the ARC of prenatal T ewes were reduced to nearly half of what was seen in control females (65), while kisspeptin cell numbers were not altered (65). Because dynorphin plays a role in mediating progesterone negative feedback by decreasing GnRH pulses in sheep (48), decreased dynorphin (and unaltered kisspeptin) may lead to a shift towards greater stimulatory drive of the GnRH system by kisspeptin and a deficit in the ability of dynorphin to inhibit pulse frequency (12). Restoring the balance of these peptides using kisspeptin antagonists or dynorphin analogs could therefore be an approach for therapeutic manipulations in PCOS and other disorders involving increased LH pulse frequency (12).

It appears that the effects of mutations in *Kiss1R* do not invariably result in an absence of puberty and sexual maturation, but rather, that in some instances this process may be delayed (66). Evidence for this comes from a long-term follow up study in a patient with a *KISS1R* mutation presenting with hypogonadotropic hypogonadism showing that reproductive maturation (pubertal development, etc.) was delayed, rather than absent (66). This sort of long term follow up was not carried out in the other reports of inactivating *KISS1R* (*GPR54*) mutations that lead to hypogonadotropic hypogonadism (61, 62), thus it is possible that pubertal development may have occurred in those patients, but much later than expected. A recent study in mice has shown that genetic ablation of kisspeptin-expressing cells in mice can still lead to a fertile adult phenotype, suggesting that there are compensatory neural mechanisms that can account for the loss of kisspeptin neurons during development (58). Whether these compensatory mechanisms are at work in patients with the *KISS1R* mutation showing delayed puberty remains to be explored.

8.4. Final Conclusions

In summary, kisspeptin and KNDy neurons in the brain are vital upstream regulators of reproduction in the brain that appear to integrate a variety of central and peripheral signals. While our studies have focused on the role that these cells play in conveying steroid signals to GnRH neurons, there is also strong evidence that the influence of stress signals, environmental cues (e.g. photoperiod), and nutritional signals on GnRH secretion are also, at least in part, mediated by kisspeptin and/or KNDy cells (12). There is also recently emerging evidence that KNDy cells, as a major target of the actions of estradiol and other sex steroids, may be involved in more than just reproduction. For example, work by Rance and colleagues in the last several years have shown that KNDy cells, and NKB released from them, play a critical role in conveying the influence of estradiol on thermoregulatory circuits, and may be responsible the symptom of “hot flushes” seen in post-menopausal women (67, 68). In addition, Kiss1R is known to be expressed in a number of brain regions not thought to be directly involved in reproductive function, such as the hippocampus and amygdala (69). Thus, future work is likely to expand the roles that kisspeptin and KNDy neurons play beyond reproduction, and may point toward these cells as integrators coordinating a set of diverse set of physiological and behavioral functions.

8.5. References

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APPENDICES

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APPENDIX B: Animal Protocol Ethics Approvals

Date: **IACUC PROTOCOL APPROVAL NOTICE TO THE INVESTIGATOR**Investigator: ACUC Protocol #: Project Title: Type of approval: Replacing protocol #: COMMENTS: Agency: Approval Period: to

The West Virginia University Animal Care and Use Committee (WVU ACUC) has granted continued approval for the above referenced project. Continued approval was based on a post approval monitoring review of the protocol and, if appropriate, an inspection of the laboratory/facility space where procedures on animals are done. Should any modifications (including an increase in the number of animals, changes in procedures, or additions/removals of personnel) be necessary, you must obtain prior approval from the WVU ACUC by submitting an amendment.

NOTE: Please remember that a copy of the complete approved protocol, which will include any amendments, must be posted in the laboratory.

Protocol start date: Protocol may be renewed through: Number of animals: Species:
 Acquisition of your animals MUST be through OLAR

 Acquisition of your animals does NOT need to be through OLAR
USDA Pain Category: Subject to inspection/review every:

Animals will be taken out of the vivarium and may or may not return. Therefore, this protocol will be monitored for re-approval every 6 months and you will be contacted by members of the WVU ACUC for a laboratory inspection every 6 months. This inspection must be completed prior to the 6 month deadline or your protocol will be closed.

Animals are not taken out of the vivarium OR this is an agricultural or wildlife protocol. Therefore, this protocol will be reviewed annually for continuing approval. You will receive a blue self inspection form 3 months prior to the 12 month deadline. This form MUST be returned by the date indicated or the protocol will be closed.

ALL personnel on WVU ACUC protocols MUST be up to date with CITI training (good for 3 years) and the Animal Health Questionnaire (good for 1 year) before protocols can be reviewed and/or approved. For assistance with this training, please go to <http://oric.research.wvu.edu>.

Mindy Hollander
 Animal Compliance and Training Officer, Office of Research Integrity and Compliance
 email: mhollander@mail.wvu.edu phone: 304-293-9368 cell: 304-216-1641

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Institutional Biosafety Committee Institutional Animal Care and Use Committee Institutional Review Board

February 5, 2007

MEMORANDUM

TO: Dr. Marcel Amstalden
Animal Science
MS 2471

FROM: Institutional Animal Care and Use Committee II

SUBJECT: Approval of AUP 2007-7
"Hypothalamic neurokinin 3 receptor activation during the estrous cycle of the ewe"
Funding Source:
AUP Approval Date: 2/5/2007
AUP Expiration Date: 2/4/2010

This AUP has been approved by the IACUC 2 for a period of 3 years. It is the responsibility of the principal investigator to assure all animal work is conducted in accordance with this AUP.

If you have indicated that you will be performing post procedural monitoring of animals at specific intervals, please provide documentation of your observations in the medical record or by using "Animal Observation" cards that are available through the Comparative Medicine Program.

A copy of this approval will be sent to the housing facility. ***You must consult with the housing facility manager prior to ordering animals to ensure that space is available.***

Pe: Housing Facility: NPC
Attending Veterinarian
IACUC

CURRICULUM VITA

Christina M. Merkley

Educational Background

- PhD** **Sept. 2008 to Present**
University of Western Ontario,
 Graduate Program in Neuroscience
 London, Ontario, Canada
Supervisor: Michael N. Lehman, Ph.D.,
 Dept Chair, Neurobiology and Anatomical Sciences, University of
 Mississippi Medical Center, Jackson, MS, USA
- Hons. BSc** **Sept. 2004 to May 2008**
Lakehead University
 Department of Psychology
 Thunder Bay, Ontario, Canada

Publications

- Merkley CM**, Porter KL, Coolen LM, Hileman SM, Billings HJ, Drews S, Goodman R.L., Lehman MN. (2012). KNDy (Kisspeptin/Neurokinin B/Dynorphin) Neurons are Activated During Both Pulsatile and Surge Secretion of LH in the Ewe. *Endocrinology*, 153(11):5406-5414.
- Lehman MN*, **Merkley CM***, Coolen LM, Goodman, RL. (2010). Anatomy of the Kisspeptin Neural Network in Mammals. Special issue: New insights into the neurobiology of reproduction and puberty. *Brain Research*, 1364, 90-102.
 *shared first authorship

Scholarships and Awards

- 2011- present: Ontario Graduate Scholarship
 2011- present: Western Graduate Research Scholarship in Neuroscience
 2012: Young Investigator Award for the World Conference on Kisspeptin Signaling in the Brain, Tokyo, Japan
 2010: Young Investigator Award for the International Congress of Neuroendocrinology Meeting, Rouen, France
 2007- 2008: In-course Academic Scholarship, Lakehead University
 2004: Lakehead University Entrance Scholarship

Abstracts

- Merkley CM**, Frazao R., Coolen, LM, Clegg DJ, Elias CF, Lehman MN. (November 6-9, 2012). Estrogen-induced plasticity in glutamatergic inputs to Kiss1 neurons in the mouse. Oral presentation at the 2nd World Conference on Kisspeptin Signaling in the Brain, Tokyo, Japan

Merkley CM, Frazao R, Coolen LM, Clegg DJ, Elias CF, Lehman MN. (October 13-17, 2012). Estrogen-induced plasticity in glutamatergic inputs to Kiss1 neurons in the mouse. Poster presented at the *Society for Neuroscience Annual Meeting, Neuroscience 2012*, New Orleans, LA.

Merkley CM, Coolen LM, Goodman RL, Lehman MN. (June 23-26, 2012). Colocalization of Met-Enkephalin, but not Galanin or Tyrosine Hydroxylase, within Kisspeptin Neurons in the Sheep. Poster presented at the *Endocrine Society Annual Meeting, ENDO 2012*, Houston, TX.

Merkley CM, Coolen LM, Goodman RL, Lehman MN. (Nov 12-16, 2011). Direct Projections of Arcuate KNDy (Kisspeptin/Neurokinin B/Dynorphin) Neurons to GnRH Neurons in the Sheep. Poster presented at the *Society for Neuroscience Annual Meeting, Neuroscience 2011*, Washington, D.C.

Merkley CM, Coolen LM, Goodman RL, Lehman MN. (June 4-7, 2011). Evidence for neuroplasticity in synaptic inputs to arcuate kisspeptin neurons and GnRH neurons across the ovine estrous cycle. Poster presented at the *Endocrine Society Annual Meeting, ENDO 2011*, Boston, MA.

Cernea MC, Padmanabhan V, Coolen LM, **Merkley CM**, Lee T, Lehman MN. (June 4-7, 2011). Morphological alternations in KNDy-GnRH neurocircuitry in a sheep model of PCOS. Poster presented at the *Endocrine Society Annual Meeting, ENDO 2011*, Boston, MA.

Merkley CM, Coolen LM, Goodman RL, Lehman MN. (May 6, 2011). Neuroplasticity across the estrous cycle: changes in synaptic inputs to kisspeptin and GnRH neurons. Poster presented at the *1st annual Michigan Alliance for Reproductive Technologies and Science (MARTS) Research Symposium*, Lansing, MI.

Merkley CM, Coolen LM, Goodman RL, Lehman MN. (July 11-15, 2010). Colocalization of glutamate within kisspeptin neurons and their projections onto GnRH neurons in the ewe. Poster presented at the *7th International Congress of Neuroendocrinology*, Rouen, France

Merkley CM, Coolen LM, Jackson L, Goodman RL, Lehman MN. (June 10-13, 2009). Evidence for transcriptional activation of kisspeptin neurons and glutamatergic inputs to kisspeptin during the preovulatory surge of the sheep. Poster presented at the *Endocrine Society annual meeting ENDO*, Washington, DC.

Merkley CM, Coolen LM, Jackson L, Goodman RL, Lehman MN. (May 8, 2009). Transcriptional activation of kisspeptin neurons and glutamatergic inputs to kisspeptin during the preovulatory surge of the sheep. Poster presented at the

29th Annual Southern Ontario Neuroscience Assembly, McMaster University, Hamilton, Ontario.

Merkley CM, Wesner MF. (May 21-26, 2008). Do depressive and non-depressive neurometric contrast sensitivity measures relate to conventional psychometric measures across seasons? Poster presented at the *20th Annual Association for Psychological Science Convention, Chicago, IL.*

Merkley CM, Pavlou D, Wesner MF. (May 24-27, 2007). Nonseasonal depression (not SAD) shows spatiotemporal contrast sensitivity changes across seasons. Poster presented at the *19th Annual Association for Psychological Science, Washington, DC.*

Seminars:

2012: E2-Induced Neuroplasticity in Glutamatergic Inputs to Kiss1 Cells in the Mouse. World Conference on Kisspeptin Signaling in the Brain, Tokyo, Japan.

2012: Graduate Program in Neuroscience Student Seminar Series

2011: Graduate Program in Neuroscience Student Seminar Series

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Society for Neuroscience Member (2010- Present)