Neuropeptide and Gonadal Steroid Action on Arcuate Kisspeptin Neurons: Implications for Central Regulation of Fertility

by

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DEDICATION

To my family.

I am proud to dedicate this work to my husband Steve, my dad Dave, my mom Julie, my sister Lauren, my mother-in-law Vickie, and although he is no longer with us, the man who would have been my father-in-law Tony. This dissertation means a lot, but you all mean infinitely more. You are my purpose, and I love you.

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A telling indicator of the type of PI running a lab is the caliber of individuals working for her. I would like to thank my Moenter lab mates, past and present, for making grad school more enjoyable. You are skilled and intelligent, and it has been a pleasure working with you. For those of you who came from UVa with Sue, you helped this new grad student start in your lab even as the dust was still settling from your own move to U-M. You made me feel welcome, and you were crucial to getting me up to speed. To the more junior members of the lab, thank you for dealing with the kinks as I have learned how to teach techniques, and

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4AP	4-aminopyridine, blocker of I_A and I_D
ACSF	artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
APV	D(-)2-amino-5-phosphonovaleric acid; NMDA receptor blocker
AR	androgen receptor
Ar	gene for AR
AVPV	anteroventral periventricular nucleus
cas	castrate
Ca_V	voltage-gated calcium channel
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione; AMPA-KA receptor blocker
DHT	dihydrotestosterone
DMSO	dimethylsulfoxide
DPN	(R)-2,3-*bis*(4-Hydroxyphenyl)-propionitrile; ERβ agonist
EOP	endogenous opioid peptide
ERE	estrogen response element
ERα	estrogen receptor α
ERβ	estrogen receptor β
Esr1	gene for estrogen receptor α
Esr2	gene for estrogen receptor β

FSCV	fast scan cyclic voltammetry
FSH	follicle stimulating hormone
GABA	γ-aminobutyric acid
$GABA_AR$	$GABA_A$ receptor
Gapdh	glyceraldehyde 3-phosphate dehydrogenase; housekeeper
GFP	green fluorescent protein
GIRK	G-protein coupled inward rectifying potassium
GnRH	gonadotropin-releasing hormone
HCN	hyperpolarization and cyclic nucleotide gated channels
I_A	A-type transient potassium current
icv	intracerebroventricular
I_D	D-type transient potassium current
I_h	hyperpolarization-activated, nonselective cation current
I_{mem}	membrane current
I_{NaP}	persistent sodium current
I_T	transient-type calcium current
K_{ir}	inward-rectifying potassium channel
Kiss1	gene for kisspeptin
Kiss1r	gene for kisspeptin receptor
KNDy	arcuate neurons coexpressing NKB, dynorphin and kisspeptin;
KOR	κ-opioid receptor
LH	luteinizing hormone
LHRH	See GnRH

MBH	mediobasal hypothalamus
mPOA	medial preoptic area
MUA	multi-unit activity
Na_V	voltage-gated sodium channel
NK1R	neurokinin-1 receptor
NK2R	neurokinin-2 receptor
NK3R	neurokinin-3 receptor
NKB	neurokinin B
NMDA	N-methyl-D-aspartate
norBNI	nor-binaltorphimine; KOR antagonist
Oprk1	gene for KOR
ovx	ovariectomized
Pdyn	gene for dynorphin
PeN	periventricular nucleus
POA	preoptic area
Ppia	peptidylprolyl isomerase A; housekeeper
PPT	4,4',4''-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol; ERα agonist
Prl	gene for prolactin
RFRP-3	RFamide-related peptide-3, also known as gonadotropin-inhibiting hormone
R_{in}	input resistance
Rps29	ribosomal protein S29; housekeeper
SK	senktide, NK3R agonist

Tac1	gene for both substance P and neurokinin A
Tac2	gene for NKB
Tac3r	gene for NK3R
Tacr3	gene for NK3R
TTX	tetrodotoxin; votage-gated sodium channel antagonist
U69593	KOR agonist

ABSTRACT

Pulsatile gonadotropin-releasing hormone (GnRH) release is essential to fertility and is modulated by gonadal steroids, most likely via steroid-sensitive afferents. Arcuate neurons coexpressing kisspeptin, neurokinin B (NKB), and dynorphin (KNDy neurons) are steroid-sensitive and have been postulated to both generate GnRH pulses and mediate steroid feedback on pulse frequency. KNDy neurons have been proposed to interact with one another via NKB and dynorphin to activate and inhibit the KNDy network, respectively, and thus alter kisspeptin output to GnRH neurons. To test the roles of NKB and dynorphin on KNDy neurons and the steroid sensitivity of these actions, targeted extracellular recordings were made of fluorescence-identified neurons from male mice that were either gonad-intact or castrate and otherwise untreated or treated in vivo with steroid receptor agonists. Senktide, an agonist for the high-affinity receptor for NKB (neurokinin-3 receptor, NK3R), increased action potential firing in KNDy neurons. Dynorphin reduced spontaneous KNDy neuron activity, but antagonism of kappa-opioid receptors (KOR) failed to induce firing in quiescent KNDy neurons. Senktide-induced activation was greater in KNDy neurons from castrate mice, whereas dynorphin-induced suppression was greater in those from intact mice. Similar to the intact condition, both estradiol and dihydrotestosterone suppressed NK3R agonist-induced KNDy neuron firing and enhanced the inhibition of firing rate caused by KOR activation. An estrogen receptor-alpha

agonist but not an estrogen receptor-beta agonist mimicked the effects of estradiol on NK3R activation. These observations support stimulation and inhibition of KNDy neuron firing by NK3R and KOR activation, respectively. Modulation of these responses by gonadal steroids may be a mechanism mediating steroid negative feedback. Overall, the work presented here supports contribution of KNDy neurons to steroid-sensitive elements of a GnRH pulse generator.

CHAPTER 1

Introduction

A note from the author: The Introduction (Chapter 1) of this dissertation reviews the state of relevant knowledge in the field at the initiation of the dissertation (in 2011), while the Conclusion (Chapter 4) integrates the present work with data from more recent publications.

1.1 Significance

Reproductive health is vital to individual quality of life as well as overall species survival. The reproductive system is responsible for proliferation of organisms via puberty onset, ovarian cyclicity, gametogenesis, fertilization, pregnancy, and lactation. Up to 20% of couples are infertile, and the cause in ~20% of these cases is unexplained (1), demonstrating the importance of continued research in normal reproductive physiology and pathophysiology. Beyond reproduction, sex hormones impact other systems, including the nervous, skeletal, and cardiovascular systems.

1.2 The HPG axis and GnRH neurons

Complex networks in the central nervous system regulate fertility. The final common pathway that integrates the output of all of these networks is the gonadotropin-releasing hormone (GnRH) neuronal population, which is located in the preoptic area (POA) of the hypothalamus and has projections to the median eminence (2). At the median eminence, GnRH neurons release GnRH into the hypothalamo-pituitary portal vasculature to stimulate the pituitary to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (3-5). LH and FSH initiate gonadal production of testosterone, estradiol, and progesterone, and these steroids feed back to regulate GnRH output and the pituitary via androgen, estrogen, and progesterone receptors (6-8). However, GnRH neurons themselves have only been rarely shown to express progesterone receptors, and have not been shown to express androgen receptors, or the α-isoform of the estrogen receptor (ERα, Figure 1.1 A), which is more critical for estradiol feedback than the β-type estrogen receptors expressed by GnRH neurons (9-17). These data implicate a critical role for steroid-sensitive afferents to mediate feedback to GnRH neurons, but knowledge of the identity and function of such inputs is incomplete. Understanding how steroids regulate these inputs will enhance our basic knowledge of normal neuroendocrine function and can help develop more effective strategies for the diagnosis, management, and prevention of central conditions that compromise fertility.

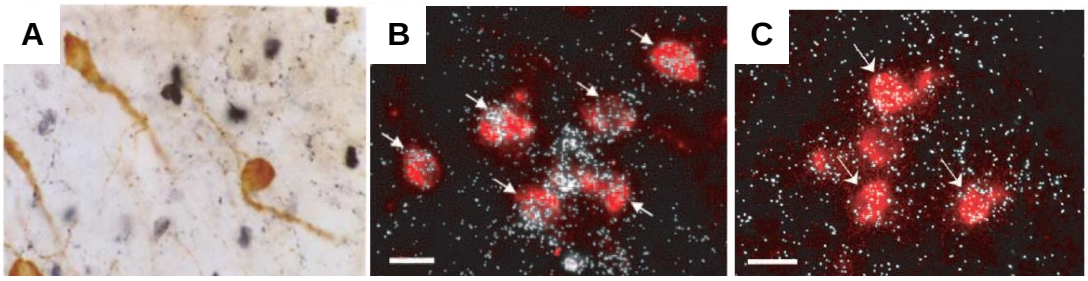

Figure 1.1 ERα is not detected in most GnRH neurons but is present on most kisspeptin neurons. A: Micrograph (x 400) of the lack of immunohistochemical colocalization of GnRH (brown cytoplasm) and ERα (black nuclei) in the organum vaculosum of the lamina terminalis of an ovariectomized female rat (13). B and C: Photomicrographs showing coexpression of *Kiss1* (red) and ERα (silver grains) mRNA in brains from female (B (18)) and male (C (19)) mice. Scale bar = 20μm.

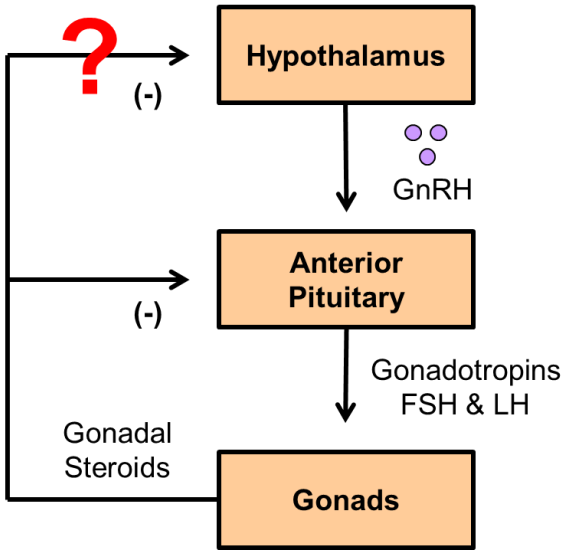

Figure 1.2 The hypothalamo-pituitary-gonadal axis. The mechanisms for gonadal steroid feedback in the brain are not well-understood.

Patterns of GnRH release

One crucial way that steroid feedback affects GnRH is by modulating its pattern of release. Normal GnRH release can be described in two fundamental patterns, pulses and surges (4,20-24), both of which are critical to reproduction. While the GnRH surge is only observed in females prior to ovulation, GnRH pulses are prevalent throughout the remainder of the female cycle and regularly in males. GnRH/LH pulses can be too frequent, as in polycystic ovary syndrome

(25,26), too infrequent, as in hypogonadotropic hypogonadism (27), or can begin too early, as in precocious puberty (28). All of these can cause infertility and associate with other long-term health problems that have a profound impact on quality-of-life.

In a test of the importance of GnRH pulses, hourly treatments, but not constant treatment, with GnRH maintained effective concentrations of circulating LH and FSH in ovariectomized monkeys with ablations of the mediobasal hypothalamus (MBH; Figure 1.3) (29). The ablations presumably interfered with the release of endogenous GnRH because they abolished LH and FSH release (29). Similar observations have been made with gonadotropin-specific subunit expression *in vivo* in ewes and rats and *in vitro* with cultured rat pituitary cells (30-36), suggesting that the pattern of GnRH release is critical for healthy release of gonadotropins.

While both LH and FSH require pulsatile GnRH secretion, specific GnRH patterns differentially regulate each gonadotropin. A higher GnRH pulse frequency favors pituitary expression and release of LH, while a lower GnRH pulse frequency favors pituitary expression and release of FSH (33-36). Although GnRH pulses influence the release of both gonadotropins, LH release requires GnRH pulses, whereas FSH can be released constitutively (37,38). Therefore, LH but not FSH is commonly used as an indicator for GnRH release (5).

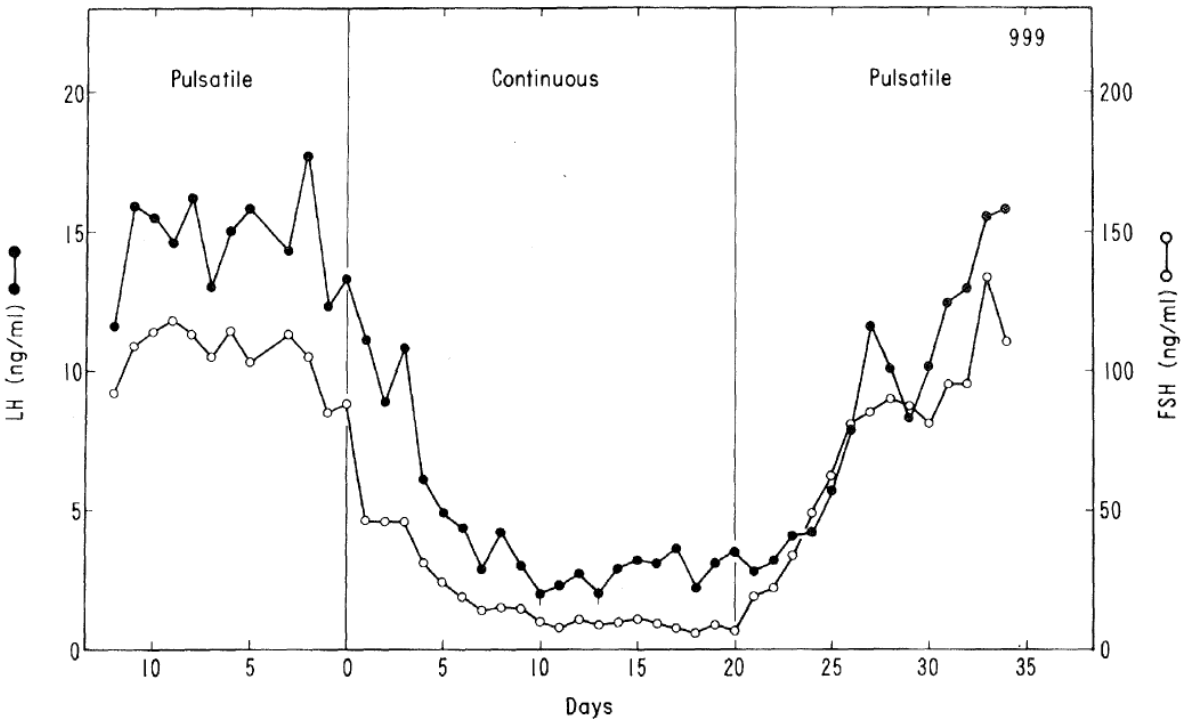

Figure 1.3 Suppression of plasma gonadotropin concentrations by continuous GnRH infusion in an ovariectomized monkey with lesioned hypothalamus. The levels of gonadotropins sustained by pulsatile (1 µg/min for 6 min once per hour) GnRH infusion were suppressed by continuous infusion of GnRH at the same rate (1 µg/min continuously) between days 0 and 20. Gonadotropin levels were recovered by re-establishing GnRH pulses beginning day 20. Dark circles = LH, open circles = FSH. (29)

Gonadal steroid regulation on patterns of GnRH release

In correspondence with the two patterns of GnRH release, gonadal steroid feedback also has two modes, negative and positive. In both males and females, negative gonadal steroid feedback corresponds with pulsatile GnRH release. In females only, positive steroid feedback is associated with the GnRH surge. The female reproductive cycle is known as the menstrual cycle in some mammals, including primates, and the estrous cycle in others, such as sheep and rodents. From mid-follicular phase, estradiol rises and becomes predominant, and, in sheep, there is roughly one GnRH pulse per hour (39). The amplitude of these pulses is inhibited by estradiol (40). Towards the end of the follicular phase the ovary produces substantially more estradiol, resulting in lower-amplitude GnRH

pulses that progressively increase in frequency until the mode of estradiol feedback switches from negative to positive (39,41,42). At this point, estradiol positive feedback causes a sudden high-concentration surge of GnRH (23,39), which causes a subsequent surge of LH that induces ovulation (23,39,43). Ovulation marks the beginning of the luteal phase, during which steroid negative feedback resumes. During this phase, estradiol is still present, but progesterone from the corpus luteum becomes dominant (44). The frequency of GnRH pulses during the follicular phase is reduced by progesterone to about one pulse every four hours (39,40,44). In the absence of pregnancy, progesterone and estrogen levels decrease, resulting in sloughing of the uterine endometrium, manifesting as menstruation in those species that have a menstrual cycle, and beginning the follicular phase of a new reproductive cycle (44). Similar steroid feedback on GnRH occurs in species such as mice that have estrous cycles, with positive steroid feedback happening during early proestrus and negative feedback occurring during the remainder of the cycle. Because of the vital nature of gonadal steroid feedback to GnRH, studying the mechanisms that may mediate this feedback is crucial to enhancing our understanding of reproductive function.

1.3 Kisspeptin

One candidate that has emerged as a potential mediator of gonadal steroid feedback onto GnRH neurons is the neuropeptide kisspeptin. Kisspeptin appears to be a key regulator of reproductive function. Mutations in the

kisspeptin receptor KISS1R can cause hypogonadotropic hypogonadism in humans (45,46), and knockout of the genes for kisspeptin (*Kiss1*) or its receptor (*Kiss1r*) reduces or eliminates reproductive function in mice (47,48), suggesting that kisspeptin plays a stimulatory role in the reproductive axis. Conversely, an activating mutation in KISS1R can lead to precocious puberty in humans (49). Consistent with these observations, the castration-induced rise in LH can be blocked by kisspeptin antagonists in the male rat and mouse (50), supporting kisspeptin as an intermediary to gonadal steroid feedback on gonadotropin release.

There is functional and anatomical evidence that GnRH neurons receive kisspeptin signals both directly and indirectly. Functionally, kisspeptin depolarizes and increases action potential firing in GnRH neurons *in vitro* and *ex vivo* (51-57), and it stimulates GnRH release in the median eminence *in vitro* and *in vivo* (3,58) (Figures 1.4 and 1.5). In rodents, higher *Kiss1* mRNA expression in the hypothalamic arcuate nucleus accompanies increased secretion of GnRH and gonadotropins, and these effects are blocked by knockout of the kisspeptin receptor gene (*Kiss1r*) (19,59).

Figure 1.4 Kisspeptin stimulates GnRH neuron action current firing. Extracellular electrophysiological recording from a GnRH neuron *ex vivo* in a brain slice preparation from an ovariectomized (OVX) adult mouse. 1) Currents recorded during an untreated control period. 2) Currents recorded during treatment with kisspeptin. Each vertical line is a trace of the current that corresponds to an action potential. (55)

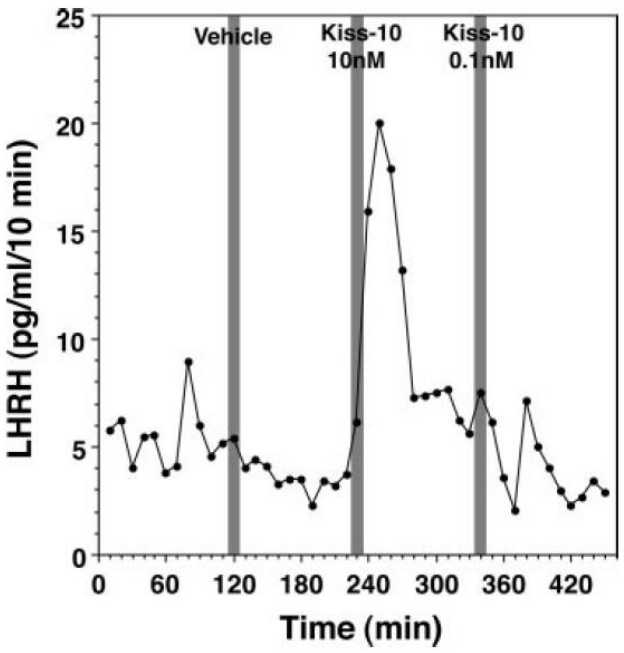

Figure 1.5 Kisspeptin stimulates GnRH (LHRH) release in the median eminence. Perfusion through a dialysis membrane with 10nM of human kisspeptin-10 (kiss-10, second gray bar) markedly increases the levels of GnRH collected by microdialysis from the median eminence of a pubertal female monkey and measured by radioimmunoassay. (58)

Anatomically, the majority of GnRH neurons in mice, rats, and sheep express Kiss1r message or protein (Figure 1.6) (53,60-63), and *Kiss1r* mRNA has been found in both the MBH and POA of monkeys (64,65). Furthermore, kisspeptin neuron fibers have been found in apposition to GnRH neurons in rodents, ungulates (large, hoofed mammals including goats, pigs, sheep, and horses), and primates (Figure 1.7) (62,66-74), supporting direct action on GnRH neuronal cell bodies and/or projections. Kisspeptin may also signal GnRH neurons via indirect pathways, including GABAergic or glutamatergic interneurons (55,75) or nonsynaptic communication in the median eminence, such as paracrine action via portal capillaries (68-70,72,76).

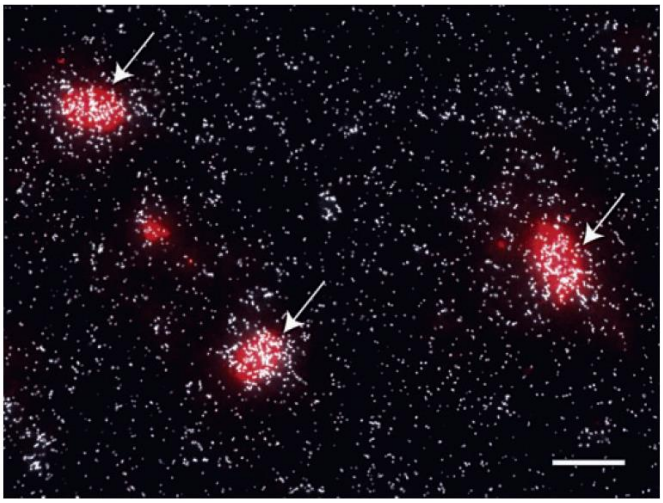

Figure 1.6 Photomicrograph showing coexpression of GnRH (red) and *Kiss1r* (silver grains) mRNA in adult male rats. The majority (77 ± 2%) of all GnRH neurons coexpress *Kiss1r*. Arrows = GnRH neurons that coexpress *Kiss1r*; scale bar = 20 µm (61)

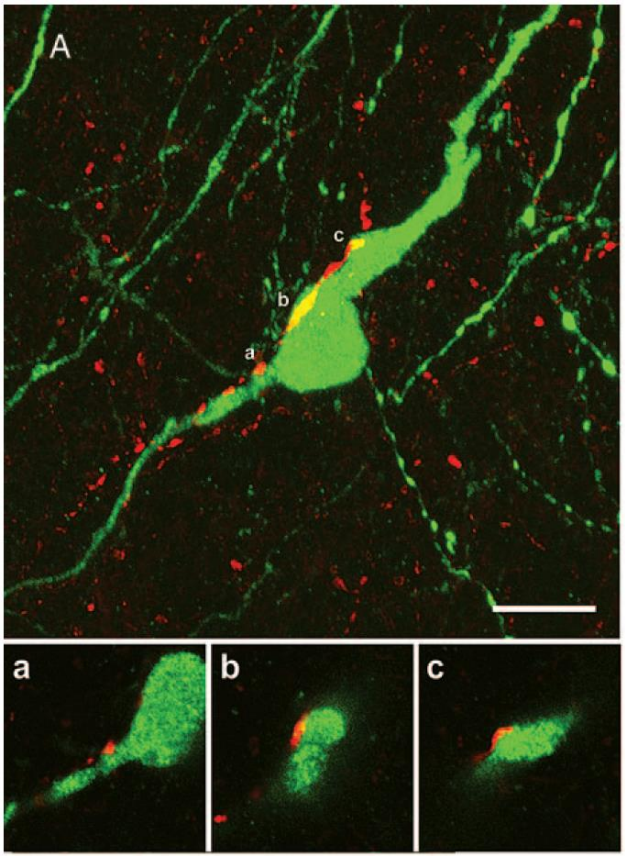

Figure 1.7 GnRH (green) neurons and kisspeptin (red) neuronal fibers are apposed in adult female mice. A: Confocal stack of 75 images. a, b, and c: individual 370-nm thick optical sections of the regions indicated by the corresponding letters in panel A. Scale bar = 10 µm. (66)

Kisspeptin neurons

Kisspeptin likely originates from neurons in several locations throughout the brain. In rodents, kisspeptin-expressing (message and peptide) neurons have been found in the hypothalamic arcuate nucleus, anteroventral periventricular

nucleus (AVPV), periventricular nucleus (PeN), anterodorsal preoptic nucleus, paraventricular nuclei, ventromedial hypothalamic nucleus, posterior hypothalamus, caudoventrolateral reticular nucleus, lateral reticular nucleus, hippocampal dentate gyrus, medial amygdala, bed nucleus of the stria terminalis, nucleus of the solitary tract, spinal trigeminal tract, and cerebral cortex (66,67,77-84). In ungulates, Kiss1-expressing somata have also been found in the arcuate nucleus, PeN, dorsomedial hypothalamus, medial preoptic area (mPOA), ventromedial hypothalamic nucleus, caudal paraventricular nucleus, and preoptic periventricular zone of the hypothalamus adjacent to the third ventricle (68-70,85). The population of kisspeptin neurons that appears to be most highly conserved in primates including humans is in the infundibulum, the analog of the arcuate nucleus (64,72,86). Otherwise, humans only have a sparse distribution of kisspeptin cell bodies in the mPOA and do not appear to have any kisspeptin neurons in the rostral periventricular region of the third ventricle, and rhesus macaques do not show any kisspeptin neurons in the POA, including the AVPV (64,72,86).

In rodents, the kisspeptin neuron populations that appear to be most directly involved in reproduction are those in the arcuate nucleus and the AVPV/PeN. Both populations extend projections to GnRH neurons (87), and both express gonadal steroid receptors (Figure 1.1) (18,19,88,89). Kisspeptin expression in both of these regions is affected by gonadal steroid feedback (18,19). However, the effects of gonadal steroids on kisspeptin neurons may differ between the arcuate vs AVPV, as supported by both anatomical and

functional evidence. While the number of kisspeptin neurons in the arcuate nucleus appears to be similar between males and females, the number of kisspeptin neurons in the AVPV is 10 to 25 times higher in female rodents than in the same region of male rodents (66,80). This sexual dimorphism in kisspeptin expression in the AVPV but not the arcuate mirrors the differentiation in positive but not negative gonadal steroid feedback. Functionally, ovariectomy increases and estradiol treatment restores low *Kiss1* mRNA expression in the arcuate nucleus of female mice and rats, whereas these treatments have the opposite effect on *Kiss1* in the murine AVPV (Figure 1.8) (18,19,90). In intact female rats, *Kiss1* mRNA levels are their lowest in the arcuate nucleus and their highest in the AVPV during the preovulatory estradiol surge (91), consistent with negative estradiol feedback in the arcuate and positive feedback in the AVPV. These distinctions are further reflected by intracellular signaling mechanisms initiated by activation of ERα. Negative estradiol feedback on serum LH concentration via ERα is mediated by “non-classical” signaling that is independent of the estrogen response element (ERE) (92). Similarly, estradiol inhibition of *Kiss1* expression in the arcuate nucleus is also mediated by ERE-independent mechanisms (92,93). In contrast, positive estradiol feedback on both LH release and AVPV *Kiss1* gene expression utilize “classical,” ERE-dependent ERα pathways (92,93). Interestingly, both positive and negative estradiol-mediated feedback on spontaneous action potential firing rate of GnRH neurons requires ERE-dependent ERα signaling (94), suggesting GnRH neuron firing uses different signaling mechanisms for ERα-mediated negative feedback than arcuate

kisspeptin neurons or the pituitary. GnRH neuron ERE-dependence notwithstanding, these observations support overall separate roles of arcuate kisspeptin neurons in negative estradiol feedback and AVPV kisspeptin neurons in positive estradiol feedback.

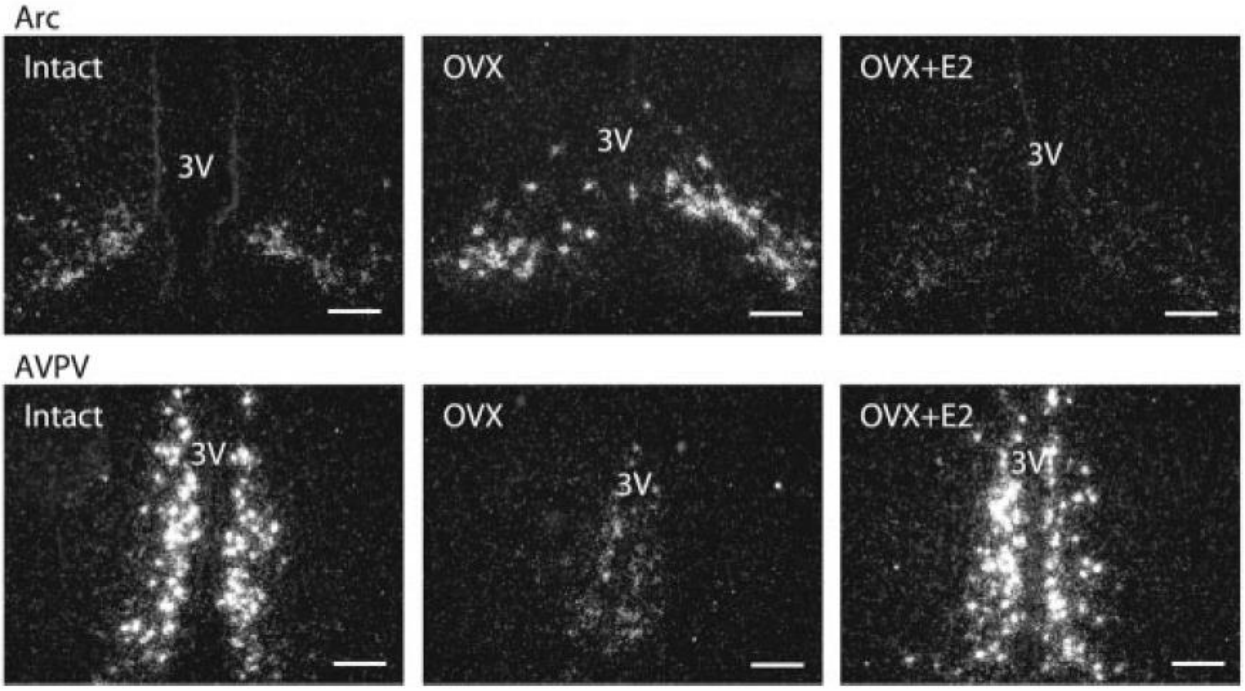

Figure 1.8 Photomicrographs of cells in the arcuate nucleus (Arc, top) or AVPV (bottom) expressing *Kiss1* mRNA (white clusters of silver grains) from female mice that are intact (left), ovariectomized (OVX, middle), or ovariectomized and estradiol-treated (OVX+E2, right). Estradiol inhibits *Kiss1* expression in the arcuate nucleus, and stimulates *Kiss1* expression in the AVPV. 3V = third ventricle; Scale bars = 100µm. (18)

Support for negative gonadal steroid feedback on arcuate kisspeptin neurons has also been observed in models other than female rodents. In male mice, rats, hamsters, and monkeys, castration increases *Kiss1* mRNA in the MBH (which contains the arcuate/infundibulum), whereas replacement with estradiol, dihydrotestosterone (DHT), or testosterone restores lower *Kiss1* mRNA (19,61,64,81,90). Estradiol replacement has also been found to inhibit ovariectomy-enhanced *Kiss1* expression in sheep and monkeys (73,86,95).

Similarly, *KISS* mRNA is increased in the infundibulum of postmenopausal women, in whom estradiol levels are low (86).

All kisspeptin neuron populations and their potential roles, especially in reproduction, must be acknowledged when interpreting observations about kisspeptin's influence on GnRH/LH release. However, this dissertation investigates negative gonadal steroid feedback and potential mechanisms affecting GnRH pulsatility in adult male mice. Therefore kisspeptin neurons in the arcuate nucleus will be the focus of the remainder of the work presented here.

1.4 GnRH pulse generator

The mediator of gonadal steroid feedback onto GnRH pulses may or may not be the same as the originator of the GnRH pulses. The concept that a steroid-sensitive originator of pulses may be located in the central nervous system was first proposed after a study that took frequent blood samples from female monkeys. This study observed a peak (pulse) in LH concentration roughly once per hour in ovariectomized monkeys, and these peaks were suppressed in intact animals (20), suggesting gonadal steroid feedback. In addition, the fluctuation in concentration that occurred with LH was not observed with another pituitary hormone (20), suggesting that there was not a broad, nonspecific effect on pituitary hormone release. The term "GnRH pulse generator" emerged to describe the possible brain unit that initiates GnRH pulses when researchers performed studies recording *in vivo* electrical activity, referred to as multi-unit

activity (MUA), using an array of multiple electrodes implanted into the brain. MUA recorded in the MBH of monkeys (Figure 1.9) (96-98), rats (99), and goats (100-102) consistently correlates with circulating LH pulses. Adding to these observations, lesions of the arcuate nucleus within the MBH block LH pulses in rats, while complete deafferentiation of the entire hypothalamus does not affect LH pulsatility (103). These data suggest that the MBH and, more specifically, the arcuate nucleus is a critical source of GnRH pulse generation that can function independently of extra-hypothalamic inputs. Importantly, this same anatomical region may be responsible for both pulse generation and steroid feedback. MUA in the MBH is also sensitive to gonadal steroids (104-107), and site-specific gonadal steroid antagonism demonstrates that the arcuate nucleus is a region critical for steroid feedback on LH pulses (108). Together, these data are consistent with a GnRH pulse generator in the arcuate nucleus that is also steroid-sensitive.

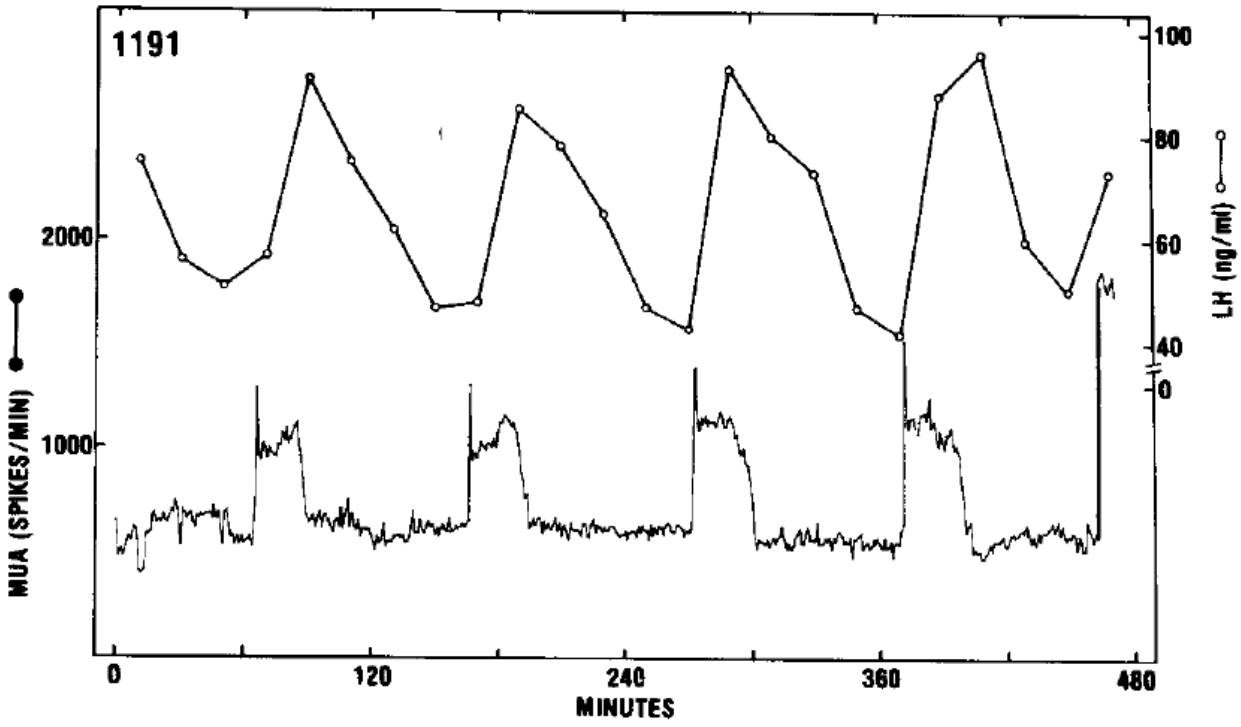

Figure 1.9 Correlation between peaks of multi-unit electrical activity (MUA) and LH pulses in an ovariectomized monkey treated with thiopental anesthesia. Top plot indicates LH levels in peripheral circulation (open circles), bottom plot indicates MUA in the arcuate nucleus. (97)

Within the arcuate nucleus, kisspeptin neurons may be key contributors to the GnRH pulse generator. Not only is *Kiss1* mRNA expression steroid-sensitive, as outlined above, but pulsatile release of kisspeptin in female rhesus monkeys correlates with most GnRH pulses (58). Similar to GnRH, it appears the pattern of kisspeptin release is critical. Pulsatile treatment with kisspeptin maintains LH pulses in male rats and monkeys (109,110), whereas continuous kisspeptin decreases LH secretion in castrate male monkeys and testicular degeneration in male rats (111-113). It should be noted that some low-concentration GnRH release and low-amplitude LH pulses are possible with mutations in kisspeptin or its receptor, resulting in sub-optimal but present reproductive maturation (46,114). However, kisspeptin signaling is required for normal regulation of

GnRH neuron activity (measured by *cFos*)(89) and pulsatile LH release (59,115), supporting the importance of this peptide in healthy reproductive function.

1.5 KNDy neuropeptides and the KNDy neuron working hypothesis

In addition to their putative role in negative gonadal steroid feedback on GnRH pulse generation, arcuate kisspeptin neurons are unique for their co-localization of neurokinin B (NKB) and dynorphin (102,116-121), two other neuropeptides that have been implicated in reproductive function. Mutations in a high-affinity receptor for NKB (neurokinin-3 receptor, NK3R), or NKB itself result in hypogonadotropic hypogonadism in humans (122,123), thus NKB may be stimulatory. In contrast, LH release is increased by antagonists for the κ-opioid receptor (KOR), which binds dynorphin (45,46,122,124), suggesting that dynorphin is inhibitory. Similar to their effects on expression of kisspeptin, gonadal steroids also inhibit expression of mRNA for NKB and dynorphin (93,120,125,126), and both NKB- and dynorphin-expressing arcuate neurons have been shown to express gonadal steroid receptors in single and dual label studies (88,119,127,128). Projections singly-labeled for neurokinin B or dynorphin have been located near GnRH somata and/or processes (118,129,130), and dual-labeling studies in sheep and rats have also shown co-localization of KNDy neuropeptides in projections apposed to GnRH neurons (121,131), supporting the arcuate kisspeptin population as the origin of these projections.

Arcuate kisspeptin neurons may act on each other, as well. Not only do these neurons express NK3R and KOR (120,130,132), but arcuate cells with NKB and/or dynorphin appear to have projections in apposition to or directly synapsing on each other (119,128,130). Interestingly, kisspeptin receptor expression has not been found in the arcuate nucleus (63), suggesting that while NKB and dynorphin may act on these putatively interconnected neurons, kisspeptin may not.

To reflect the key roles these peptides appear to play in reproduction, the Goodman lab has proposed that the arcuate kisspeptin-, NKB-, dynorphin-expressing neurons be renamed “KNDy neurons” (133). This alternative name can be used interchangeably with “arcuate kisspeptin neurons” and will be used from this point on to describe this neuronal population. KNDy neurons are hypothesized to contribute to GnRH pulse generation in the following model (Figure 1.10) (117,120,131,134): They are proposed to act in a network that projects both to GnRH neurons and to other KNDy neurons. In this network, NKB acts on NK3R on KNDy neurons to stimulate and synchronize these cells and cause kisspeptin release, which increases GnRH neuron activity. Dynorphin then acts on KOR to inhibit KNDy neuron activity, which would decrease release of NKB and kisspeptin, and thus GnRH. Communication among KNDy neurons may also be mediated by fast synaptic transmission, as the vesicular glutamate transporter vGluT2 and glutamic acid decarboxylase GAD-67 are coexpressed with KNDy neuropeptides in arcuate neuron cell bodies and terminals (131,135).

To better understand KNDy neurons in the context of central control of reproduction, it is critical to understand what we know about NKB and dynorphin.

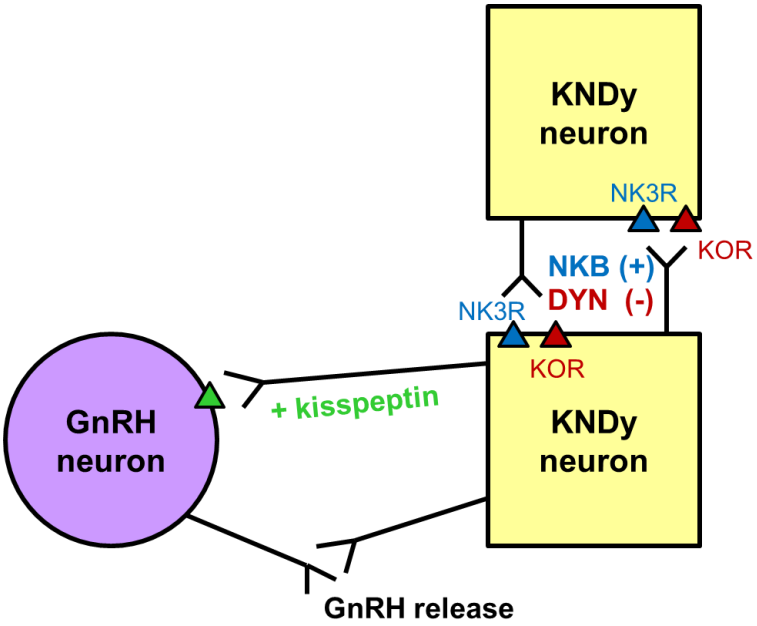

Figure 1.10 KNDy working model. KNDy neurons are located in the arcuate nucleus and are hypothesized to mediate negative gonadal steroid feedback onto GnRH neurons through modulations of their neuropeptidergic output (more details in text). +, stimulatory; -, inhibitory. NKB, neurokinin B; DYN, dynorphin; KNDy neuron, neuron that colocalizes kisspeptin, NKB, and DYN; GnRH, gonadotropin releasing hormone; NK3R, neurokinin-3 receptor; KOR, κ-opioid receptor.

Neurokinin B

In many mammalian species, NKB appears to be stimulatory to GnRH release. For example, NK3R agonists increase both LH secretion and MBH electrical activity in ewes and goats, respectively (102,136). The NK3R-enhanced LH secretion is also observed in castrate male monkeys, and it is blunted by kisspeptin receptor desensitization, suggesting that action of NKB is upstream of kisspeptin in this model (137). In rodents, the role of NKB is less clear because stimulation of NK3R enhances, inhibits, or has no effect on LH secretion in rats and mice (120,125,138,139). A possible explanation for the disparity between the

results of the rodent studies compared with those from other mammals is that, in testing responses to NK3R activation, the rodent work only measures pituitary output. Despite the importance of LH, measuring it does not necessarily reflect central actions of NK3R. In this regard, NKB may act at various loci upstream of the pituitary. This is hinted by an increase in expression of *cFos* (an immediate early gene associated with increased firing activity) in arcuate kisspeptin neurons from female rats when treated with the NK3R agonist senktide (125). In addition to its action on KNDy neurons, NKB may also influence GnRH neurons. Despite observation of little to no NK3R on GnRH neuron cell bodies in sheep and rats (121,132,140), NK3R immunoreactivity has been found in apposition to GnRH nerve fibers in the rat median eminence (121,140), suggesting that NKB may still ultimately influence peptide release from GnRH neurons.

Dynorphin

Endogenous opioid peptides (EOPs) appear to mediate steroid negative feedback on GnRH secretion. EOPs are linked to progesterone negative feedback on LH release in human studies (141,142), and EOP receptor antagonists increase LH pulse frequency in intact luteal phase monkeys (143) and ewes (144) and pregnant rats (145), as well as ovariectomized and ovariectomized, steroid-treated ewes (144,146,147). These data support EOP inhibition of LH pulses. At the hypothalamic level, EOP receptor antagonism also increases the frequency of peaks of electrical activity in the MBH of ovariectomized rats (148) and increases both the amplitude and duration of each

GnRH pulse in ovariectomized ewes (149), supporting EOP action at the hypothalamus that can also act independently from gonadal steroids. Furthermore, EOPs appear to decrease the frequency of hypothalamic electrical volleys. Treatment with morphine, which can act via multiple opioid receptors (δ, μ, and κ) decreases the frequency of MBH MUA associated with LH pulses in monkeys (150), while treatment with the opioid receptor antagonist naloxone increases MUA frequency in rats (99,148), further corroborating a potential inhibitory action of EOPs on the putative GnRH pulse generator.

In most mammalian species, the specific EOP involved in steroid negative feedback seems to be dynorphin (124,151), and its relevant action appears to be localized to the arcuate nucleus (151). Preprodynorphin mRNA is decreased in the arcuate nucleus of ewes and the infundibulum of women by ovariectomy and menopause, respectively (151,152). Intracerebroventricular injection of dynorphin in goats inhibits the frequencies of both LH pulses and MUA peaks, whereas a KOR antagonist increases these frequencies (102). When tested more locally, KOR-specific antagonist treatment targeted to the MBH of ewes increases LH secretion during the luteal phase, when progesterone feedback is prevalent (124). This outcome is not replicable with antagonists specific to other EOP receptors (124), supporting a dynorphin-specific effect. At the cellular level, expression of KOR message is found on almost all KNDy but no GnRH neurons (120,153). These data support arcuate dynorphin as a major mediator of gonadal steroid negative feedback, at least in primates, goats, and sheep.

The role of EOPs in rodents is less clear. Pharmacologically, KOR-specific agonism in mice inhibits an ovariectomy-induced LH rise (120), and KOR antagonism in rats stimulates LH release (154), supporting an inhibitory role of dynorphin. However, knockout of KOR decreases LH secretion (120), and ovarian steroids inhibit preprodynorphin mRNA expression in ovariectomized mice (120) in an apparent contradiction of results. Much like the rodent NK3R studies, though, these KOR-specific studies do not directly test a functional response at the level of the brain, leaving an incomplete picture of dynorphin's hypothalamic actions in rodents. It should be noted that one study that did take direct measurements from hypothalamic neurons supports another opioid receptor as influencing GnRH output. GnRH neurons from guinea pigs are hyperpolarized by agonism of the μ-opioid receptor (155), suggesting that multiple EOP receptors may be involved with regulation of GnRH in rodents. Together, the data discussed here provide mixed evidence for stimulation by NKB on GnRH pulse generation and inhibition by dynorphin. Studies of the actions of these peptides at the level of the KNDy neuron may help to clarify their roles in pulse generation and gonadal steroid feedback.

1.6 Dissertation project preview

Before the studies presented in this dissertation, most of the data on KNDy neurons as the putative pulse generator had tested mechanisms either up- or downstream of KNDy neurons. There had been no direct tests of KNDy

neuron function, particularly regarding modulation of their electrophysiological activity. Chapter 2 investigates how activation of NK3R and KOR affects KNDy neuron action potential firing and whether the responses to activation of these receptors are modulated by gonadal secretions in male mice. Chapter 3 expands these observations to test which specific gonadal steroids and gonadal steroid receptors are active in the responses observed in Chapter 2. Together these studies provide insight into potential mechanisms for negative gonadal steroid feedback on a potential contributor to the GnRH pulse generator.

CHAPTER 2

Regulation of arcuate neurons coexpressing kisspeptin, neurokinin B, and dynorphin by modulators of neurokinin 3 and κ-opioid receptors in adult male mice

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2.1 Abstract

Pulsatile GnRH release is essential to fertility and is modulated by gonadal steroids, most likely via steroid-sensitive afferents. Arcuate neurons coexpressing kisspeptin, neurokinin B (NKB), and dynorphin (KNDy neurons) are steroid-sensitive and have been postulated to both generate GnRH pulses and mediate steroid feedback on pulse frequency. KNDy neurons are proposed to interact with one another via NKB and dynorphin to activate and inhibit the KNDy network, respectively, and thus alter kisspeptin output to GnRH neurons. To test the roles of NKB and dynorphin on KNDy neurons and the steroid sensitivity of these actions, targeted extracellular recordings were made of *Tac2*(NKB)-GFP-identified neurons from castrate and intact male mice. Single-cell PCR confirmed

most of these cells had a KNDy phenotype. The neurokinin 3 receptor (NK3R) agonist senktide increased action potential firing activity of KNDy neurons. Dynorphin reduced spontaneous KNDy neuron activity, but antagonism of κ-opioid receptors (KOR) failed to induce firing activity in quiescent KNDy neurons. Senktide-induced activation was greater in KNDy neurons from castrate mice, whereas dynorphin-induced suppression was greater in KNDy neurons from intact mice. Interactions of dynorphin with senktide-induced activity were more complex; dynorphin treatment after senktide had no consistent inhibitory effect, whereas pretreatment with dynorphin only decreased senktide-induced activity in KNDy neurons from intact but not castrate mice. These data suggest dynorphin-mediated inhibition of senktide-induced activity requires gonadal steroid feedback. Together, these observations support the hypotheses that activation of NK3R and KOR, respectively, excites and inhibits KNDy neurons and that gonadal steroids modulate these effects.

2.2 Introduction

GnRH neurons form the final common pathway for the central regulation of fertility. GnRH is secreted in a pulsatile pattern (4,156-159) that is essential for pituitary synthesis and release of LH and FSH (29,33,159-161), which activate steroidogenesis and gametogenesis. Although gonadal steroids feed back to regulate GnRH pulse pattern (7), detection of steroid receptors other than

estrogen receptor β is rare in native GnRH neurons (9,10,162,163), suggesting feedback is largely mediated via steroid-sensitive afferents (11,164).

One steroid-sensitive afferent population is the arcuate neurons coexpressing kisspeptin, neurokinin B (NKB, aka *Tac2*), and dynorphin (KNDy neurons (18,19,69,88,119,127,128,131,134)). Several studies suggest these cells are important in reproductive neuroendocrine regulation; mutations in the kisspeptin receptor KISS1R, NKB, or the NKB receptor NK3R cause hypogonadotropic hypogonadism in humans (45,46,122), and knock out of the genes for kisspeptin (*Kiss1*) or KISS1R (*Kiss1r*) reduce or eliminate reproduction in mice (47,48). Furthermore, κ-opioid receptor (KOR, the dynorphin receptor) antagonists increase LH release (124).

KNDy neurons have been hypothesized to regulate the pattern of GnRH release as a self-activating and self-inhibiting network (120,131). In this model, kisspeptin provides excitatory KNDy network output to GnRH neurons, which express *Kiss1r* (52,53,55,60,62,78,165). NKB is postulated to stimulate KNDy neurons (122,132,140), generating kisspeptin release onto GnRH neurons to initiate a pulse of GnRH secretion (131,136). NKB-induced activation of the KNDy network also putatively activates dynorphin release, and subsequent action of dynorphin is proposed to inhibit NKB-stimulated KNDy activation, stop kisspeptin output to GnRH neurons, and thus terminate the GnRH pulse (120,124,131,149).

Functional studies of KNDy neurons are limited but have provided initial support for some elements of this model (124,166,167). Animal studies using the

NK3R agonist senktide, however, have produced variable results, with both increases and decreases in LH depending on steroid milieu and species (102,120,125,136,138,139,166,168-170). The present study examined how NKB and dynorphin interact to alter the activity of GFP-identified KNDy neurons in the arcuate nucleus from castrate and intact adult male mice directly at the central level.

2.3 Materials and methods

Animals

Tac2-enhanced green fluorescent protein (GFP) BAC transgenic mice [015495-UCD/ STOCK Tg (Tac2-EGFP)381Gsat] were obtained from Mouse Mutant Regional Resource Center (http://www.mmrrc.org/). GnRH-GFP mice (171) were propagated in our colony. Mice were maintained under a 14h light:10h dark photoperiod with 2916 chow (Harlan, Indianapolis, IN) and water available *ad libitum*. Male mice age 38-120d were either left intact or were castrated under isoflurane anesthesia 3-7d before study; bupivacaine was provided as an analgesic. All procedures were approved by the University Committee on the Use and Care of Animals at the University of Michigan.

Brain slice preparation

Reagents were purchased from Sigma Chemical Company (St. Louis, MO) unless noted. Solutions were bubbled with 95%O_2, 5%CO_2 throughout

experiments and for ≥15 min before use. Brain slices were prepared with modifications (172) as previously described (173). Brains were rapidly removed and placed in ice-cold high-sucrose saline solution containing (in mM) 250 sucrose, 3.5 KCl, 26 $NaHCO_3$, 10 D-glucose, 1.25 Na_2HPO_4, 1.2 $MgSO_4$, and 3.8 $MgCl_2$. Coronal (300µm) slices were cut with a Vibratome 3000 (Ted Pella, Inc., Redding, CA). Slices were incubated 30min at 30-32C in 50% high-sucrose saline and 50% artificial cerebrospinal fluid (ACSF) containing (in mM) 135 NaCl, 3.5 KCl, 26 $NaHCO_3$, 10 D-glucose, 1.25 Na_2HPO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$ (pH 7.4), then transferred to 100% ACSF solution at room temperature for 0.5-7h before recording.

Cell Harvest for single-cell PCR and cDNA synthesis

Patch pipettes (2-3MΩ) were filled with 5-8µl of a solution containing (in mM): 140 K gluconate, 5 KCl, 10 HEPES, 5 EGTA, 4.0 MgATP, 0.4 NaGTP, 1.0 $CaCl_2$, pH 7.3, 305 mOsm. The whole-cell configuration was obtained and cytoplasm aspirated into the pipette. Pipette contents were expelled into a microcentrifuge tube containing 2X reverse transcription buffer, and volume adjusted to 20µl with molecular grade water (Invitrogen, Carsbad, California). Similarly-prepared pipettes (n=5) were lowered into the slice but positive pressure was maintained and no cell contents were intentionally harvested; these false harvests were processed as above and used to estimate background contamination (174). The components for the reverse transcription reaction were (in mM): 20 Tris (pH 8.4), 50 KCl, 5 $MgCl_2$, 10 DTT, 10 dNTPs, plus 500ng oligo

dT (Invitrogen), 50ng random hexamers (Roche Life Sciences, Indianapolis, Indiana), 200U Superscript III (Invitrogen), and 40U RNase inhibitor (Roche). Mouse hypothalamic and liver RNA (10ng diluted in pipette solution) and pipette solution stock were also reverse transcribed as positive and negative controls, respectively. Additionally, a standard curve of mouse hypothalamic RNA (5, 0.5, 0.05, 0.005ng/µl final concentration) was reverse transcribed. cDNA synthesis was performed as recommended. cDNA was stored at -80C until PCR reactions.

Single-Cell PCR

Single-cell cDNA, controls and the standard curve were preamplified using TaqMan PreAmp Master Mix (Invitrogen). TaqMan PrimeTime qPCR assays for mRNAs of *Gapdh*, *Tac2*, *Kiss1*, *Pdyn*, *Oprk1*, and *Tac3r* were purchased from Integrative DNA Technologies (Coralville, IA, Table 2.1). All assay primers span an intron to minimize amplification of genomic DNA. PrimeTime qPCR assays were reconstituted to 20X (5µM probe, 10µM each primer) in Tris-EDTA. The components for the preamplification reaction were: 5µl cDNA, 0.05X final concentration of each primer-probe, 10µl 2X preamplification buffer and water to a final volume of 20µl. cDNA was preamplified for 18 cycles according to the manufacturer's recommended cycling conditions. The preamplified DNAs were then diluted 1:10 with Tris-EDTA; a second dilution to 1:50 was also created for more abundant transcripts (*Gapdh, Tac2*), both were stored at -20C until used for quantitative PCR.

Table 2.1 qPCR assays for single-cell PCR

gene	IDT Assay ID	ref seq #	exons	amplicon (bp)	amplicon location (bp)
Kiss1	Mm.PT.45.16269514	NM_178260	1-2	105	66-170
Pdyn	Mm.PT.45.9486062	NM_018863	1-2	100	89-188
Oprk1	Mm.PT.42.8829407	NM_001204371, v1 NM_011011, v2	2-3 2a-3	94 94	398-491 368-461
Tacr3	Mm.PT.51.8434948	NM_021382	4-5	114	1266-1379
Gapdh	Mm.PT.39.1	NM_008084	2-3	150	50-199

PrimeTime® qPCR Assays from Integrated DNA Technologies, Coralville, IA.
www.idtdna.com/pages/products/gene-expression/primetime-qpcr-assays-and-primers

Quantitative PCR was performed utilizing 2-5µl of diluted preamplified DNA per reaction, in duplicate, for 40-50 cycles (TaqMan Gene Expression Master Mix; Invitrogen). PCR reaction efficiencies were calculated from the slope of the standard curve. To confirm the linearity and parallelism of the preamplification step, 2µl of non-preamplified standard curve cDNA was also amplified in singlicate. On average, the threshold cycle for preamplified standards was 10 cycles earlier than cDNA, but PCR efficiencies were nearly identical, indicating cDNAs were amplified linearly and without bias. Single cells were considered positive for a transcript if their threshold was a minimum of 4 cycles earlier than the preamplification blank. Amplicon size was confirmed by agarose gel electrophoresis.

Electrophysiological recordings

Targeted single-unit extracellular recordings were used, as this configuration has minimal impact on the cell's intrinsic properties, including response to fast synaptic transmission (175,176). Recording pipettes (1.5-3MΩ) were pulled from borosilicate glass (Schott #8250, World Precision Instruments, Sarasota, FL) with a P-97 puller (Sutter Instrument, Novato, CA). Pipettes were

filled with HEPES-buffered solution containing (in mM): 150 NaCl, 10 HEPES, 10 glucose, 2.5 $CaCl_2$, 1.3 $MgCl_2$, 3.5 KCl, and low-resistance (23±3MΩ) seals were formed between the pipette and neuron. Recordings were made in voltage-clamp with a 0mV pipette holding potential and signals filtered at 10kHz using an EPC8 amplifier and PatchMaster software (v2x42, HEKA Instruments Inc., Bellmore, NY).

Experimental designs

Slices were transferred to a recording chamber with constant perfusion of ACSF at 28-33C. All treatments were diluted in ACSF and administered by bath; vehicles had no effect on firing rate. After a ≥5min stabilization period, neuronal activity was recorded under control and treatment conditions as detailed below. At the end of each experiment, inactive cells were treated with high-potassium ACSF (20mM K^+); cells that exhibited action currents in response were verified to be alive and all data, including quiescence, were used; for cells not responding to K^+, data analysis was truncated at the last action current.

Experiment 1

To study effects of NKB on KNDy neurons and if steroid milieu modifies effects, the NK3R agonist senktide (Phoenix Pharmaceuticals, Inc., Burlingame, CA, in ≤0.1% dimethyl sulfoxide) was bath-applied to brain slices from castrate (1, 10, or 100nM) or intact adult male mice (10 or 100nM) for 5-7min. Senktide was used because it is more specific for NK3R than NKB (177); preliminary

studies indicated senktide has similar effects to NKB in KNDy neurons (n=3 cells from each castrate and intact males, not shown). Effects of 10nM senktide on GnRH neurons from castrate males were also determined and compared to untreated GnRH neurons.

Experiment 2

To examine if activation of NK3R in KNDy neurons from castrate males requires fast synaptic transmission, ionotropic GABA and glutamate receptors were blocked. Cells were first treated with 10nM senktide as above to verify response. In preliminary trials, repeated treatment of the same cell with 10nM senktide generated comparable responses under control conditions in KNDy neurons from both castrate and intact mice (n=3 each, not shown). Following washout (5-30min to allow for return towards basal firing rate), a cocktail of blockers of $GABA_A$ (100μM picrotoxin in 0.2% ethanol), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (40μM CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione in 0.2% dimethyl sulfoxide), and N-methyl-D-aspartic acid (20μM APV, D(−)2-amino-5-phosphonovaleric acid) receptors was applied for 5min before a second 5-7min treatment with 10nM senktide in the blocker cocktail.

Experiment 3

To study effects of dynorphin on KNDy neurons, 1μM dynorphin A (dynorphin, Tocris Bioscience, Ellisville, MO) was applied for 5min to spontaneously-active KNDy neurons from castrate and intact males.

Spontaneously-active neurons were used because we hypothesized dynorphin would inhibit KNDy neurons. Since extracellular recordings monitor firing activity, inhibition cannot be observed with this method in quiescent cells.

Experiment 4

To test the hypothesis that dynorphin reduces senktide-induced KNDy neuron activation in castrate males, senktide was applied for 5-7min, followed by senktide alone, senktide and dynorphin (1μM), or senktide and the KOR agonist U69593 (1μM, in 0.01% ethanol) for an additional 5-7min. To test if endogenous KOR activation affected the firing pattern in the presence of prolonged senktide, cells were treated for 10min with senktide and a KOR antagonist nor-binaltorphimine (norBNI, 10μM, Tocris Bioscience). To determine if pretreatment with dynorphin blocked the ability of senktide to activate KNDy neurons, senktide was applied to confirm responsiveness (as in Experiment 2), and then after the wash period 1μM dynorphin was applied for 5min, followed by 5min of dynorphin plus 10nM senktide.

Experiment 5

To test the hypothesis that quiescent KNDy neurons are under suppression by endogenous dynorphin, 10μM norBNI was applied to quiescent KNDy neurons for 5-6min. The ability of 10μM norBNI to block 1μM dynorphin-induced inhibition of firing was tested as a positive control.

Analyses

Targeted extracellular recordings detect action currents, which are the currents underlying action potentials. Thus their frequency reflects action potential firing rate. Action currents were identified using custom software written in IgorPro (WaveMetrics, Inc., Lake Oswego, OR). Control action current frequency was averaged for the last 2min before treatment. The first 3min of treatment were not included in the analysis to allow time for solution exchange and drug penetration of the slice; the action current firing frequency was averaged during min 4 and 5 after initiating treatment. Cells with basal firing frequency ≤0.1Hz were considered quiescent. Cells that remained quiescent during treatment were considered non-responsive if they subsequently generated action currents in response to elevated K^+. Spontaneously-active cells with a change in firing frequency of >20% were considered responsive. The percentage of responsive cells is reported, but for statistical rigor both responsive and non-responsive cells were included in statistical analyses. No more than three cells from a given animal were included in the same experiment, and n indicates number of cells.

Data are reported as mean±SEM. Non-parametric or parametric two-tailed comparisons were utilized as appropriate for data distribution. Responses to single treatments were analyzed by Wilcoxon matched pairs test or paired t-test. Data from cells exposed to multiple treatments were analyzed by one-way Friedman test, followed by a Dunn's multiple-comparison test. Comparisons among groups were analyzed by Friedman two-way repeated-measures analysis

of variance, Mann-Whitney, or by unpaired t-test. All tests were two-tailed, significance was set at $p<0.05$.

2.4 Results

GFP expression driven by the Tac2 promoter identifies KNDy neurons in the arcuate nucleus

Single-cell PCR was performed on *Tac2*-GFP-identified cells from the arcuate nucleus of castrate (23 cells) and intact (18 cells) males (Table 2.2). Single-cell cDNA was amplified for *Gapdh* to determine RNA quality; of 41 cells, 3 did not amplify for *Gapdh* and were eliminated from further analysis. No transcripts of interest were amplified from false harvests (n=5). *Tac2* mRNA was detected in 87% of all GFP-identified cells, indicating that GFP expression is highly correlated with endogenous *Tac2* expression. The half-life of eGFP protein is ~26h (178). It is thus likely that GFP-identified cells in intact males not expressing *Tac2* mRNA when harvested had expressed this mRNA within the previous few days. All cells from castrate and 71% of cells from intact mice expressed *Tac2*, indicating steroid negative feedback suppresses *Tac2* expression, consistent with earlier reports (120,166,179,180). Consistent with this, 16/17 *Tac2*-GFP-identified cells from intact mice expressed either kisspeptin (*Kiss1*) or prodynorphin (*Pdyn*) mRNA, indicating a KNDy phenotype (120,166). In contrast to a previous report (166), a substantial population of *Tac2*-GFP neurons that did not express *Kiss1* was not observed (18% intact mice, 0%

castrate mice). This discrepancy may be due to relative sensitivity of methods utilized, or the mode of cell identification. The percentage of *Pdyn* mRNA positive cells was 90% in castrate and 76% in intact mice. With regard to G-protein coupled receptors, 19% of cells from castrate and 41% of cells from intact mice expressed transcript for the dynorphin receptor KOR (*Oprk1*). Most (86%) cells from castrate mice expressed mRNA for the NKB receptor NK3R (*Tac3r*) vs. 35% of cells from intact mice.

Table 2.2 *Single-cell PCR results*

transcript	intact (n=17) **# cells expressing transcript**	castrate (n=21) **# cells expressing transcript**
Tac2	12 (71%)	21 (100%)
Kiss1	14 (82%)	21 (100%)
Pdyn	13 (76%)	19 (90%)
Tac2 + Kiss1	11 (65%)	21 (100%)
Tac2 + Pdyn	10 (59%)	19 (90%)
Pdyn + Kiss1	11 (65%)	19 (90%)
Tac2+Kiss1 +Pdyn	10 (59%)	19 (90%)
Kiss1 OR Pdyn	16 (94%)	21 (100%)
Oprk1	7 (41%)	4 (19%)
Tac3r	6 (35%)	18 (86%)

Number of cells expressing transcript (percent GFP-identified cells)

Spontaneous activity of KNDy neurons from intact and castrate males

Eighty percent of KNDy neurons from castrate mice (78/98) were quiescent during the control period (defined as a firing frequency of ≤ 0.1 Hz),

whereas 57% (25/44) of KNDy neurons from intact mice were quiescent. The firing rate of spontaneously-active KNDy neurons was not different between castrate (2.0±0.3Hz, n=20 cells) and intact mice (1.9±0.5Hz, n=19 cells).

The NK3R agonist senktide increases activity to a greater extent in KNDy neurons from castrate than those from intact males

To study the effects of activation of NK3R on KNDy neurons, the NK3R agonist senktide was bath-applied to brain slices from castrate adult males during extracellular recordings. Figure 2.1A shows a representative response to 10nM senktide in cells from castrate males. Senktide increased the frequency of action currents in a dose-dependent manner (1nM, $p=0.0625$, n=8; 10nM, $p<0.05$, n=11; 100 nM, $p<0.05$, n=6, Figure 2.1C). The proportion of cells responding with >20% change in firing rate also increased with dose (3 of 8 at 1nM, 10 of 11 at 10nM, and 6 of 6 at 100nM).

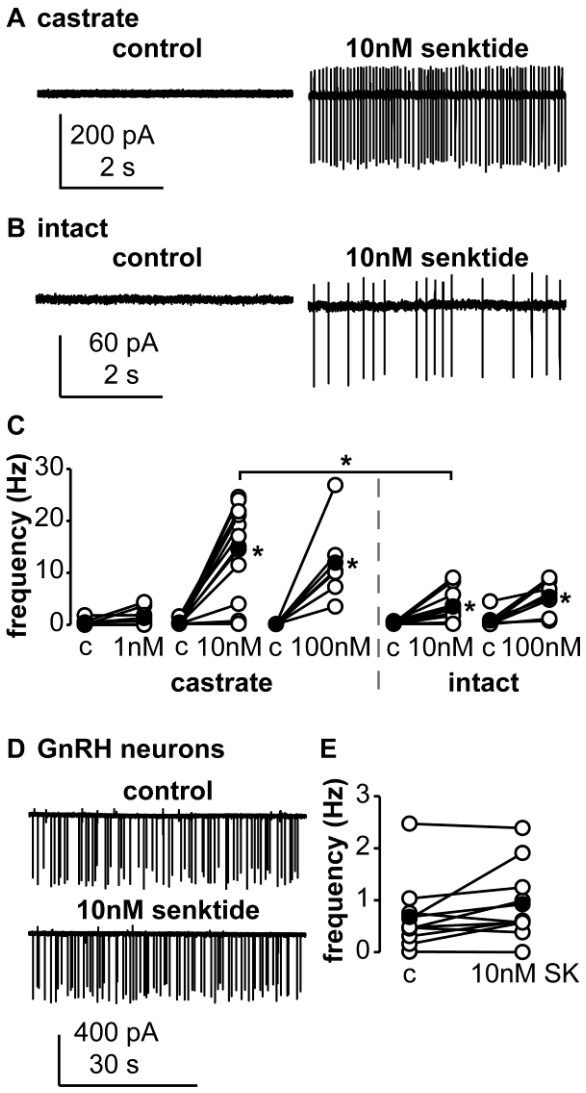

Figure 2.1 *The NK3R agonist senktide increases the activity of KNDy neurons and does not change firing activity of GnRH neurons.* A & B. Representative extracellular recordings of a KNDy neuron from a castrate (A) or an intact (B, trace smoothed to reduce baseline drift) mouse during control (left) and 10nM senktide (right) conditions; variable current amplitudes reflect small changes in pipette position during the recording and do not provide information about changes in cell function. C. Action current frequency of KNDy neurons during control (c) and senktide treatment at doses indicated in castrate (left) and intact (right) mice. D. Representative extracellular recordings of a GnRH neuron from a castrate mouse during control (top) and 10nM senktide (bottom) conditions. E. Action current frequency of GnRH neurons during control (c) and 10nM senktide (SK) treatment. Open circles show data from individual cells, filled circles show means: lines connect data from the same cell. *p<0.05.

To test if gonadal steroids affect the response to senktide in KNDy neurons, 10 or 100nM senktide was bath-applied to brain slices from intact male mice; Figure 2.1B shows a representative response to 10nM senktide. Firing frequency increased in all KNDy neurons from intact mice in response to both 10nM ($p<0.05$, $n=11$, Figure 2.1C) and 100nM senktide ($p<0.05$, $n=7$, Figure 2.1C). Senktide (10nM) caused a greater increase in KNDy neuron activity in cells from castrate mice versus those from intact mice ($p<0.05$, Figure 2.1C),

whereas the response to 100nM senktide was similar between cells from castrate and intact mice (Figure 2.1C). These data indicate that KNDy neurons from both castrate and intact mice are stimulated by activation of the NK3R, but that neurons from castrate animals are more sensitive. For subsequent senktide experiments, 10nM was used because it was the lowest dose tested that consistently elicited a response, and this response was different between castrate and intact mice.

Senktide does not alter firing of GnRH neurons

To test if senktide alters GnRH neuron activity, senktide was bath-applied to coronal preoptic area brain slices from castrate GnRH-GFP mice. Senktide (10nM) had no consistent effect on firing frequency of GnRH neurons (n=10, Figure 2.1D, E), with senktide treatment either increasing (n=5), decreasing (n=2), or eliciting no change (n=3) in firing frequency. Additionally, we compared senktide-treated cells with untreated GnRH neurons recorded for the same duration. Changes in firing frequency pattern in the absence of treatment (n=5, not shown) were similar to those during senktide treatment. These data support the hypothesis that activation of NK3R within a coronal slice through the preoptic area does not consistently alter the activity of GnRH neurons at the level of the soma.

The senktide-induced response in KNDy neurons is independent of fast synaptic transmission

The activation of KNDy neurons by senktide could be direct and/or via NK3R-expressing afferents that remain in the brain slice. To begin to differentiate between these possibilities, we asked whether the senktide-induced firing activity in KNDy neurons required and/or was modulated by fast synaptic transmission. Cells were first verified to respond to senktide (10nM), washed with ACSF, and then treated a second time with senktide in the presence of blockers of ionotropic GABA and glutamate receptors (Figure 2.2). Blocking these receptors after the wash period did not alter firing rate of KNDy neurons (0.6±0.4Hz wash vs. 0.9±0.6Hz blocked, n=8), nor did it alter senktide-induced activity (8.5±2.4Hz unblocked vs. 13±3.0Hz blocked, n=8, Figure 2.2). These data suggest activation of NK3R excites KNDy neurons by a mechanism that does not require fast synaptic transmission.

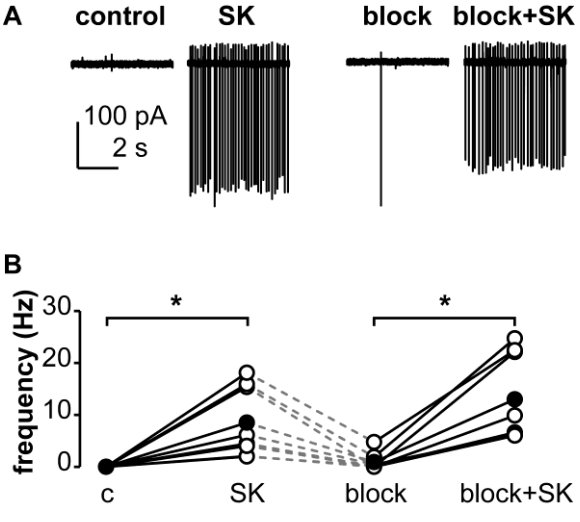

Figure 2.2 *The senktide-induced activation of KNDy neurons is independent of fast synaptic transmission.* A. Representative extracellular recordings from a cell during control and treatment periods. B. Action current frequency during various treatments. Open circles show data from individual cells, filled circles show means; lines connect data from the same cell, dashed lines indicate a variable wash period with ASCF after the first senktide treatment; c=control; SK=10nM senktide; block=blockers of ionotropic GABA and glutamate receptors (APV, CNQX, picrotoxin). *p<0.05.

Spontaneous activity of KNDy neurons is inhibited by dynorphin to a greater extent in cells from intact than castrate mice

To test the hypothesis that dynorphin decreases activity of KNDy neurons, we first examined spontaneously-active (>0.1Hz) KNDy neurons. Bath-applied dynorphin (1μM) decreased the firing frequency in 6/7 cells from castrate mice ($n=7$, $p<0.05$, Figure 2.3A) and in all cells examined from intact mice ($n=6$, $p<0.05$, Figure 2.3A). Dynorphin reduced the firing frequency of responsive cells from castrate mice by 50% and by 91% in cells from intact mice. The difference between the firing rate during dynorphin treatment vs. the control firing frequency was greater in KNDy neurons from intact than those from castrate mice ($p<0.05$, Figure 2.3B). The inhibitory effect of dynorphin was blocked by 10μM norBNI, a KOR antagonist, in all KNDy neurons from both castrate (4.5±1.1Hz norBNI vs. 5.5±1.2Hz norBNI+dynorphin, $n=5$) and intact (1.1±0.6 Hz norBNI vs. 1.5±0.9Hz norBNI+dynorphin, $n=3$) mice. These data indicate endogenous activity of KNDy neurons is inhibited by dynorphin via KOR, and this inhibition is more pronounced in the presence of gonadal steroids.

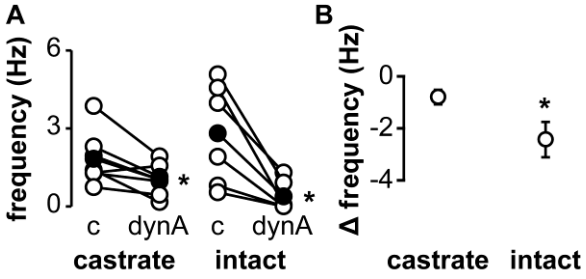

Figure 2.3 *Dynorphin attenuates spontaneous activity in KNDy neurons.* A. Action current frequency during periods of control and treatment with 1μM dynorphin in neurons from castrate (left) or intact (right) animals. Open circles show data from individual cells, filled circles show means; lines connect data from the same cell; c=control; dynA=1μM dynorphin. B. Difference between action current frequency during treatment and control periods in cells from castrate or intact mice. *$p<0.05$.

The KOR antagonist norBNI does not induce activity in quiescent KNDy neurons

Because the majority of KNDy neurons from castrate mice were quiescent during the control period, and because dynorphin inhibited spontaneous activity in most KNDy neurons, we hypothesized that activity in quiescent cells was inhibited by endogenous dynorphin. To test this, quiescent KNDy neurons were treated with the KOR antagonist norBNI (10μM). NorBNI did not induce activity (castrate: 0.0±0.0Hz control vs. 0.0±0.0Hz treated, n=5; intact: 0.0±0.0Hz control vs. 0.0±0.0Hz treated, n=5).

The pattern of firing activity in KNDy neurons induced by prolonged senktide treatment does not change with subsequent addition of KOR agonists in cells from castrate mice

We next tested the hypothesis that senktide-induced KNDy neuron activation is inhibited by dynorphin. KNDy neurons from castrate males were either treated with 10nM senktide for a full 10min (n=8) or were treated with senktide for 5min followed by senktide plus dynorphin (1μM, n=8) or senktide plus the KOR agonist U69593 (1μM, n=9) for an additional 5min. Neither dynorphin nor U69593 reduced the senktide-induced activation at the end of the second 5-min treatment compared with activity at the end of the 10min senktide-only treatment (Figure 2.4).

Examination of individual cells treated with dynorphin during the last 5min of the 10min senktide treatment reveals that all cells exhibited >20% decrease in firing frequency after addition of dynorphin compared to senktide treatment alone

(Figure 2.4). In contrast, only a subset of cells (4/8) treated with senktide alone for 10min showed >20% decrease in firing frequency during minutes similar time periods of recording (Figure 2.4). To test if the decrease in firing frequency in this subset of cells is due to senktide-induced dynorphin release, norBNI (10μM) was included during a 10min senktide treatment (Figure 2.4). Similar to the observations with senktide alone, norBNI failed to maintain firing rate throughout the 10min senktide treatment, with 5/9 cells exhibiting >20% decrease in firing frequency.

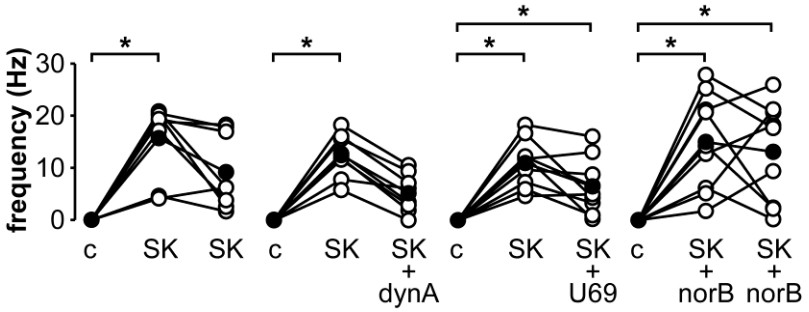

Figure 2.4 *Activation of KOR does not reduce ongoing senktide-induced activity in KNDy neurons from castrate mice.* Action current frequency during various treatments, which are detailed in text. Open circles show data from individual cells, filled circles show means; lines connect data from the same cell. c=control; SK=10nM senktide, an NK3R agonist; dynA=1μM dynorphin; U69=1μM U69593, a KOR agonist; norB=10μM norBNI, a KOR antagonist. *p<0.05.

Pretreatment with dynorphin blocks senktide-induced activation in KNDy neurons from intact but not castrate mice

To test if treatment with dynorphin before, rather than during, senktide application is able to block senktide-induced activity, cells from both castrate and intact mice were first verified to respond to senktide (10nM), washed with ACSF, and then treated with dynorphin (1μM) followed by a second treatment with senktide in the presence of dynorphin (Figure 2.5). Senktide treatment increased firing frequency of KNDy neurons from castrate mice to the same extent

regardless of whether or not dynorphin was present (0.0±0.0Hz control vs. 18.1±3.0Hz senktide, $p<0.05$; 2.1±1.2Hz dynorphin vs. 13.4±2.8Hz senktide+dynorphin, $p<0.05$, n=7, Figure 2.5). In contrast, pretreatment with dynorphin decreased the senktide-induced firing frequency in KNDy neurons from intact mice (0.0±0.0Hz control vs. 7.2±2.9Hz senktide, $p<0.05$; 0.0±0.0Hz dynorphin vs. 1.2±0.8Hz senktide+dynorphin, n=6, Figure 2.5). Spontaneous activity was suppressed in KNDy neurons (n=2 castrate, n=2 intact, not shown) throughout a 10-min treatment with dynorphin alone, confirming cells remained responsive to dynorphin throughout the duration tested. These data provide further support for enhancement of dynorphin-mediated inhibition by gonadal steroids.

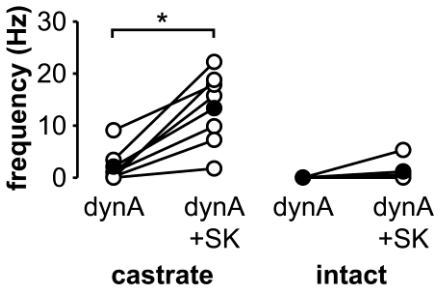

Figure 2.5 *Pretreatment with dynorphin A decreases senktide-induced activity in KNDy neurons from intact but not castrate mice.* Action current frequency in KNDy neurons from castrate (left) or intact (right) animals during treatments with 1µM dynorphin (dynA) and dynorphin plus 10nM senktide (SK); treatments shown follow a previous treatment to confirm responsiveness to senktide (not shown). Open circles show data from individual cells, filled circles show means; lines connect data from the same cell. *$p<0.05$.

2.5 Discussion

Targeted extracellular recordings revealed that the NK3R agonist senktide activates KNDy neurons by a mechanism that is independent of fast synaptic transmission and is attenuated in cells from intact compared to castrate males. In contrast, dynorphin inhibited spontaneous and senktide-induced activity of KNDy neurons to a greater extent in cells from intact than castrate mice.

Based on genetics studies in infertile patients (122) and the coexpression of NKB and NK3R in arcuate neurons (132,140), NKB has joined kisspeptin as a focus of studies of afferent control of GnRH release. Unlike the effect of kisspeptin, which consistently activates GnRH neurons, studies have yielded conflicting results concerning the role and site of NKB action. Peaks of multi-unit activity (MUA) recorded near the arcuate nucleus in the medial basal hypothalamus are highly correlated with LH pulses, an indirect marker of GnRH release. Senktide increased the frequency of MUA peaks in multiple steroid milieux (102). In castrate and gonad-intact male nonhuman primates and in follicular-phase but not luteal-phase ewes, activation of NK3R caused robust single discharges of LH (136,168). These data support a stimulatory role of NKB in several steroid conditions.

Rodent studies have had less consistent outcomes. Intracerebroventricular (icv) treatment with senktide decreased serum LH in ovariectomized (OVX) mice (120) and rats (125,169) and in OVX rats replaced with estradiol in a model of negative feedback (139,169,170). Senktide (icv) also decreased the frequency of MUA peaks in OVX rats (169). In contrast, senktide (icv) increased serum LH in intact female rats and OVX female rats replaced with estradiol (AM vs. PM not indicated) (125). In intact males, senktide (icv) was reported to have either no effect (138) or to increase serum LH (166). The lack of consistent changes in LH in response to senktide even within a species, sex, or steroid model may be due to differences in response at many levels including the KNDy neuron, the GnRH neuron, and/or the pituitary.

To avoid the complexities of these intermediate systems influencing the response to activation of NK3R, we examined the response to senktide directly at KNDy or GnRH neurons using electrophysiology to record activity at the soma of these cells in acute brain slices from the male mouse. Males were chosen for study because they are not subject to the variability of steroid feedback that occurs during the ovarian cycle in intact females. Further, GnRH pulse generation is a feature of both sexes, and in males this is unencumbered by co-existence of a surge mode of release. While the steroids involved in feedback regulation of pulse frequency could differ between sexes, we postulate that many core mechanisms of pulse generation itself are shared between males and females. Senktide induced a dose-dependent increase in firing rate of KNDy neurons from castrate males, but had no effect on GnRH neurons. These data are consistent with a recent electrophysiological study of kisspeptin-GFP-identified neurons in slices from castrate and intact adult male mice (166). The present data further identify a role for gonadal steroids in modulating the response to senktide, as the activation induced in KNDy neurons from intact males was suppressed. This is contrary to the previous study, which found no evidence for steroid dependence of NK3R-mediated activation of KNDy neurons (166). This discrepancy may be attributable to recording configuration, as the previous study used whole-cell recordings, which alter the intracellular milieu unlike extracellular recordings.

One possible mechanism for the reduced response to senktide observed in cells from intact males in the present study may be altered expression of

NK3R in KNDy neurons. The transcript for NK3R (*Tac3r*) was expressed in the majority of KNDy neurons from castrate animals but in less than a third of those from intact animals. Further, a stimulatory effect of NK3R activation may be amplified by positive feedback from other KNDy neurons that synapse on the recorded cell (119,128,130).

In addition to possible network effects of NKB from other KNDy neurons, KNDy neurons receive other types of inputs, including those from non-KNDy cells that may be responsive to senktide. Some connections with such neurons could be maintained within the slice preparation, and thus the effects observed may be due to action of senktide that is not directly on the neuron targeted for recording. One mode of signaling from these putative upstream neurons to KNDy neurons is fast synaptic transmission mediated by GABA and glutamate. KNDy neurons receive spontaneous GABAergic and glutamatergic transmission (181,182), which may arise in part from KNDy neurons themselves because they express both the vesicular glutamate transporter 2 (vGLUT-2) (118,135) and glutamic acid decarboxylase (GAD), an enzyme involved in GABA synthesis (135). The present observations show that NK3R-mediated stimulation of KNDy neurons is independent of fast synaptic transmission mediated by ionotropic glutamatergic and GABAergic signaling.

Gonadal steroids may have an effect on spontaneous activity in KNDy neurons. Eighty percent of cells from castrate mice vs. 57% of those from intact mice were quiescent. The latter is similar to that observed in whole-cell recordings of arcuate kisspeptin neurons from intact males (182). Previous

extracellular recordings of these neurons from intact males, however, reported only 10% were silent (183). This contrast may in part be due to a different definition of quiescence. Specifically, the present study considered cells to be quiescent if spontaneous firing frequency was <0.1 Hz, whereas the previous extracellular study only considered cells to be silent if the cell did not fire a single action potential throughout the recording duration, which would reduce the percentage of silent cells. Regardless, all datasets appear to challenge the hypothesis that gonadal steroids inhibit endogenous activity in KNDy neurons. It is important to point out that the present experiments were designed to examine the effects of neuromodulators on firing activity; recordings were thus of short duration. Changes in long-term firing pattern relevant to the frequency of GnRH release may well reveal effects of steroid feedback. It is nonetheless interesting to speculate that the regulation of mRNA expression and the regulation of firing activity by steroids in KNDy neurons may be different.

A potential cause for the quiescence observed in most KNDy neurons is endogenous dynorphin within the slice. Antagonists of KOR, the high-affinity receptor for dynorphin, support an inhibitory effect of dynorphin on LH release in mice, rats, and sheep (120,124,154), and KOR antagonists increase multi-unit activity in the medial basal hypothalamus in goats (102). In the present study in acutely prepared brain slices, the KOR antagonist norBNI did not initiate activity in KNDy neurons from either intact or castrate mice, suggesting that under these conditions, endogenous dynorphin is not responsible for quiescence.

Unlike the lack of effect of a KOR antagonist on quiescent KNDy neurons, the KOR agonist dynorphin inhibited spontaneously-active KNDy neurons. Greater inhibition was observed in intact than castrate males, supporting the hypothesis that the inhibitory effect of dynorphin on KNDy neurons is greater in the presence of gonadal steroids. This may be due to higher responsiveness to dynorphin, as single-cell PCR revealed the percentage of KNDy neurons expressing KOR in intact mice was twice that in castrate mice in the present study. In contrast, *in situ* hybridization demonstrated no change in the percentage of cells expressing the transcript for KOR in castrate vs. testosterone-replaced males (166). This discrepancy may result from a difference in feedback in intact vs. testosterone-replaced mice or method of mRNA detection.

Dynorphin has been hypothesized to inhibit KNDy neurons after stimulation by NKB (120,131,149,169,170). Interestingly, in KNDy neurons from castrate males, neither pretreatment with dynorphin nor treatment with dynorphin subsequent to senktide treatment altered the average firing frequency pattern relative to that of senktide alone. In contrast to KNDy neurons from castrate mice, pretreatment with dynorphin attenuated senktide-induced activity in cells from intact males. These data further support stronger dynorphin-mediated inhibition in the presence of gonadal steroids. The inhibitory effect may be further enhanced by the reduced effectiveness of senktide in cells from intact mice. Because spontaneous activity in cells from both castrate and intact males was reduced by dynorphin, the lack of effect of dynorphin on senktide-induced activity

in cells from castrate animals raises the question of what initiates spontaneous activity in KNDy neurons, since dynorphin cannot counteract activation of NK3R. Other transmitters, such as glutamate or GABA, may play a role in this regard (118,135).

When senktide treatment was extended to ten minutes, some cells sustained or increased their firing frequency, whereas others decreased firing frequency. This raised the question of whether, in the latter subgroup, senktide stimulated release of endogenous dynorphin within the slice, thereby causing delayed inhibition of KNDy neuron activity during extended senktide treatment. In the presence of the KOR antagonist norBNI, however, a similar subpopulation of KNDy neurons decreased firing frequency after activation with senktide. This indicates activation of KOR by senktide-stimulated dynorphin release was not inhibiting the activity in this subpopulation. These observations suggest heterogeneity within the KNDy neuron population, in which some cells become desensitized to NK3R activation over time and others do not.

Disruption of the pulsatile pattern of GnRH release results in infertility in diseases including polycystic ovary syndrome and idiopathic hypogonadotropic hypogonadism (26,29,184); understanding mechanisms of control of GnRH release by steroid feedback is thus crucial to developing treatments. Our work begins to elucidate the role of KNDy neurons in this critical role. We provide evidence for regulation of KNDy neurons in males by activation of both NK3R and KOR in a gonadal steroid-dependent manner, with steroids reducing overall activity of KNDy neurons in response to these treatments. The

electrophysiological recordings presented here used an extracellular configuration, which does not interfere with the intracellular environment, but does not allow for exploration of intracellular mechanisms. Future work will test the mechanisms linking activation of KOR or NK3R to changes in activity.

2.6 Acknowledgements

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CHAPTER 3

Both estrogen and androgen modify the response to activation of neurokinin-3 and κ-opioid receptors in arcuate kisspeptin neurons from male mice

The work in this chapter was submitted for review to Endocrinology *in August 2015.*

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3.1 Abstract

Gonadal steroids regulate the pulsatile pattern of GnRH that is essential to reproduction, but GnRH neurons appear to lack most receptors necessary for this steroid feedback. The arcuate kisspeptin, or KNDy (kisspeptin, neurokinin B, dynorphin), neuron population may convey steroid feedback to GnRH neurons. KNDy neurons increase action potential firing upon activation of neurokinin B receptors (neurokinin-3 receptor, NK3R) and decrease firing upon activation of dynorphin receptors (κ-opioid receptor, KOR). In KNDy neurons from intact male mice, NK3R-mediated stimulation is attenuated and KOR-mediated inhibition enhanced compared to castrate mice, suggesting gonadal secretions are involved. Estradiol suppresses spontaneous GnRH neuron firing in male mice,

but the gonadal secretions that mediate effects on firing in KNDy neurons are unknown. To test the effects of gonadal steroids, targeted extracellular recordings were made from KNDy neurons in brain slices from castrate adult male mice that were treated *in vivo* with steroid receptor agonists or left untreated. Estradiol inhibited spontaneous firing in KNDy neurons; firing rate in DHT-treated mice was intermediate between castrate and estradiol-treated mice. Both estradiol and DHT, however, suppressed NK3R agonist-induced KNDy neuron firing and enhanced the inhibition of firing rate caused by KOR activation. An estrogen receptor α agonist but not an estrogen receptor β agonist mimicked the effects of estradiol on NK3R activation. These observations suggest modulation of responses to activation of NK3R and KOR as mechanisms for gonadal steroid negative feedback in KNDy neurons, supporting contribution of these neurons to steroid-sensitive elements of a GnRH pulse generator.

3.2 Introduction

GnRH neurons form the final common output from the central nervous system that integrates signals from the brain to control reproduction. These neurons release GnRH in a pulsatile pattern (4,156,158,159) to stimulate pituitary release of LH and FSH, which is essential for reproduction (29). Gonadal steroid hormones regulate GnRH pulse patterns (7), but only the β isoform of estrogen receptor (ERβ), which is not critical for fertility, has been consistently detected in

these cells (10,11,162,163). Steroid-sensitive afferent neurons thus likely convey feedback signals to GnRH neurons.

Several observations support a steroid-sensitive “GnRH pulse generator” in the hypothalamic arcuate nucleus (97,102,148,169,185). A likely contributor to this pulse generator is the arcuate KNDy neuron population, which is named for its colocalization of the neuropeptides kisspeptin, neurokinin B, and dynorphin (131). KNDy neurons project to GnRH neurons (131), and all three KNDy neuropeptides are important for reproductive function. Mutations in the genes encoding kisspeptin receptor, neurokinin B, or the high-affinity receptor for neurokinin B (neurokinin-3 receptor, NK3R) have all been found in human patients with hypogonadotropic hypogonadism (45,46,122). Consistent with these observations, kisspeptin and NK3R agonists increase GnRH neuron activity and/or GnRH/LH release (55,62,166,186,187). In contrast, antagonism of the high-affinity receptor for dynorphin (κ-opioid receptor, KOR) in mice increases LH release (124).

The neuropeptides made by KNDy neurons also regulate activity of these neurons. Activation of NK3R and KOR modifies frequency of action potential firing in KNDy neurons (188,189) and these modifications are sensitive to gonadal hormones (189). Specifically, NK3R-induced stimulation of action potentials is attenuated in KNDy neurons from intact vs. castrate males, whereas KOR-mediated inhibition of action potential firing is enhanced in KNDy neurons from intact compared to castrate males. Together, these data suggest potential mechanisms through which gonadal secretions have a negative feedback effect

on KNDy neurons and thus the GnRH pulse generator. However, the identity of the gonadal secretions involved remains unclear.

Estrogens and androgens are both candidate mediators of the feedback effects on NK3R and KOR activation in KNDy neurons. KNDy neurons express both androgen receptor (AR) and estrogen receptors (19) and may also be regulated by steroid-sensitive afferents (190). The brain expresses aromatase, which converts testosterone to estradiol (191,192). Both androgens and estrogens inhibit arcuate kisspeptin mRNA expression (18,19), but steroidal modulation of mRNA expression may not necessarily reflect changes in kisspeptin protein levels (193), let alone KNDy neuron action potential firing rate and/or downstream GnRH neuron function. In this regard, some studies have shown that estradiol is a more potent suppressor of GnRH/LH release in males (194-197). To better understand the role of gonadal steroids in the neuropeptide-mediated stimulation and inhibition of KNDy neurons, the present study tested the hypothesis that estradiol is the main steroid modifying action potential firing of individual KNDy neurons in male mice.

3.3 Materials and methods

Animals

Tac2-enhanced GFP BAC transgenic mice (015495-UCD/STOCK Tg [*Tac2*-EGFP]381Gsat) were obtained from the Mouse Mutant Regional Resource Center (http://www.mmrrc.org/) and propagated in our colony. Mice were

maintained under a 14-hour light, 10-hour dark photoperiod (lights on 3AM EST) with 2916 chow (Harlan) and water available ad libitum. Male mice aged 79 to 123 days were used either intact or following bilateral castration under isofluorane anesthesia (VetOne); Bupivacaine (0.25%, 8µl per site, Hospira) was provided as a postoperative analgesic. Silastic (Dow Corning) implants containing steroids (194) were placed subcutaneously in the scapular region at the time of surgery. Mice were used 4-7 days after castration. All procedures were approved by the University Committee on the Use and Care of Animals at the University of Michigan.

Experimental design

Reagents were purchased from Sigma Chemical Company unless otherwise noted. Intact and castrate (4-7 days post surgery) male mice were used. Treatments were administered beginning on the day of surgery. Mice were placed in one of six treatment groups: 1) castrate no further treatment, 2) castrate plus an implant containing 0.625µg estradiol suspended in sesame oil, 3) castrate plus two implants containing 400µg DHT each in sesame oil (800µg total), 4) castrate plus daily subcutaneous injections of 1mg/kg of the ERα agonist 4,4',4''-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol (PPT; Tocris Bioscience), 5) castrate plus daily subcutaneous injections of 1mg/kg of the R enantiomer of the ERβ agonist (R)-2,3-*bis*(4-Hydroxyphenyl)-propionitrile (DPN, Tocris Bioscience), or 6) castrate plus daily subcutaneous injections of 2mg/kg DPN. All injected treatments were suspended in a vehicle of 5%

dimethylsulfoxide (DMSO), 95% sesame oil, administered between 9:30AM and 12:00PM EST, and terminated the day before recording. All treatments were administered *in vivo* and were not present in recording solutions.

Seminal vesicle mass

To confirm endocrine status, the ratio of seminal vesicle to body mass was determined at the time of brain slice preparation. The ratios from castrate mice (2.3±0.2 mg/g, n=20 mice) were lower ($p<0.05$) than those from intact control males (7.0±0.7 mg/g, n=8 mice). Only the group with the DHT implants (8.0±0.4 mg/g, n=12 mice) had a similar ratio to that from the intact control mice, whereas the remaining groups were similar to the castrate mice (PPT, 3.0±0.5 mg/g, n=5 mice; 1mg DPN/kg, 2.8±0.3 mg/g, n=4 mice; 2mg DPN/kg, 4.0±0.4 mg/g, n=4 mice).

Determination of estrogen receptor agonist dose

Groups of castrate or intact adult male mice were treated daily with vehicle, PPT, or DPN for 4-6 days. PPT was administered at 1mg/kg (198). DPN was administered 1, 2, or 4mg/kg. Injections began the day of surgery, and continued until the day before tissue harvest. Agonist concentration was devised to give a 50-100µL injection per 25g mouse and volume adjusted by mass. At the time of euthanasia, mice were anesthetized under isoflurane and decapitated. Pituitaries were collected and RNA extracted via RNeasy (Qiagen). Approximately 250ng pituitary RNA was reverse transcribed (20µL/reaction) as

described (199) along with a standard curve of mouse pituitary RNA (5, 0.5, 0.05, 0.005ng/µL final concentration). PrimeTime qPCR assays for mRNAs of prolactin (*Prl*), and the housekeeping genes *Ppia*, and *Rps29* were purchased from Integrative DNA Technologies (Table 3.1). Quantitative PCR was performed using 10ng cDNA per reaction in duplicate. Relative expression of *Prl* was normalized to the average relative expression of *Ppia* and *Rps29* via the $\Delta\Delta$ Ct method. Amplicon size was confirmed by agarose gel electrophoresis. All assay primers span an intron to minimize amplification of genomic DNA. PCR reaction efficiencies were calculated from the slope of the standard curve.

Castration reduced pituitary *Prl* expression and PPT treatment restored expression to the same level as that observed in pituitaries from intact animals (Supplementary Figure 3.1). While all doses of DPN resulted in reduced ($p<0.05$) pituitary *Prl* expression compared to intact animals, the highest DPN dose tested (4mg/kg) caused higher ($p<0.05$) *Prl* expression compared with the castrate group (Supplementary Figure 3.1). Based on these findings, only 1 and 2mg/kg DPN were tested in electrophysiological experiments.

Table 3.1 qPCR Assays for PCR[a]

Gene	IDT Assay ID	Accession No.	Exons	Amplicon, bp	Amplicon Location, bp
Prl	Mm.PT.58.21865636	NM_011164	3-4	142	266-407
Ppia	Mm.PT.39a.2.gs	NM_008907	4-5	85	355-439
Rps29	custom	NM_009093	1-2	127	119-245
Ar	Mm.PT.47.17416675	NM_013476	4-5	125	2065-2189
Esr1	Mm.PT.47.16003033	NM_007956	6-7	100	1456-1555
Esr2	Mm.PT.47.17681375	NM_207707	4-5	115	1036-1150

[a]PrimeTime qPCR assays were from Integrated DNA Technologies, Coralville, Iowa (www.idtdna.com/pages/products/gene-expression/primetime-qpcr-assays-and-primers).

Cell harvest for cDNA synthesis and single-cell PCR for transcripts of gonadal steroid receptors

Cells used for single-cell PCR were harvested from intact or castrate adult male mice as described (189). Briefly, the whole-cell patch-clamp configuration was achieved and cytoplasm aspirated into the pipette. False harvests in which a pipette was lowered into the tissue but no aspiration of cell contents occurred were used to estimate background contamination (174). Additionally, a standard curve of mouse hypothalamic RNA (5, 0.5, 0.05, 0.005ng/µL final concentration) was reverse transcribed. Single-cell cDNA, controls, and the standard curve were preamplified using TaqMan PreAmp Master Mix (Invitrogen) as described (189). TaqMan PrimeTime qPCR assays for mRNAs of *Ar*, *Esr1*, and *Esr2* were purchased from Integrative DNA Technologies (Table 3.1). Quantitative PCR was performed utilizing 2-5µl of diluted preamplified DNA per reaction, in duplicate, for 40-50 cycles (TaqMan Gene Expression Master Mix; Invitrogen). Single cells were considered positive for a transcript if their threshold was a minimum of 4 cycles earlier than the preamplification blank.

Brain slice preparation

Solutions were bubbled with 95% O_2/5% CO_2 throughout experiments and for ≥15min before use. Brain slices were prepared with modifications (172) as described (173). Brains were rapidly removed and placed in ice-cold high-sucrose saline solution containing (in mM) 250 sucrose, 3.5 KCl, 26 $NaHCO_3$, 10 d-glucose, 1.25 Na_2HPO_4, 1.2 $MgSO_4$, and 3.8 $MgCl_2$. Coronal (300µm) slices

were cut with a VT1200 S (Leica Biosystems). Slices were incubated 30min at room temperature in 50% high-sucrose saline and 50% artificial cerebrospinal fluid (ACSF) containing (in mM) 135 NaCl, 3.5 KCl, 26 $NaHCO_3$, 10 d-glucose, 1.25 Na_2HPO_4, 1.2 $MgSO_4$, and 2.5 $CaCl_2$ (pH 7.4) and then transferred to 100% ACSF solution at room temperature for 1 to 6 hours before recording.

Electrophysiological recordings

Targeted single-unit extracellular recordings were used because this configuration has the least impact on the intrinsic properties of the recorded cell (175,176). Recording pipettes (2-4.5 MΩ) were pulled from borosilicate glass (Schott 8250; World Precision Instruments) with a P-97 puller (Sutter Instrument). Pipettes were filled with HEPES-buffered solution containing (in mM) 150 NaCl, 10 HEPES, 10 glucose, 2.5 $CaCl_2$, 1.3 $MgCl_2$, and 3.5 KCl, and low-resistance (<25 MΩ) seals were formed between the pipette and neuron. Recordings were made in voltage clamp with a 0mV pipette holding potential and 2mV/pA gain and signals were filtered at 5kHz using an EPC8 amplifier and PatchMaster software (version 2x42; HEKA Instruments, Inc).

Slices were transferred to a recording chamber with constant perfusion of ACSF at 29-33C and stabilized for ≥5min before recording from a cell. All acute treatments were diluted in ACSF and administered by bath. Vehicles were determined to have no effect on firing rate (189). After pipette seal formation and another ≥5-min stabilization period, neuron activity was recorded for a 5-min untreated control period, followed by 4-5min of treatment. At the end of each

experiment, inactive cells were treated with high-potassium ACSF (20mM K^+). Cells that exhibited action currents in response were verified to be alive, and all data, including quiescence, were used. For cells not responding to K^+, data analysis was truncated at the last action current.

To test the effect of the *in vivo* treatments on NK3R-mediated responses in quiescent KNDy neurons, the NK3R agonist senktide (10nM, in ≤0.1% DMSO, Phoenix Pharmaceuticals, Inc) was bath-applied for 4-5min to brain slices from each *in vivo* treatment group. A cell was considered responsive if it exhibited >0.1Hz frequency of action potential firing during senktide treatment.

To examine the effect of steroid hormones on KOR-mediated responses in KNDy neurons, dynorphin A (dynorphin; 1μM; Tocris Bioscience) was bath-applied for 5min to brain slices from mice treated *in vivo* with either estradiol or DHT. Spontaneously active neurons were used because we hypothesized dynorphin would inhibit KNDy neurons. Because extracellular recordings monitor firing activity, inhibition of quiescent cells cannot be observed with this method. Percent change of firing frequency was used to compare dynorphin responses among hormonal treatment groups because of the range of pre-treatment spontaneous activity with different hormonal conditions (Figure 3.1).

Analyses

Results were unaffected by time of day, time from brain slice preparation, mouse age, duration of in vivo treatments, and location of the cell within the arcuate nucleus. Targeted extracellular recordings detect action currents, which

are the currents underlying action potentials. Their frequency thus reflects action potential firing rate. Action currents were identified using custom software written in IgorPro (WaveMetrics, Inc). Control action current frequency was averaged for the last 2min before treatment. The first 3min of treatment were not included in the analysis to allow time for solution exchange and drug penetration of the slice. Treatment action current firing frequency was determined during the next 2min. Cells with basal firing frequency ≤0.1 Hz were considered quiescent. Cells that remained quiescent during treatment were considered nonresponsive if they subsequently generated action currents in response to elevated K^+. Spontaneously active cells with a change in firing frequency of >20% were considered responsive; quiescent cells that generated action currents at a frequency of >0.1Hz during treatment were also considered responsive. The percentage of responsive cells is reported, but for statistical rigor, both responsive and nonresponsive cells were included in statistical analyses. No more than two cells from a given animal and no fewer than four animals were included in the same experiment, and n indicates number of cells.

A subset of cells was not treated because they did not have the firing required for an intended experiment (e.g., quiescent vs. active). In these cases, the final 1-2min recorded from cells that had stable recordings for ≥5min and were verified to be alive were included in analysis of spontaneously-firing vs. quiescent cells from different hormonal treatment groups.

Data are reported as mean ± SEM. Nonparametric or parametric comparisons were used as appropriate for data distribution. Responses within

groups were analyzed by two-tailed Wilcoxon matched-pairs signed rank test. Comparisons among groups were analyzed by Friedman two-way repeated-measures ANOVA with Bonferroni's multiple comparisons test (senktide), Kruskal-Wallis test with Dunn's multiple comparisons test (seminal vesicle mass, spontaneous firing, percent change with dynorphin), or one-way ANOVA with uncorrected Fisher's least significant difference test (*Prl* expression). Significance was set at $p<0.05$.

3.4 Results

Spontaneous activity of KNDy neurons differs with in vivo *treatment*

Spontaneous firing activity in KNDy neurons in brain slices is an indicator of the *in vivo* influences of treatments through various mechanisms and was evaluated for all cells. These data include the control periods of the cells presented in the experiments below, as well as stable recordings from cells that were not further studied because they did not have the firing rate required for the intended experiment. The proportion of cells that were spontaneously active is shown in Figure 3.1. Roughly half of the KNDy neurons recorded from the castrate (49%, 18/37 cells) and castrate plus DHT-treated (54%, 13/24 cells) groups and 38% (9/24) of cells from the estradiol-treated group exhibited spontaneous action potential firing (>0.1Hz firing frequency, Figure 3.1 A). Estradiol but not DHT lowered spontaneous firing frequency compared to that in cells from castrate mice (Figure 3.1 B, $p<0.05$). These observations suggest that

estradiol inhibits spontaneous action potential firing rate in KNDy neurons. The number of spontaneously firing cells from the other treatment groups was too low for statistical comparison (firing rate: PPT 0.3±0.1Hz 2 cells, 1mg/kg DPN 0.4±0.3Hz 2 cells; 2mg/kg DPN 0.3Hz 1 cell).

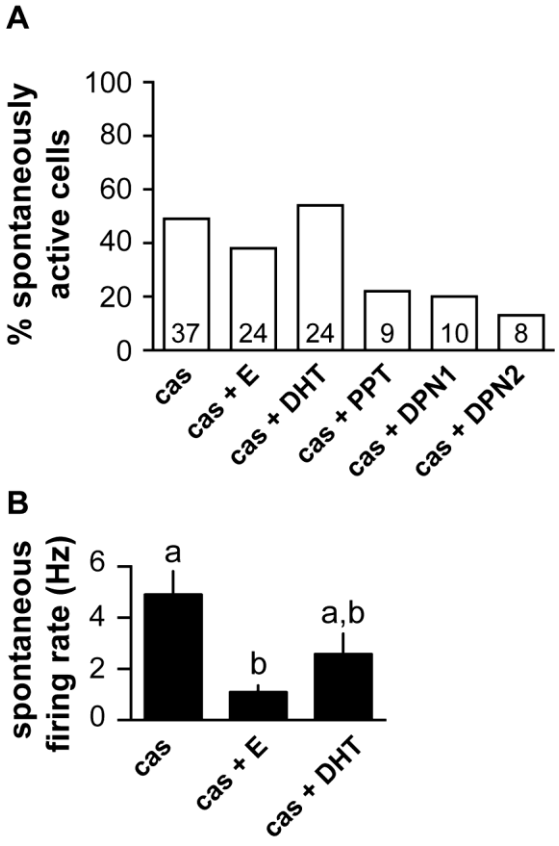


Figure 3.1 *Spontaneous activity in KNDy neurons is altered by* in vivo *treatment.* A, Spontaneous firing in KNDy neurons from the indicated *in vivo* treatment groups; numbers in bars indicate total number of cells, % indicates proportion that were spontaneously active. B, Action potential firing rate in spontaneously-active KNDy neurons from indicated *in vivo* treatment groups. Data include control periods of cells from experiments below, plus cells that were not acutely treated. cas, castrate; E, estradiol; PPT, ERα agonist; DPN1, 1mg/kg ERβ agonist; DPN2, 2mg/kg ERβ agonist; different letters indicate statistical difference, $p<0.05$, Kruskal-Wallis test with Dunn's multiple comparisons test.

Both estradiol and DHT attenuate the NK3R-mediated increase in action potential firing frequency in KNDy neurons

To test the hypothesis that estradiol is the main steroid modifying the response of KNDy neurons to activation of NK3R, we compared the response in castrate adult males left otherwise untreated or treated with estradiol or DHT implants. The NK3R agonist senktide (10nM) was bath applied to brain slices

during targeted extracellular recordings of quiescent (≤0.1Hz firing frequency) KNDy neurons. Representative traces from these recordings are shown in Figure 3.2 A-C. Senktide increased action potential firing in 100% (6/6) of cells recorded from castrate mice, similar to previous reports (189), and senktide increased firing in cells from estradiol-treated (70% 7/10 cells) and DHT-treated mice (75% 6/8 of cells). Both responding and non-responding cells were included for statistical analyses. Senktide increased firing frequency ($p<0.05$ vs. pre-senktide control) in castrate and steroid treated mice (Figure 3.2 G). Contrary to our hypothesis, this increase in firing frequency was attenuated by either estradiol or DHT treatment (both $p<0.05$ vs. castrate only).

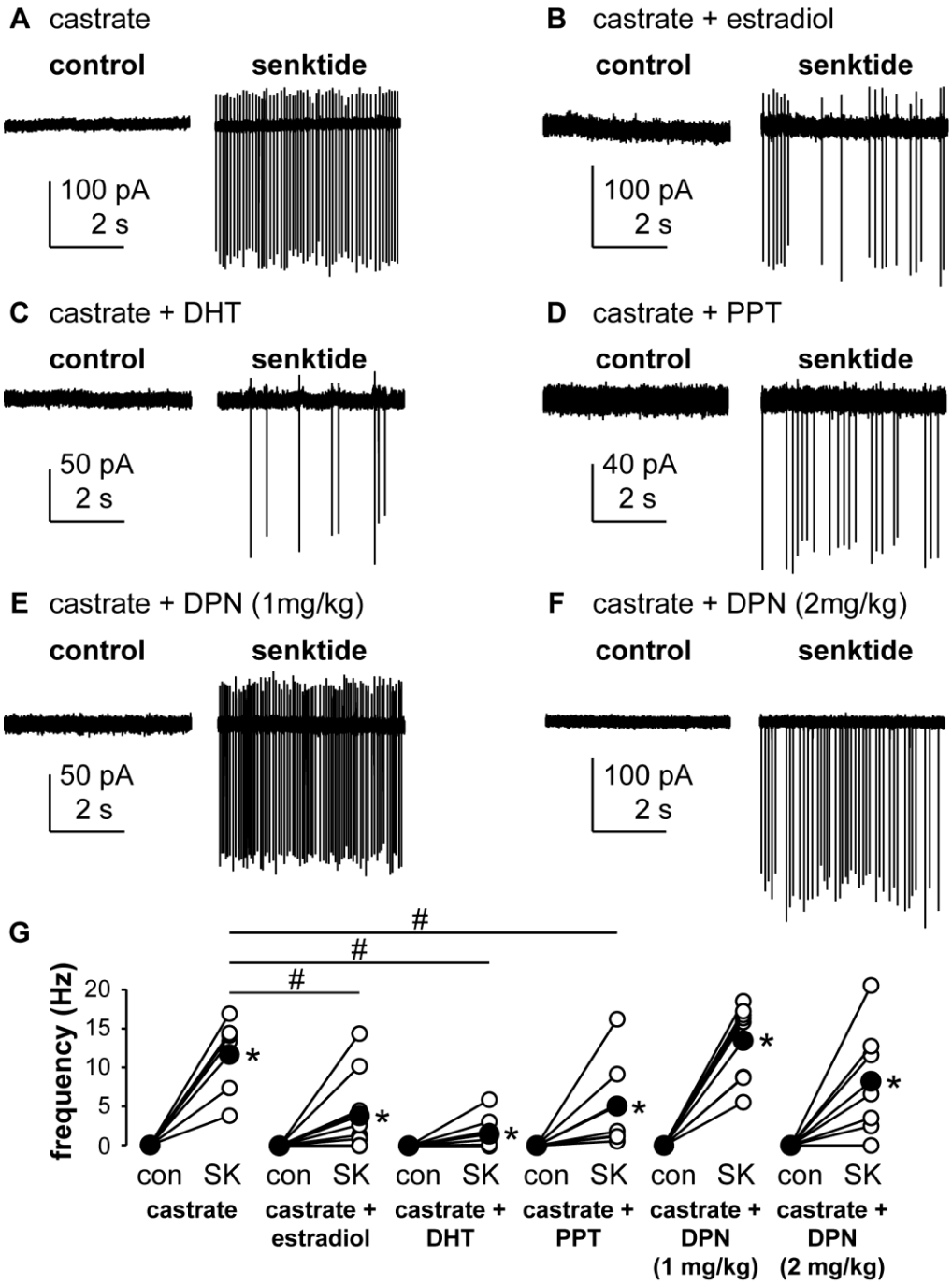


Figure 3.2 *Estradiol (via ERα) and DHT attenuate the NK3R-induced increase in KNDy neuron firing frequency.* A-F, Representative raw current traces from KNDy neurons before (left) and during (right) acute treatment with the NK3R agonist senktide (10nM) in the various groups as labeled. Variable current amplitudes reflect small changes in pipette position during the recording and do not provide information about changes in cell function. G, Firing frequency of individual KNDy neurons during control period (con) and senktide (SK) treatment. Open circles show data from individual cells, filled circles show means, and lines between open circles connect data from the same cell; *, $p<0.05$, comparisons within groups, Wilcoxon matched-pairs signed rank test; #, $p<0.05$, comparisons among groups, Friedman two-way repeated-measures ANOVA with Bonferroni's multiple comparisons test.

Estradiol acts via ERα but not ERβ to inhibit the NK3R-mediated response in KNDy neurons

We next tested if ERα or ERβ mediates the suppression of senktide-induced firing rate. Because mice with kisspeptin-targeted knock out of ERα exhibit altered steroid feedback (200), we hypothesized that ERα, not ERβ, mediates the estrogen attenuation of the KNDy neuron response to activation of NK3R. Senktide was bath applied to brain slices from castrate adult male mice that had been treated with daily injections of either the ERα agonist PPT (1mg/kg) or the ERβ agonist DPN (1 or 2mg/kg). Representative traces are shown in Figure 3.2 D-F. Senktide increased firing frequency ($p<0.05$ compared to pre-senktide control) in cells from both estrogen receptor agonist groups (Figure 3.2 G). A response to senktide was observed in 100% of KNDy neurons from the groups treated with PPT (7/7) and 1mg/kg DPN (8/8) and in 86% (6/7) of cells from animals treated with 2mg/kg DPN. PPT attenuated the senktide-induced increase in mean firing rate of KNDy neurons ($p<0.05$ compared to castrates). In contrast, DPN had no effect on the senktide-induced increase in firing frequency (1mg/kg, $p>0.9$; 2mg/kg, $p>0.9$ compared to castrates). Together, these data support the hypothesis that ERα mediates estradiol's inhibition of the NK3R-mediated increase in action potential firing in KNDy neurons.

Both estradiol and DHT enhance the KOR-mediated decrease in action potential firing frequency in KNDy neurons

Dynorphin inhibits KNDy neuron firing activity in a gonad-dependent manner, with inhibition being greater in intact than castrate males (189). The effect of dynorphin on firing rate of spontaneously-active KNDy neurons from castrate male mice treated with estradiol or DHT was examined to test the hypothesis that estradiol enhances dynorphin-mediated inhibition of KNDy neuron firing rate. Quiescent neurons were excluded from this experiment because inhibition of firing could not be observed. Representative traces from these recordings are shown in Figure 3.3 A-C. Dynorphin decreased firing (>20% decrease in frequency compared with pretreatment spontaneous activity) in 63% (5/8) of KNDy neurons from the castrate-only group and in 100% of cells from both the estradiol- (9/9) and DHT-treated (7/7) animals. One cell (12.5% of 8 cells) in the castrate group increased firing frequency (>20% increase compared with pretreatment spontaneous activity) during dynorphin treatment. Statistical analyses within each *in vivo* treatment group indicated that dynorphin decreased action potential firing within each group ($p<0.05$ vs. pre-dynorphin control, Figure 3.3 D). Because of the range of spontaneous firing frequency among groups (Figure 3.1), percent change of frequency before and during treatment was used to normalize the data for comparison among groups. The percent decrease in firing frequency during dynorphin treatment was greater ($p<0.05$) in cells from both estradiol- (92±6% decrease) and DHT- (91±6% decrease) treated mice compared to castrates (36±16% decrease).

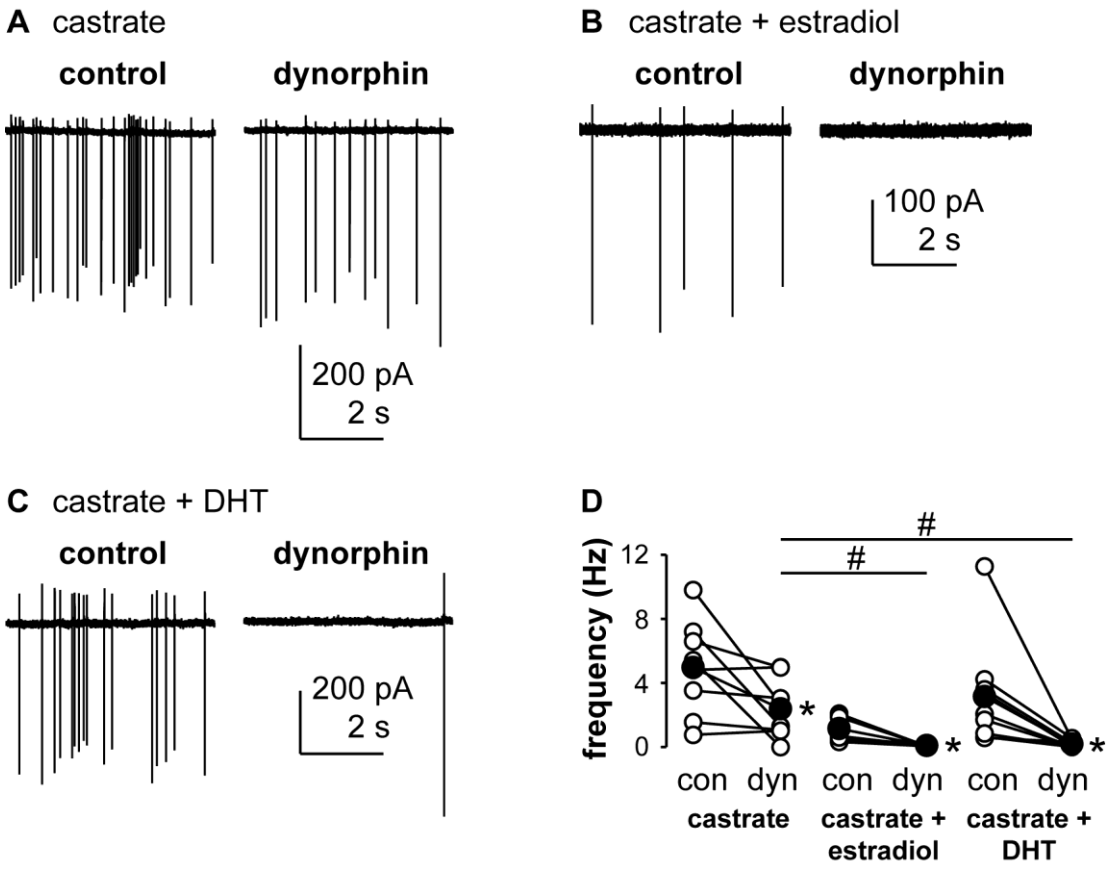


Figure 3.3 *Estradiol and DHT enhance the KOR-induced inhibition of KNDy neuron firing frequency.* A-C, Representative raw current traces from KNDy neurons before (left) and during (right) acute treatment with dynorphin (1μM) from the various groups as labeled. D, Firing frequency of individual KNDy neurons during control period (con) and dynorphin (dyn) treatment. Open circles show data from individual cells, filled circles show means, and lines between open circles connect data from the same cell; *, $p<0.05$, comparisons within groups, Wilcoxon matched-pairs signed rank test; #, $p<0.05$, comparisons among groups (percent change), Kruskal-Wallis test with Dunn's multiple comparisons test.

KNDy neurons from both castrate and intact male mice express AR and/or ERα but not ERβ message

ERβ mRNA (*Esr2*) expression in KNDy neurons had not been examined in male mice. Single-cell PCR was performed on cell contents from fluorescently-labeled arcuate neurons from intact or castrate *Tac2*-GFP adult male mice to determine expression of *Esr2* as well as AR (*Ar*) and ERα (*Esr1*) mRNA. GFP reliably identifies KNDy neurons in these animals (189). Two cells did not amplify

for GAPDH and were thus eliminated from further analysis, leaving 17 cells from intact and 22 cells from castrate mice for analysis (Table 3.2). The expression of *Esr1* and *Ar* detected by single-cell PCR was consistent with that reported using in situ hybridization (19). In contrast, *Esr2* was not detected in the cells studied from either intact or castrate mice. Of interest, there was considerable overlap of *Ar* and *Esr1* expression. Of the cells that expressed *Ar*, 10 cells (59%) from intact and 12 cells (55%) from castrate animals also expressed *Esr1*.

Table 3.2 Single-Cell PCR Results[a]

Transcript	**Intact (n=17)** No. Cells Expressing Transcript, n (%)	**Castrate (n=22)** No. Cells Expressing Transcript, n (%)
Ar	11 (65)	15 (68)
Esr1	15 (88)	16 (73)
Esr2	0 (0)	0 (0)
Ar + *Esr1*	10 (59)	12 (55)

[a]Results are shown as the number of cells expressing transcript (percentage of GFP-identified cells).

3.5 Discussion

Pulsatile GnRH release is controlled by gonadal steroid feedback, but GnRH neurons typically lack most receptors critical for this feedback. Here, we show that contrary to our hypothesis, agonists of both ERα and AR modify response of KNDy neurons to activation of either NK3R or KOR in castrate adult male mice. This response is manifested as an attenuation of NK3R-induced stimulation and strengthening KOR-mediated inhibition of KNDy neuron action potential firing, both of which are consistent with steroid negative feedback to reduce overall reproductive neuroendocrine output.

Estradiol's action on the response of KNDy neurons to activation of NK3R in males appears to be mediated by ERα. Estradiol-mediated inhibition of the NK3R-induced increase in KNDy neuron firing was mimicked by specific agonism of ERα but not ERβ. Single-cell PCR identified only ERα, suggesting KNDy neurons from male mice may lack ERβ in contrast to these cells in females (18). The apparent absence of ERβ in KNDy neurons in males agrees with previous work that showed little to no expression of ERβ in the arcuate nucleus of males (201-203). It is important to point out that *in vivo* steroid-induced changes in synaptic connectivity or cellular function anywhere within the brain slice could alter KNDy neuron activity, thus expression of the receptor within these cells is not a prerequisite for response. The present observations are, however, consistent with previous studies using global knockouts, which indicated that ERα but not ERβ is necessary for fertility in both males and females (14,204,205). Likewise, estradiol-induced down regulation of kisspeptin and neurokinin B mRNA expression in the female mouse arcuate nucleus is mediated via ERα (18,206). Further, kisspeptin-specific ERα knockout advanced pubertal onset, disrupted cyclicity, and diminished the ovariectomy-induced increase in LH secretion in female mice (200).

One possible caveat to studies with the ERβ agonist is that we were unable to identify a reliable positive control for efficacy of DPN on a putative ERβ-specific target (207-210). This was despite using the pure active R-enantiomer DPN at a molar concentration >3x higher than that of PPT. Our strategy was thus to use doses of DPN that did not increase pituitary prolactin

mRNA levels, an ERα-specific target, compared to the castrate only group (198). DPN has only ~70-fold selectivity for ERβ over ERα, compared with the 400-fold selectivity of PPT for ERα (211). Although neither tested dose of DPN attenuated the senktide-induced response compared to the castrate-only condition, cells from mice treated with the higher DPN dose (2mg/kg) had a distribution of senktide-induced responses resembling those of both the estradiol and PPT-treated groups, in that all of these groups contained a subset of cells with low or no response to senktide. Such cells were absent from the castrate and lower DPN dose groups. This indicates the 2mg DPN/kg treatment may have some off-target action on ERα to influence KNDy neuron action potential firing. This was also suggested by the small but significant difference between 1 and 2mg DPN/kg on pituitary prolactin expression, although neither dose elicited changes in prolactin expression compared castrate only. The failure of DPN to alter the response to senktide is of interest with regard to the receptor mediating the response to DHT. While DHT cannot be aromatized into estradiol, it can be metabolized into 5α-androstane-3β,17β-diol, which can act on some isoforms of ERβ (212). ERβ-specific agonism did not mimic the effects of DHT, indicating DHT action via AR rather than its metabolite's action on ERβ expressed in other neurons that transsynaptically alter KNDy neuron response to senktide.

In addition to the influence of gonadal steroids on NK3R- and KOR-mediated responses, estradiol decreased the firing frequency of spontaneously active neurons, whereas the spontaneous firing frequency with DHT was intermediate to those in the estradiol and castrate-only groups. Previous studies

observed no change in spontaneous firing frequency of KNDy neurons between castrate and intact male or female mice (183,189). It should be noted that these past and the present comparisons were made from short duration recordings and may not reflect long-term patterns of activity of these cells. The present data may be biased towards spontaneously active neurons as some cells with no spontaneous firing activity were excluded from these analyses because verification of viability (high K^+) was not done to permit subsequent studies on other cells in that slice. Despite these possible limitations, the present findings suggest that androgens and/or estrogens may influence spontaneous activity of KNDy neurons monitored over short duration.

There are several mechanisms through which estradiol and DHT may influence spontaneous KNDy neuron firing activity and/or the response to senktide or dynorphin. First, firing activity could be altered by steroid-mediated changes in expression or modulation of ion channels and other molecules contributing to action potential firing in KNDy neurons. Second, inputs other than KNDy neuropeptides, such as fast synaptic transmission, may mediate steroid-mediated inhibition of KNDy neuron activity (190,213). Third, estradiol and DHT may change expression levels of NK3R or KOR. Previous work using single-cell PCR found that half as many KNDy neurons from castrate mice expressed KOR mRNA and twice as many expressed NK3R mRNA than KNDy neurons from intact males (189). This differs from another study in male mice using in situ hybridization that found testosterone did not affect the percentage of arcuate kisspeptin neurons expressing either receptor message (166). Because these

studies focused on mRNA, tests of protein co-localization may ameliorate this apparent contradiction. Altered subcellular localization of either NK3R or KOR is another possible mechanism for steroid-induced changes in responsiveness. Finally, steroids may alter expression of KNDy neuropeptides, as both estradiol and DHT suppress arcuate expression of kisspeptin, neurokinin B, and/or dynorphin steady-state mRNA levels (18,19,166,206). The present studies cannot distinguish among these and other possible mechanisms, leaving this area open for further investigation.

The present study focuses on KNDy neurons, which form an important input to GnRH neurons, but steroid feedback likely regulates GnRH release at multiple levels in the central nervous system. In this regard, the inhibitory actions of both estradiol and androgen at the KNDy neuron are not necessarily reflected by GnRH neurons. Inhibition of NK3R-mediated stimulation and enhancement of KOR-mediated inhibition in KNDy neurons by both estradiol and DHT would presumably decrease the amount of kisspeptin released to stimulate GnRH neurons, which express kisspeptin receptor and are directly depolarized by kisspeptin (52,53,55,60,61). In a study using the same experimental model used here, however, the increase in peaks of action potential firing observed in GnRH neurons after castration was inhibited by estradiol whereas DHT had no effect (194). Androgens only appear to inhibit GnRH neuron action potential frequency in male mice at pharmacologically high doses that mimic steroid abuse (214). The contrasting effect of DHT in KNDy vs. GnRH neurons supports past (215) and recent (185) evidence that additional central circuits beyond kisspeptin-

expressing neurons alter GnRH output. Estradiol may also have a differential role in GnRH neurons via ERβ. Acute treatment with high physiological levels of estradiol excites GnRH neurons from female mice via ERβ (11,216).

A candidate for non-kisspeptin relay of steroid feedback to GnRH neurons and for which some mechanistic evidence exists is γ-aminobutyric acid (GABA)ergic neurons. GABA excites GnRH neurons because of their intracellular chloride concentration (217-219). Estradiol inhibits GABAergic post-synaptic currents in GnRH neurons from males (220), consistent with its inhibitory effect on action potential firing rate of these cells (194); GABAergic currents in females vary with time of day but also correspond directly with changes in GnRH neuron firing activity (221,222). Interestingly, estradiol plus DHT increases GABAergic currents in GnRH neurons from female mice (223); this combination has not been tested in males. One source of GABAergic input to GnRH neurons may be KNDy neurons, as half of them coexpress mRNA for glutamic acid decarboxylase-67, an enzyme that synthesizes GABA (135). While inhibition of KNDy neurons is a plausible pathway to explain estradiol negative feedback via reduction of kisspeptin and/or GABA release, it does not account for the apparent stimulation of GnRH neurons by estradiol plus DHT. In this regard, there are GABAergic inputs to GnRH neurons that are independent of KNDy neurons and regulated by other factors (224-228) that may increase excitatory drive to GnRH neurons independent of kisspeptin.

Based on the present observations and previous work highlighted here, we propose a model for gonadal steroid feedback on GnRH neurons via KNDy

neurons and other inputs (Figure 3.4). In males, estradiol plays an overall inhibitory role, attenuating NK3R-mediated stimulation and enhancing KOR-mediated inhibition of KNDy neuron firing activity. This decreases the amount of stimulatory kisspeptin released to GnRH neurons. Estradiol also decreases GABAergic excitation of GnRH neurons, ultimately decreasing GnRH release. The effects of DHT on NK3R- and KOR-mediated responses in and kisspeptin release from KNDy neurons mirror those of estradiol. However, these effects are counteracted in the GnRH neuron by a DHT-mediated increase in GABAergic excitation, as well as by possible activation of ERβ by the diol metabolite of DHT. We propose that in the intact male, the excitatory central effects of androgens are outweighed by inhibitory actions of estradiol at the central level, and by androgen inhibition at the level of the pituitary (194,229). This is supported by high levels of aromatase in the hypothalamus to convert testosterone to estradiol (191), as well as the ultimate inhibition of LH release by both estradiol and androgen (166,194).

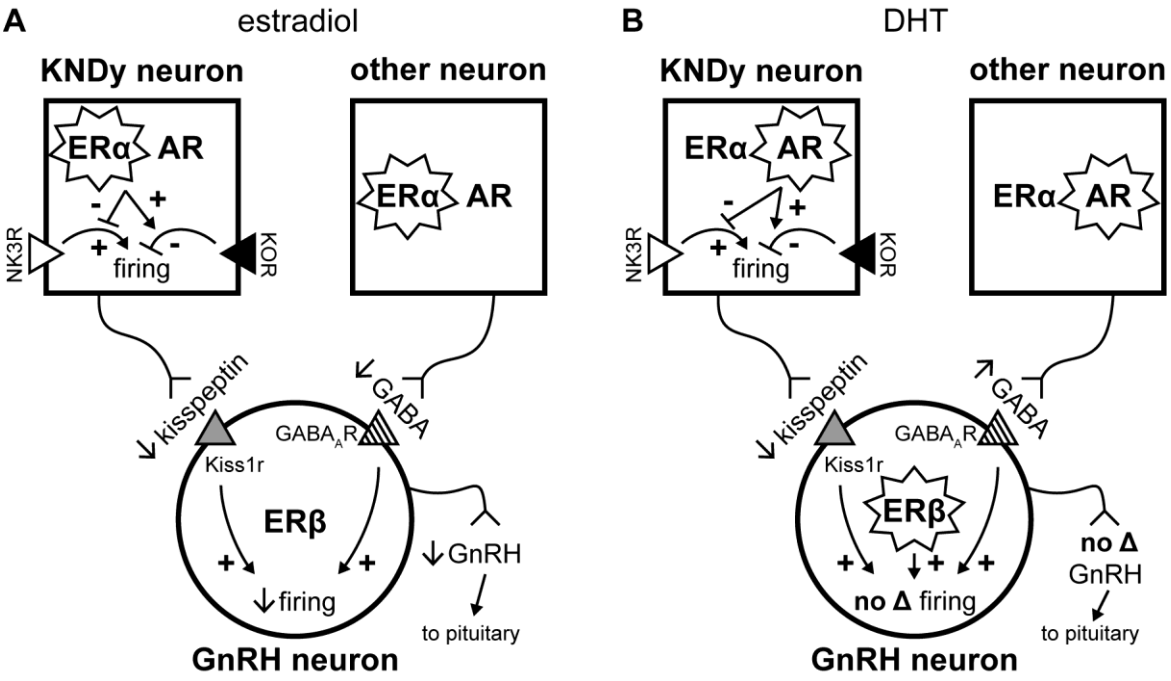


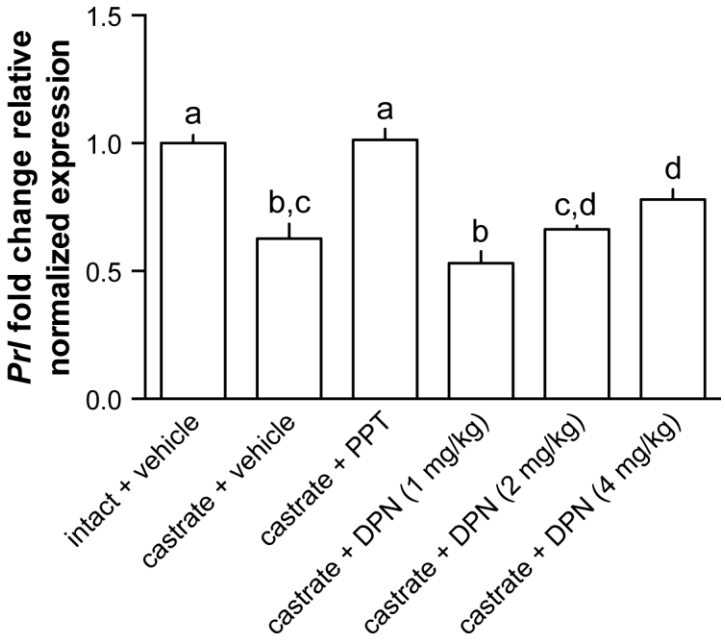
Figure 3.4 *Proposed model for gonadal steroid feedback on KNDy, GnRH, and other neurons in male mice.* Different pathways are activated by estradiol (A) and DHT (B) to differentially affect GnRH output (see text for details). We propose that in the brain of the intact male, estradiol negative feedback prevails. A, The low levels of estradiol in males do not act through ERβ to affect GnRH output. B, ERβ may be activated by a diol metabolite of DHT. A star surrounding a steroid receptor indicates activation; +, stimulation; -, inhibition. AR, androgen receptor; ERα, estrogen receptor α; ERβ, estrogen receptor β; $GABA_AR$, $GABA_A$ receptor; Kiss1r, kisspeptin receptor; KOR, κ-opioid receptor; NK3R, neurokinin 3 receptor.

The current studies demonstrate an inhibitory role of both estradiol and androgen on KNDy neurons via inhibition of action potential firing induced by NK3R activation and enhancement of inhibition of firing by KOR activation. These findings elucidate in part mechanisms of steroid feedback to a potential key component of the GnRH pulse generator. The present data also suggest that components beyond kisspeptin neurons are important for the overall regulation of GnRH release.

3.6 Acknowledgements

We thank Elizabeth Wagenmaker for excellent technical assistance and editorial comments, and Dr. R. Anthony DeFazio, Tova Berg, and Luhong Wang for editorial comments.

3.7 Supplementary material



Supplementary Figure 3.1 *Determination of estrogen receptor agonist doses.* Fold change (compared to intact + vehicle) of relative normalized expression of prolactin (*Prl*) mRNA, determined by quantitative PCR. Vehicle, 95% sesame oil, 5% DMSO; PPT, ERα agonist (1mg/kg); DPN, ERβ agonist; different letters indicate statistical difference; $p<0.05$, ordinary one-way ANOVA with uncorrected Fisher's least significant difference test.

CHAPTER 4

Conclusion

4.1 Contribution of the present work to the KNDy neuron model

The work presented in Chapters 2 and 3, as well as other recent studies demonstrate that action potential firing in KNDy neurons is stimulated by NK3R activation and inhibited by KOR activation (166,188,189,230), and these observations support the mechanisms for GnRH pulse initiation and cessation proposed in the KNDy model described in Chapter 1. In addition to a potential role as a contributor to the GnRH pulse generator, the work presented in this dissertation also supports KNDy neurons as mediators of negative gonadal steroid feedback. The effects on firing rate of NK3R agonism are attenuated, and the effects of KOR agonism are enhanced in KNDy neurons from intact compared with castrate male mice (Chapter 2) (189). The gonadal secretions mediating these changes appear to be both estradiol via ERα and androgen (Chapter 3) (230). Other work initiated since the beginning of this dissertation adds further detail to some aspects of the KNDy model while challenging others. This work has addressed several questions relevant to KNDy neurons, such as their electrophysiological properties, afferents, and output to GnRH neurons.

4.2 Electrophysiological mechanisms for modifying KNDy neuron activity/output

Recent studies have further tested electrophysiological properties of KNDy neurons, revealing possible mechanisms for their contribution to pulse generation and/or steroid feedback. These studies have characterized nascent pacemaker currents, tested membrane current modulation by neuropeptides, and examined GABAergic and glutamatergic currents.

Pacemaker currents

If KNDy neurons are part of the GnRH pulse generator that controls the rate of pulses, then it would stand to reason that they would express pacemaker currents that could control periodic bursts of action potential firing. These types of currents and the ion channels that carry them have been identified in KNDy neurons from female guinea pigs and mice. Specifically, KNDy neurons exhibit a hyperpolarization-activated, nonselective cation current (I_h), and to varying degrees they express each of the four known subunits of hyperpolarization and cyclic nucleotide gated channels (HCN) associated with I_h (167,231). KNDy neurons also express mRNA for low-threshold voltage-gated calcium channels $Ca_V3.1$ and 3.2, and they exhibit the transient (T)-type calcium current (I_T) mediated by these channels (167). In addition, both α and β subunits of sodium channels (Na_V) associated with persistent sodium current (I_{NaP}) and I_{NaP} itself are present in KNDy neurons (232). I_h, I_T, and I_{NaP} all contribute to rhythmic firing and burst activity (233-235). Regenerative patterns of action potential firing are not

only important for cells, like KNDy neurons, that are hypothesized to maintain a rhythmic pace of activity, but burst firing is also critical for release of neuropeptides (236-238). Thus, the pacemaker currents may be key components of KNDy neurons' contribution to the GnRH pulse generator by controlling a rhythmic pattern of KNDy neuropeptide release.

Interestingly, there is not yet evidence that pacemaker currents in KNDy neurons play a role in sensitivity to gonadal steroid feedback. While both I_h and I_T have been found to be regulated by estradiol in other cell types, including GnRH neurons (239-241), the response of these currents to gonadal steroids has yet to be tested in KNDy neurons. I_{NaP} is the only pacemaker current that has been tested in this regard, and estradiol affects neither I_{NaP} current density nor mRNA expression of $Na_V1.1\alpha$ or $Na_V\beta2$ in KNDy neurons (232). Together these data suggest that, although pacemaker currents may contribute to KNDy neuron burst firing and neuropeptide release, there are likely other mechanisms through which gonadal steroid feedback directly modifies KNDy neuron activity or output.

KNDy neuron membrane currents modified by activation of NK3R and KOR and modulation of these currents by gonadal secretions

One possible site of steroid regulation of KNDy neuron action potential firing may be through the pathways stimulated by activation of KNDy neuropeptide receptors, in particular NK3R and KOR. However, the mechanisms through which activation of NK3R and KOR modify action potential firing in KNDy neurons are unknown. In non-hypothalamic cell types, NK3R activation has been

linked to various currents including those mediated by sodium, calcium, potassium, and non-specific cation channels (242-246). In AVPV kisspeptin neurons and other cell types, KOR agonism has been associated with alteration of potassium currents possibly via the G-protein coupled inward rectifying potassium (GIRK) channel $K_{ir}3.1$ (247-249). Yet, these types of currents have not specifically been tested in KNDy neurons.

In order to change firing activity in KNDy neurons, we hypothesize that activation of NK3R and/or KOR initiates pathways that modulate membrane currents to affect action potential firing rate. We further hypothesize that gonadal steroids modify these pathways to alter the receptor-modulated membrane currents. To begin to test these hypotheses, preliminary whole cell voltage-clamp recordings were made of KNDy neurons from intact and castrate male mice. Voltage-clamp recordings monitor changes in membrane current. Using a pipette solution mimicking the physiological intracellular milieu, we compared membrane current and conductance near resting membrane potential at -70 or -60mV under control conditions and during treatment with senktide or dynorphin.

In KNDy neurons from intact and castrate mice, the NK3R agonist senktide induced a net inward current, but the current amplitude was markedly larger in cells from castrate mice (Figure 4.1). These data are consistent with Chapter 2's observations that the senktide-induced increase in action potential firing frequency is greater in cells from castrate mice (189). A relative inward current is generated if the net flow, or flux, of positive ions into the cell increases with channels opening, or if the net flux of positive ions out of the cell decreases

with channels closing (or if net flux of negative ions out of the cell increases with channels opening, or if net flux of negative ions into the cell decreases with channels closing). An initial gauge of channel opening or closing is provided by membrane conductance (inverse of input resistance). Senktide increased input resistance (i.e., decreased conductance, Figure 4.1), suggesting NK3R activation closed channels. Together, these data indicate decreased conductance via channels that, when open, would normally allow net flux of positive ions out of the cell (i.e., the net inward current is the result of reducing an outward current). Because the membrane potential during the experiment was near the resting membrane potential of the cell, the channel would need to be open near this potential. Both A- and D-type transient potassium currents (I_A and I_D, respectively), which can be blocked by 4-aminopyridine (4AP), fit this description (250,251). 4AP decreased the response to senktide in a cell from a castrate mouse (Figure 4.1; for technical reasons the 4AP experiment was done with the voltage-gated sodium channel blocker tetrodotoxin, which did not independently alter current response, also shown in Figure 4.1), suggesting that senktide acts by blocking channels conducting transient potassium currents. In contrast to the inward current generated by senktide, dynorphin initiated an outward membrane current accompanied by a decrease in input resistance (increase in membrane conductance) in a subset of KNDy neurons from intact mice (Figure 4.2). Dynorphin's effect on KNDy neurons from castrate mice has yet to be tested. These data demonstrate that intrinsic properties of KNDy neurons can be altered by NK3R and KOR activation and that, for at least the former, this response is

modulated by gonadal secretions. Modulation of the response to neuropeptides therefore remains a major candidate to control KNDy neuron firing, including the burst patterns required for release of KNDy neuropeptides.

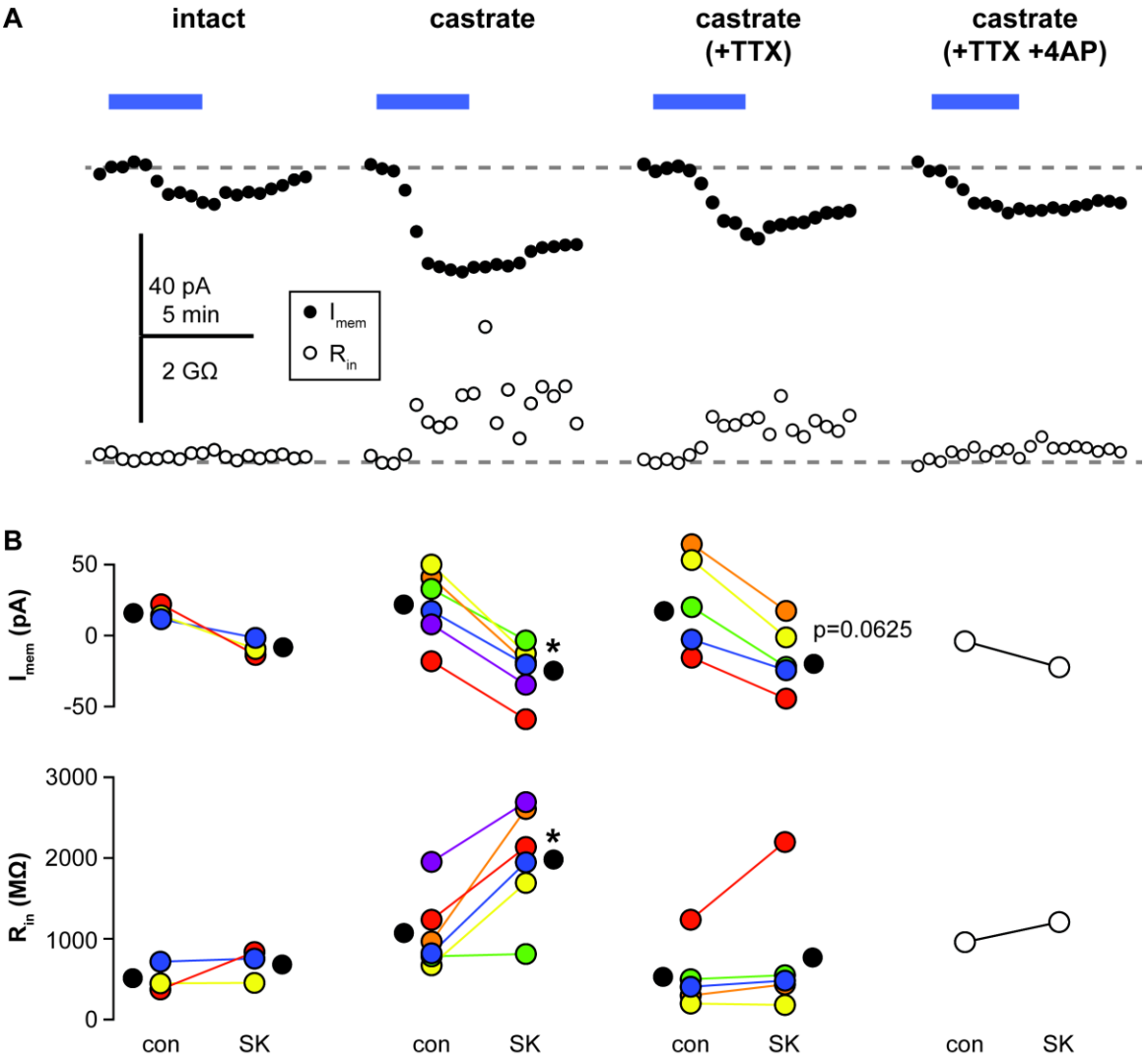


Figure 4.1 Senktide induces an inward membrane current (I_{mem}) and increased input resistance (R_{in}) that are attenuated in KNDy neurons from intact vs. castrate male mice. A: Sample I_{mem} (closed circles) and R_{in} (open circles) of KNDy neurons from the indicated treatment groups. Blue bars, senktide treatment; TTX, tetrodotoxin, votage-gated sodium channel antagonist; 4AP, 4-aminopyridine, I_A and I_D blocker. B: Plots of I_{mem} and R_{in} measured during the control period (con) and senktide (SK) treatment from the groups indicated at the top of "A." To isolate signals intrinsic to the recorded cell, communication within all brain slices was reduced by treatment with blockers of receptors mediating fast-synaptic transmission (AMPA-KA, NMDA, and $GABA_A$ receptors: 10μM CNQX, 20μM APV, 100μM picrotoxin, respectively)(220,226). Lines connect data from the same cell; points that are the same color in an I_{mem} plot and the R_{in} plot below it indicate data from the same cell; black circles, means; *p<0.05.

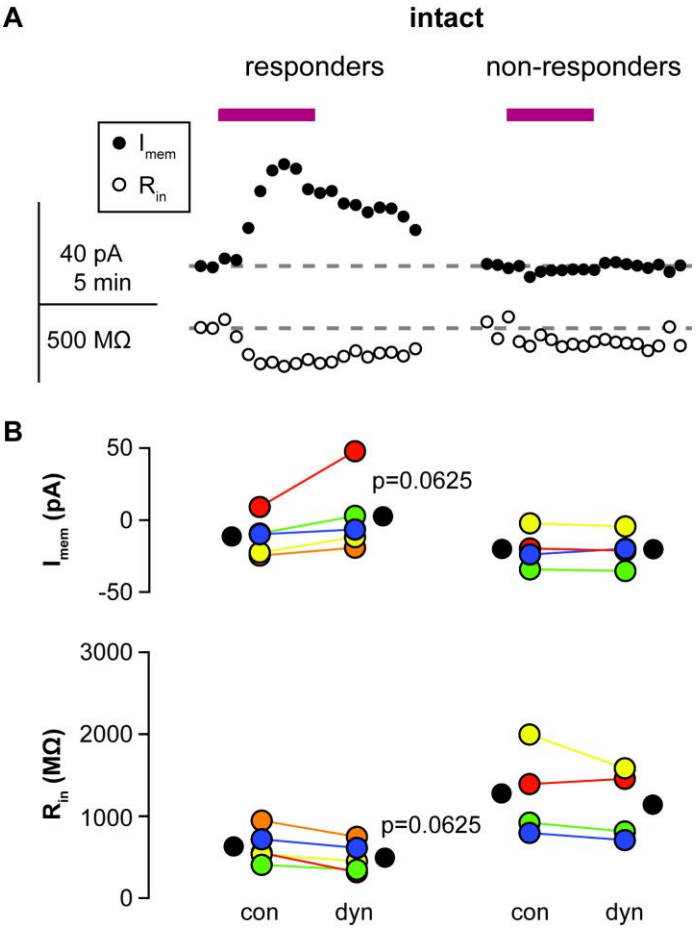


Figure 4.2 Dynorphin induces an outward membrane current (I_{mem}) and decreased input resistance (R_{in}) in a subset of KNDy neurons from intact male mice. A: Sample I_{mem} (closed circles) and R_{in} (open circles) of KNDy neurons that appeared to respond (left) or not respond (right) to dynorphin treatment (magenta bars). B: Plots of I_{mem} and R_{in} measured during the control period (con) and dynorphin (dyn) treatment from the groups indicated at the top of "A." Lines connect data from the same cell; points that are the same color in an I_{mem} plot and the R_{in} plot below it indicate data from the same cell; black circles, means.

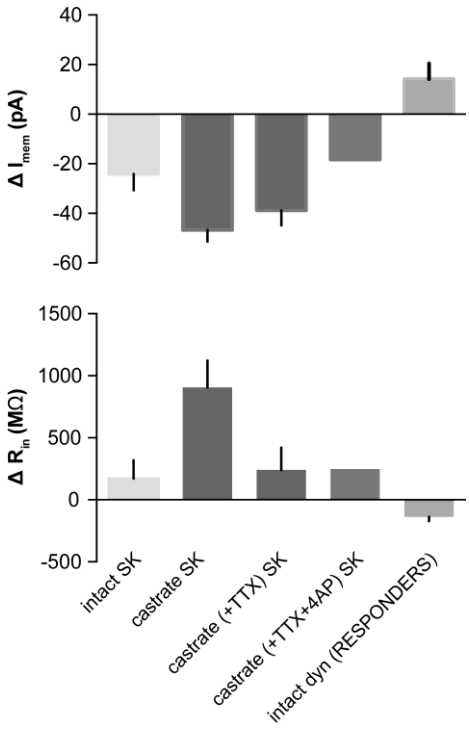


Figure 4.3 Changes (Δ) in membrane current (I_{mem}) and input resistance (R_{in}) in response to treatment from all groups shown in Figures 4.1 and 4.2.

GABAergic and glutamatergic currents

Other mechanisms that may mediate gonadal steroid feedback onto KNDy neuron firing are GABA and glutamate. KNDy neurons express ionotropic $GABA_A$ and glutamate receptors (167,182,231,248,252), and immunocytochemistry shows vGlut2-labeled varicosities apposed to the majority of KNDy neurons (185). Treatment of rodent arcuate brain slices with the glutamate receptor agonist N-methyl-D-aspartate (NMDA) stimulates burst firing in KNDy neurons (167,231), supporting activation by glutamate that may be released at the vGlut2-positive varicosities *in vivo*. While this study tested exogenous agonism, another study in ewes indirectly tested whether glutamate action typically occurs *in vivo* by measuring *cFos*, an immediate early gene associated with increased firing activity. In this study, increased expression of *cFos* in arcuate glutamate neurons is temporally associated with naturally-occurring LH pulses (185), suggesting not

only that the arcuate nucleus is capable of exhibiting glutamatergic activity, but also that it is correlated with the LH pulse. This observation supports a possible non-peptidergic mechanism for pulse generation but does not reveal if this mechanism originates from KNDy neurons (131,135) or other glutamatergic afferents to KNDy neurons. While stimulation of arcuate glutamate neurons is associated with pulsatile LH, neither LH pulse frequency nor amplitude is affected by local arcuate microimplantation of an NMDA receptor antagonist *in vivo* (253), suggesting that action via these receptors is not necessary for maintenance of LH pulses. Still, the observation with NMDA receptor antagonism does not eliminate possible glutamate action via other ionotropic receptors, such as AMPA and kainate receptors, or via metabotropic glutamate receptors.

In terms of GABA, estradiol blunts the frequency and amplitude of GABAergic postsynaptic currents in KNDy neurons (182,190), and the change in amplitude is mediated by kisspeptin neuron-specific ERα (182). The amplitude changes and mediation by ERα support postsynaptic modification of the KNDy neuron response to GABA by estradiol, and the changes in frequency suggest there are possible pre-synaptic modulations by estradiol, as well. The effect of bath-applied GABA is inhibitory to burst firing in KNDy neurons from rodent arcuate brain slices (167), but GABA has also been found to depolarize KNDy neuron membrane potential (190), an effect traditionally associated with activating action potential firing by bringing the membrane closer to firing threshold. While these observations appear to contradict one another, the GABA-induced depolarization is sub-threshold and may be associated with inactivation

of ion channels to cause “depolarization inhibition” of KNDy neurons (190). As with glutamate, the origin of GABAergic inputs to KNDy neurons may be either KNDy neurons themselves (135) or other afferent populations, but these inputs have yet to be identified. Together, these observations suggest that both GABAergic and glutamatergic inputs to KNDy neurons may influence their ability to fire in bursts of activity to release neuropeptides, as well as mediate alterations of KNDy neuron firing by gonadal steroids.

Modification of responses to activation of receptors for neuropeptides and amino acids may be key mechanisms for gonadal steroid feedback on action potential firing including burst activity. However, the bursts required for neuropeptide release act on a scale of seconds, while individual GnRH pulses last for minutes and occur at a frequency of minutes to hours. This difference in timing indicates that other mechanisms are required to control pulse generation. It is possible these mechanisms integrate the electrophysiological properties discussed here, perhaps via a coordinated network effect, to achieve the timing appropriate for pulse generation.

4.3 Afferents to KNDy neurons

KNDy neuron network

As discussed in Chapter 1, KNDy neurons are likely inputs for each other as part of a putative KNDy neuron network, and recent work has provided more support for KNDy neuron fibers within the arcuate nucleus (254-256), as well as

bilateral NKB connectivity and synchronized bilateral arcuate electrical activity (257).

KNDy neuron network: NKB may act via multiple tachykinin receptors

Within the KNDy neuron network, new studies have revealed possible mechanisms for neurokinin B action besides activating NK3R. While NK3R is the highest affinity receptor for NKB, NKB can act with lower affinity on the other tachkykinin receptors NK1R and NK2R (258,259). Interestingly, blocking NK1R, NK2R, or NK3R alone does not prevent the stimulatory effects of NKB on KNDy neuron potential firing, but a cocktail of antagonists for all three tachykinin receptors does (188). In terms of mRNA, expression of the message for NK1R and NK3R, but not NK2R, is found in at least a subset of KNDy neurons (260). NKB may therefore act directly on KNDy neurons via NK1R and NK3R and possibly indirectly through NK2R-expressing afferents.

Besides NKB, the endogenous high-affinity ligands for NK1R and NK2R, substance P and neurokinin A, respectively (258,259), may also influence reproduction. Intracerebroventricular injection of agonists specific for NK1R or NK2R increases LH secretion in male mice and in ovariectomized, estradiol-replaced female mice. In addition, arcuate expression of the common gene for both substance P and neurokinin A (*Tac1*) is suppressed by estradiol replacement in ovariectomized mice (260). Whether the *Tac1* mRNA splice variants specific to each peptide are differentially affected is unknown. In a test of how substance P or neurokinin A affects KNDy neuron membrane potential, an

acute localized puff of either peptide depolarizes KNDy neurons in arcuate brain slices (188). The treatment paradigm in this test minimizes intercellular communication by using tetrodotoxin plus blockade of ionotropic GABA and glutamate receptors, suggesting the effects are direct on KNDy neurons rather than via afferents. It is unclear, though, whether the observed depolarizations are a result of the different tachykinins acting with lower affinity on NK3R or even NK1R. If substance P and neurokinin A do affect KNDy neuron activity, the sources of substance P and neurokinin A are unlikely to be KNDy neurons themselves because *Tac1* expression does not overlap with *Kiss1* expression in the arcuate (260). Furthermore, the physiological significance of activating the high affinity receptors for these peptides may be minimal. A peak in arcuate MUA accompanied by a pulse of LH release is readily stimulated by intravenous treatment of low concentrations of senktide in goats (261), supporting stimulation via NK3R. However, agonists for NK1R and NK2R in the same study are only able to mimic the responses to senktide in a subset of animals when used in concentrations 100x that used for senktide (261). These observations suggest that the impact on GnRH pulse generation by NK1R and NK2R activation in goats may be limited. Together these data support possible NKB action via all three tachykinin receptors to affect KNDy neuron activity. They also suggest that substance P and neurokinin A may affect KNDy neurons, but that the impact of this action on the reproductive axis may be minimal.

KNDy neuron network: Kisspeptin and/or RFRP-3 may modify the KNDy neuron network

In addition to the influence of NKB and dynorphin on KNDy neuron activity supported in Chapters 2 and 3 (189,230), we must consider the possibility that the KNDy neuron network is affected by kisspeptin. As discussed in Chapter 1, KNDy neurons appear to lack kisspeptin receptor (63), and, not surprisingly, kisspeptin has no effect on KNDy neuron action potential firing (188). However, several recent studies have forced researchers to revisit the possibility that kisspeptin may still play a role in the arcuate nucleus. LH secretion is increased by treatment with a kisspeptin agonist targeted to the arcuate nucleus in rats (262), and it is suppressed by kisspeptin antagonism targeted to the same hypothalamic region in ewes (253), suggesting that the arcuate nucleus may be responsive to stimulation by kisspeptin. The kisspeptin receptor agonists and antagonists in these studies were administered by localized microimplants or microinjections to the arcuate region. Therefore, the area affected by the treatment may not be perfectly limited to the arcuate nucleus, and the strategy for treatment may damage the targeted tissue. Therefore, other studies are needed to further test the possible involvement of kisspeptin in this region. One such study uses a kisspeptin receptor knockout mouse, and finds that kisspeptin-induced changes action potential firing rate are unaltered in unidentified arcuate neurons from this knockout mouse (263), suggesting that the effects observed in this study as well as those with microimplants and microinjections may be independent of the kisspeptin receptor. One possible explanation is that

kisspeptin may act through another receptor type, in particular neuropeptide FF (NPFF) receptors (264-266), which have been found on KNDy neurons (267). NPFF receptors are primarily known for being receptive to RFamide-related peptide-3 (RFRP-3), also known as gonadotropin-inhibiting hormone for inhibiting gonadotropin release (268,269). RFRP-3 immunoreactive fibers have been found in apposition to KNDy neurons (267). Despite opposing effects of kisspeptin and RFRP-3 on GnRH release and neuronal excitability (51-53,55,57,199,270-272), the effects of these two peptides on electrical activity in unidentified arcuate neurons appear to be similar to each other (263). Future work is needed to identify whether these arcuate neurons are KNDy neurons or other cell types. The proportion of KNDy neurons expressing mRNA for NPFF receptors is not changed by gonadectomy in male and female mice, suggesting that the availability of these receptors is unaltered by gonadal steroids (267). Still, it has yet to be tested whether steroids affect other NPFF receptor-induced factors, such as intracellular signaling. While kisspeptin may not act directly on the KNDy neuron network, it may act indirectly, and both kisspeptin and RFRP-3 may act through NPFF receptor-expressing afferents to influence KNDy neuron activity.

Other inputs to KNDy neurons

KNDy neuron activity and output may be modulated by other afferents, from neuronal areas associated with regulation of fertility regulation as well as other functions. Regarding regions of the brain associated with reproduction, KNDy neurons may receive inputs from GnRH neurons and AVPV kisspeptin

neurons. Tract tracing and immunoreactivity studies have shown GnRH varicosities and terminals in apposition to KNDy neurons (273,274), and GnRH neurons make asymmetric synaptic contacts with KNDy neurons, suggesting that these are excitatory connections (274). LH release is unaffected by GnRH antagonism in the arcuate nucleus (253), but the input from GnRH neurons indicates that there may be reciprocal communication between GnRH neurons and KNDy neurons, perhaps serving to amplify signals initiated by either population to affect the other. Another neuronal population linked to reproduction, the AVPV kisspeptin population, projects to KNDy neurons (87), suggesting possible communication with mechanisms for positive gonadal steroid feedback, as well (275,276).

Other types of inputs to KNDy neurons that have been identified include those that relay messages of metabolism and energy balance. Leptin stimulates KNDy neurons by activating TRPC channels (231), and cocaine- and amphetamine-regulated transcript has been shown to directly stimulate KNDy neuron activity (277). KNDy neurons may also receive inputs from pro-opiomelanocortin and neuropeptide Y neurons (277,278), but whether and how these inputs influence KNDy neuron electrical activity or neuropeptide output is unknown. It is possible that any of the putative afferents to KNDy neurons, including GnRH neurons, AVPV kisspeptin neurons, and other KNDy neurons, as well as neuronal populations involved with non-reproductive functions can ultimately influence KNDy neuron activity and its influence on the rest of the reproductive axis.

4.4 KNDy neuron output to GnRH neurons

While it is important to consider regulation at the KNDy neuron level, as well as inputs to KNDy neurons, it is especially important to consider how efferents from KNDy neurons act downstream, particularly in the context of their possible contribution to a gonadal steroid-sensitive GnRH pulse generator. There is compelling support for KNDy neuron projections to and synapses on GnRH neurons (279-281), but the understanding of the locus of KNDy action on GnRH neurons has been shifted since the original KNDy neuron hypothesis was proposed. Despite the strong stimulation of GnRH neuron firing by kisspeptin that was mentioned in Chapter 1 (51-57), studies in both rodents and ungulates have found only rare kisspeptin projections to GnRH cell bodies in the POA (87,131,255,273,282). Rather, much more evidence has illustrated kisspeptin- and/or NKB-expressing fibers projecting to GnRH neuron fibers in the median eminence (255,256,273,282-284), suggesting that KNDy neuron action on GnRH neurons may be localized mostly to GnRH neuron projections rather than cell bodies.

The limited KNDy connectivity with the GnRH neuron cell bodies does not eliminate the relevance of KNDy neurons to the control of GnRH release. Indeed, GnRH neuron activity initiated near the soma may not even be necessary for GnRH release. One study in ewes found that expression of *cFos* is increased in GnRH neurons by treatment with opioid antagonists, which increases LH

pulsatility (285). However, in other work that studied naturally-occurring LH pulses, *cFos* expression in GnRH neurons did not increase near the time of a natural LH pulse, although *cFos* did increase in arcuate kisspeptin neurons at this time (185). These data suggest via gene expression that an LH pulse may be temporally associated with increased firing activity in KNDy neurons, but that this increase in activity in KNDy neurons may not necessarily coincide with increased activity in GnRH neurons. The output from these more active KNDy neurons may elicit GnRH release by acting at the level of GnRH neuron projections, as kisspeptin treatment targeted to the median eminence by microinjection elicits a pulse of LH (185). This possibility is further supported by measurements of GnRH using fast scan cyclic voltammetry (FSCV) in mouse brain slices, which find that local kisspeptin treatment can evoke GnRH release in the median eminence whether or not action potentials are blocked (187), demonstrating kisspeptin-stimulated release of GnRH in the presence and absence of GnRH neuron activity.

Kisspeptin is not necessarily the only KNDy neuropeptide that modulates GnRH release; NKB may play a similar role at the median eminence. As mentioned in Chapter 1, little to no NK3R has been detected on GnRH neuron somata (121,132,140,166), but NK3R immunoreactivity has been found in apposition to GnRH nerve fibers in the median eminence (121,140). This difference in NK3R expression is reflected by recent functional studies, which show that GnRH neuron action potential firing is not altered by senktide or NKB (166,189) and that GnRH release measured by FSCV is induced by local

senktide treatment to the median eminence (186). Importantly, the senktide-induced GnRH release is still observable in kisspeptin knockout mice (186). Together, these data suggest that NKB can act in the median eminence to evoke GnRH release independent of kisspeptin.

4.5 Further support and challenges to the KNDy neuron model

Besides the observations relevant to the KNDy neuron model that have already been discussed, there is additional recent work that either supports or contradicts different components of the model. Anatomically, in situ hybridization has corroborated coexpression of mRNA for kisspeptin, NKB, and dynorphin in the arcuate of male mice (166,189), and immunohistochemistry has confirmed that the majority of kisspeptin neurons also express NKB in ewes (286). It should be noted, though, that another study using immunoreactivity in the arcuate nucleus of male rats found a substantial number of NKB neurons that did not colocalize kisspeptin (287), potentially challenging the view of consistent colocalization of all three KNDy neuropeptides in the arcuate nucleus of all mammals.

Some studies have also tested the contribution of KNDy neurons to the control of gonadotropin levels by using targeted knockdown or ablation of arcuate neurons expressing kisspeptin or NK3R in adult rodents (275,288,289). Two of the studies knock down arcuate kisspeptin using microinjections of recombinant adeno-associated virus encoding a kisspeptin antisense targeted to the arcuate

nuclei of adult female rats (275,289). Although these studies only achieve roughly 30% decrease of arcuate kisspeptin neurons compared to untreated controls, they still demonstrate decreased LH pulse frequency and abnormal estrous cyclicity (275,289), suggesting that a fully-intact KNDy population is required for the combination of consistently normal LH pulsatility with normal cyclicity. Another study achieves high levels (93-98%) of ablation of KNDy neurons by injecting an NK3R agonist conjugated to saporin (a ribosome inactivating toxin) into the arcuate nuclei of female rats (288). This study does not report on cycles, but it does show that KNDy neuron ablation causes overall lower serum gonadotropin concentrations (288), further supporting the necessity of KNDy neurons to sustain normal gonadotropin levels. These data are consistent with an important role for KNDy neurons in normal LH pulsatility and overall secretion.

In addition to testing their role in pulse generation, recent studies have also tested KNDy neurons' contribution to gonadal steroid feedback. The initially-hypothesized role in negative feedback was largely based on studies showing steroid-mediated downregulation of expression of the genes for KNDy neuropeptides (18,19,90,91,93,120,125,126). This effect is corroborated in many recent mammalian studies testing mRNA expression for kisspeptin, NKB, and dynorphin (166,254,290,291), including the single-cell PCR studies in Chapter 2 (189). The mechanisms for regulation of the expression of KNDy mRNA were largely unknown until two recent studies that revealed that estrogen regulation of arcuate kisspeptin expression is mediated by epigenetic mechanisms interacting

with the 5’ promoter region of kisspeptin (292,293). Future investigation is needed to build on these findings for kisspeptin, as well as to test mechanisms of steroid regulation of NKB and dynorphin mRNA expression.

In further investigation of steroid feedback, a recent tracing study in female mice found that all arcuate kisspeptin neurons shown to connect with GnRH neurons also express ERα (281). In addition, estradiol is unable to inhibit kisspeptin mRNA expression in female mice with kisspeptin-specific ERα knockout (294). One of the previously-mentioned ablation studies also examined KNDy neurons’ contribution to gonadal steroid feedback. While arcuate NK3R cell ablation prevents the rise in LH normally observed with ovariectomy, it does not block the inhibitory effects of estradiol on gonadotropin secretion (288). In support of this observation, estradiol can still inhibit LH secretion in kisspeptin cell-specific knockout of ERα female mice (294). These data suggest that negative estradiol feedback to GnRH release can bypass KNDy neurons.

There are two major caveats to the conclusions made based on kisspeptin-specific knockout of ERα. First, the knockout is effective in these animals from the moment the *Kiss1* promoter activates Cre recombinase (294), leaving open the possibility for compensatory mechanisms to occur throughout development. This concern is partially addressed in the arcuate NK3R and kisspeptin ablation studies because these are performed after birth during adulthood. Yet, these experiments do not study knockout of ERα specifically in KNDy neurons. The second caveat is that ERα knockout would indeed affect kisspeptin neurons in the arcuate nucleus, but it would also cause ERα knockout

in other kisspeptin neurons, including those in the AVPV, making it difficult to distinguish effects specifically on the arcuate. Future work could address this challenge by utilizing mice with *Tac2*-specific knockout of ERα (Tac cre x ERα floxed; TERKO) produced by the Myers lab. This knockout will affect the arcuate kisspeptin population rather than all kisspeptin neurons, as well as a small number of cells in other brain regions not typically associated with reproductive neuroendocrine function (Greenwald-Yarnell, unpublished). The TERKO mice would also help to determine if the ERα-mediated change in the KNDy neuron response to NK3R activation shown in Chapter 3 is mediated directly at the KNDy neuron or by estrogen-sensitive afferents. Similarly, a *Tac2*-specific knockout of AR would also help to elucidate the locus of action of androgens in KNDy neuron responses.

4.6 Conclusions and future directions

Much of the work discussed here supports KNDy neurons as components of the GnRH pulse generator that may also help to relay negative gonadal steroid feedback to GnRH release. However, we propose modifications to the KNDy neuron model. Much of the evidence still supports an interconnected network of KNDy neurons in the arcuate nucleus that is stimulated by NKB and inhibited by dynorphin. However, NKB may act by receptors other than NK3R, such as NK1R, and dynorphin's actions may be relatively weak and largely dependent on gonadal steroids. In addition, other factors like glutamate, GABA, GnRH, RFRP-

3, and kisspeptin itself, not to mention inputs from other systems including metabolism, may contribute to the ultimate activity and output of KNDy neurons. Regarding output, there may only be scarce KNDy neuron projections to GnRH neuron cell bodies in the POA, while their biggest impact on GnRH release is at the GnRH terminals in the median eminence. It is possible that stimulation of GnRH neurons near their projection terminals may initiate action potentials that propagate towards the GnRH soma, but the importance of KNDy neurons in stimulation of GnRH action potential firing may be minimal. Instead, KNDy neuron influence on vesicular release of GnRH may be much more physiologically relevant. In addition to kisspeptin, KNDy neurons may also release NKB at the median eminence to induce GnRH release. In terms of gonadal steroid feedback, evidence still exists for KNDy neurons as partial mediators of negative gonadal steroid feedback, in part by modulation of their responses to activation of NK3R and KOR. Gonadal steroids may also influence endogenous currents in KNDy neurons, as well as KNDy neuron morphology.

With the modified model in mind, though, KNDy neurons appear to be neither the entire GnRH pulse generator nor the only mechanism for negative gonadal steroid feedback onto GnRH release. A great deal of work still needs to be done to determine exactly how KNDy neurons fit into the larger framework of a negative-feedback mediating GnRH pulse generator. While there is support for a KNDy network, there is limited evidence for synchronized activity within the network. One preliminary study from our lab started to test possible synchrony using dual simultaneous long-term extracellular recordings of KNDy neurons in

brain slices from adult male mice (295). It found limited synchrony in firing between pairs of KNDy neurons (295), contrary to a hypothesized KNDy neuron network with coordinated activity. Future studies using similar strategies to record multiple KNDy neurons will be needed to expand these data and further test the proposed synchrony in firing activity. In addition, if the state of the art permits, the relationship, if any, between KNDy neuron firing and the release of neurotransmitters and peptides from KNDy neurons needs to be tested. Further investigation is also necessary to identify the currents and ion channels relevant to KNDy neuron firing patterns and steroidal regulation, particularly those affected by activation of NK3R and KOR.

KNDy neurons appear to interact with other areas of the brain besides GnRH neurons and each other (281). Regarding areas directly implicated in reproduction, KNDy neurons may project to AVPV kisspeptin neurons and the ependymal layer of the third ventricle (256,273). Outside of a direct reproductive role, KNDy neurons may also influence POA regions involved in temperature regulation (296). In terms of afferents, KNDy neurons may receive inputs from neuronal areas implicated in circadian/diurnal rhythms, limbic responses, and, as mentioned earlier, energy balance (190,231,277,278,297). Recent studies have also suggested that KNDy neurons may even be involved in positive gonadal steroid feedback in some species (275,276), emphasizing the need for future studies in both females and males. Understanding how all of these systems may influence reproduction will require determining how KNDy neurons interact with each system and integrating this knowledge to reflect the complexity of the

organism as a whole. To achieve this goal, there are many more avenues of future work that have yet to be explored.

4.7 Impact

The work presented in this dissertation advances the field of reproductive neuroendocrinology. In particular, it elucidates mechanisms through which KNDy neurons may contribute to the GnRH neuron pulse generator as well as mediate negative gonadal steroid feedback onto GnRH release. It opens many questions for future investigation to study hypothalamic influence on reproductive function.

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