Kisspeptin Neurons as Drivers of Reproductive Development

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Abstract

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A network of hypothalamic neurons integrates signals from the environment, the brain and the endocrine system to initiate puberty and sustain fertility. In the last decade, kisspeptin neurons have emerged as essential players in this network. In this dissertation, I review some of the landmark studies that shaped our understanding of how the brain regulates reproduction. I then describe three lines of investigation from my research. First, I characterize transgenic mice that were developed to understand the function of kisspeptin neurons. Second, I set out to resolve a controversy about the requirement of kisspeptin neurons for puberty and the ability of compensatory pathways to overcome their ablation. I discovered that the gene that encodes kisspeptin (*Kiss1*) is expressed in excess of levels required for fertility in mice, reflecting a failsafe to guarantee reproductive success. I also found that females require larger quantities of kisspeptin to reproduce than males, due to their need of kisspeptin for ovulation. Finally, I provide evidence that kisspeptin neurons

implicated in ovulation are scarcer in males than females as a result of lower *Kiss1* expression rather than due to sex differences in the death or birth of kisspeptin neurons. I conclude with implications of this work and explore possible future directions for understanding the development of kisspeptin neurons.

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Chapter I. Introduction

Discovery of the Reproductive Axis

The brain drives reproduction through its regulation of the pituitary gland. Our understanding of the pituitary is founded on experiments from the 19th and early 20th centuries, which demonstrated that removal of the pituitary caused atrophy of the gonads, thyroid, and adrenals (1-3), and transplantation of anterior pituitary tissue restored their function. Furthermore, pituitary extract triggered ovulation in the rabbit (1). These studies provided evidence for the secretion of gonadotrophic hormones from the anterior pituitary, which drive sex steroid synthesis and gametogenesis in the gonads. The two primary gonadotropins were subsequently isolated and termed luteinizing hormone (LH) and follicle stimulating hormone (FSH) (1).

As early as 1927, evidence emerged that hormonal secretion from the pituitary is controlled by the hypothalamus. Smith showed that selective lesions of the hypothalamus caused genital atrophy (2, 4), and in 1941 Dey demonstrated that lesions in the basal forebrain caused either constant estrus or diestrus in the guinea pig (5). In the late 1940s Sawyer, Markee, and Hollinshead found that ovulation could be blocked by the administration of centrally acting anesthetics (6), and Harris demonstrated that electrical stimulation of the hypothalamus triggers ovulation in the rabbit (7, 8). These studies provided the early foundations for our understanding of the brain as a central processor of reproduction.

The anatomical basis for hypothalamic-pituitary communication emerged in the 1930s. The portal blood circulation that serves as a conduit for hypothalamic releasing factors to the anterior pituitary was first described by Popa and Fielding, although the authors incorrectly deduced that the direction of blood flow was from the pituitary to the hypothalamus (9), which was subsequently corrected by Houssay *et al*. (1). Epithelial cells that synthesize LH and FSH, called gonadotropes, are located in the distal lobe of the anterior pituitary gland. The gonadotrophs are surrounded by other secretory cells and by fenestrated capillaries, which deliver releasing-hormones from the hypothalamus

and allow secreted pituitary hormones to enter the circulation (3). Green and Harris demonstrated that hypothalamic neuron terminals release factors into the capillary-rich median eminence (ME) at the base of the hypothalamus (10, 11). These factors flow to the distal lobe of the anterior pituitary, where they either stimulate or inhibit hormonal synthesis and secretion (8, 12). Cutting the pituitary stalk disrupted ovulatory cycles and gonadal function, but these functions were restored when portal vessels were allowed to regenerate (13, 14). Furthermore, replacement of distal anterior pituitary tissue in hypophysectomized animals restored release of gonadotropins (as well as other anterior pituitary hormones) if the replacement tissue was implanted beneath the ME (15, 16). These studies established the link between the brain and the endocrine system and thus marked the beginning of reproductive neuroendocrinology.

The hypothalamic stimulus that drives gonadotropin secretion originates from neurons that produce gonadotropin-releasing hormone (GnRH, formerly called LHRH) whose cell bodies reside in the basal forebrain. These neurons fire synchronously to release GnRH into the pituitary portal circulation (17-19). Initial evidence that a releasing factor drives gonadotropin secretion came from experiments showing that hypothalamic and median eminence extracts stimulate LH release from the pituitary *in vitro* (20-22) and *in vivo* (1, 2, 8, 23). The structure of GnRH was delineated by Roger Guillemin and Andrew Schally in 1971, who later shared the Nobel Prize for this work (24, 25). Their discovery kindled investigations into the temporal pattern of GnRH release, which has remarkable physiological implications.

Pulsatile Gonadotropin Release

The release of LH is not continuous, but periodic, occurring every hour in primates and every 15-30 minutes in rodents (26, 27). Pulses of LH are invariably preceded by bursts of electrical activity from the medial basal hypothalamus (MBH) (28, 29), which likely represent discharge from GnRH neuron terminals or cells driving GnRH neuronal activity. GnRH pulses correlate with LH pulses as shown by push-pull perfusates in rats, monkeys, and sheep (30-35). Furthermore, the periodic nature of GnRH release is required for normal pituitary function. GnRH can rescue gonadotropin

secretion in animals with lesioned GnRH neurons if it is administered intermittently but not continuously (36). The desensitization of the pituitary to continuous GnRH is thought to result from down-regulation of receptors on the surface of gonadotropes (36-39). Lesion studies and multiple unit recordings suggest that the site of the GnRH pulse generator is in the arcuate nucleus, the region of the MBH directly above the ME and pituitary portal circulation (18).

Negative Feedback of Sex Steroids on the Brain

Gonadotropin secretion is regulated by negative feedback from sex steroids in the circulation. Early evidence of negative feedback emerged in 1905 when Fichera showed that castration caused enlargement of the pituitary, which had large vacuolated cells (3). In 1932, Moore and Price proposed that sex steroids inhibited activity of the anterior pituitary. They discovered that injection of gonadal extract into either male or female rats disrupts spermatogenesis and estrous cycles, but that simultaneous injection of gonadotropin-containing extracts reverses some of these inhibitory effects (40). Moore and Price's model did not include feedback effects of sex steroids on the hypothalamus.

Holweg and Junkmann postulated that circulating sex steroids provide tonic inhibitory feedback on a hypothalamic center (12). They found that the pituitary response to castration disappears when the anterior lobe of the pituitary is separated from the ME (3) and concluded that the MBH must be responsible for the pituitary response. In the early 1940's, Dey also found that lesions of the anterior hypothalamus in guinea pigs caused enlarged genitalia, whereas those located caudally caused atrophic genitals, suggesting that the caudal region of the hypothalamus was needed for tonic LH secretion. Flerko and Szenthagothai supported this model by demonstrating that ovarian grafts implanted in the hypothalamus, but away from the anterior pituitary, decreased uterine weight (41). They also postulated that the arcuate and/or ventro-medial nuclear region of the MBH supplies tonic stimulatory input to the reproductive axis. Their conclusion was partially based on findings by Flerko & Bardos, who showed that lesions of this area interrupt ovulatory cycles and produce atrophy of the ovaries,

diminishing their capacity to produce estradiol (E_2) (42). Furthermore, Halász found that if the pituitary was transplanted into different brain regions, only the MBH could maintain its structure and function (43-46) and that the MBH was sufficient to maintain tonic gonadotropic activity. If the MBH was isolated from the rest of the brain by complete deafferentation, the pituitary still increased LH production when rats were ovariectomized (OVX) (46). However, the LH response of the pituitary was diminished, suggesting that inputs from the anterior hypothalamus also played a role.

Sex steroids inhibit the frequency (and to some degree, amplitude) of GnRH and LH pulses (3). This is demonstrated by the fact that removal of the gonads increases the frequency of LH pulses and multi-unit electrical activity (MUA) volleys in the MBH in rats, monkeys, sheep and goats (3, 47). In males, testosterone (T) inhibits GnRH pulse amplitude and frequency (48, 49). T acts through the nuclear androgen receptor (AR) and exerts additional effects after it is converted to E_2 or dihydrotestosterone (DHT), which binds AR but cannot be converted to E_2 (50). The male requires both AR and the classical estrogen receptor (ERα) for negative feedback on gonadotropin secretion (48, 49, 51). In the female, negative feedback is primarily mediated by E_2 (52, 53) acting on ERα, as mice lacking ERα mice have elevated LH levels and GnRH mRNA (54, 55).

The negative feedback actions of sex steroids on GnRH secretion are unlikely to occur directly on GnRH neurons, because GnRH neurons do not express AR or ERα. Despite the fact that GnRH neurons express the nuclear estrogen receptor, ERβ (56-58), this receptor is not required for negative feedback (54, 55). ERα-expressing neurons in the arcuate project to the rostral preoptic area in the rat (59) and to the rostral preoptic area and diagonal band of Broca in the ewe (60), areas where many of the cell bodies of GnRH neurons reside. In fact, viral tracing studies in the mouse have shown that GnRH neurons receive synaptic inputs from ERα-expressing neurons in the arcuate (61). In the rat, there are AR-expressing neurons in the arcuate (62), which marks this region as a node for the action of sex steroids on the brain (63). Evidence has accumulated to support the idea that a group of neurons that secrete the neuropeptide kisspeptin sustain GnRH release and relay negative feedback signals from the gonads.

Kisspeptins as Drivers of GnRH Secretion

The essential role of kisspeptin neuropeptides and their receptor (Kiss1r, formerly called GPR54) became evident when it was discovered that humans and mice with inactivating mutations in *KISS1/Kiss1* or *KISS1R/Kiss1r* are infertile (64-71). These individuals fail to reach puberty and suffer from hypogonadotropic hypogonadism, the lack of gonadal maturation due to insufficient gonadotropin release (65-71). Kisspeptins are C-terminal cleavage products of a 145-amino-acid propeptide, which contains a predicted signal peptide (72). Although the longest active form of kisspeptin is 54 amino acids in length, the 10 amino acids at the C-terminal are also effective activators of the G_q-coupled receptor *Kiss1r* (73-75) (Figure 1). Kisspeptins stimulate GnRH secretion (76-79) when administered at minute doses into the brain and are the most potent stimulators of GnRH neuronal activity (80). Kisspeptin depolarizes and increases the firing rate of GnRH neurons for over 30 minutes (80-82). The effects of kisspeptin on GnRH neurons are direct, as evidenced by the sustained depolarization of GnRH neurons in the presence of an action potential blocker (80), direct membrane contacts between kisspeptin neurons and GnRH axons in the ME (83-86), and the expression of *Kiss1r* on GnRH neurons (77, 80, 87). Finally, *Kiss1* mRNA is expressed in regions of the brain known to regulate reproduction (76), including the arcuate.

Kisspeptin neurons in the arcuate as the node of negative feedback

Evidence suggests that *Kiss1* neurons in the arcuate provide tonic drive to GnRH neurons and that this inductive action is modulated by the negative feedback effects of E_2 (Figure 2). The arcuate (or its homologue) contains kisspeptin neurons in all mammalian species studied thus far, including the mouse and monkey. Kisspeptin release in the median eminence of pubertal monkeys is pulsatile and synchronized to LH release (88). Kisspeptin antagonists inhibit pulsatile GnRH secretion in ovariectomized ewes as well as the post-castration rise in LH in the male rat and mouse (89). MUA in the goat MBH (thought to originate from kisspeptin neurons) correlates with LH pulses, and E_2 inhibits the frequency of both MUA and coincident LH pulses (90). Mice with targeted deletions of *Kiss1r* (*Kiss1r* KO) fail to show the normal rise in

plasma LH in response to ovariectomy (91). E_2 suppresses *Kiss1* expression in the arcuate by acting on ERα, and almost all *Kiss1* neurons express ERα (92). The natural variation of *Kiss1* expression during the estrous cycle of the rat is consistent with these observations, as *Kiss1* mRNA levels in the arcuate are lowest at the time when E_2 levels are highest (93).

As in the mouse, ovariectomy in the ewe induces the expression of *Kiss1* mRNA in the arcuate, and this effect is fully reversed by E_2 replacement (94). E_2 likely affects *Kiss1* expression directly in the ewe, since nearly all kisspeptin immunoreactive neurons in the arcuate also express ERα (95). Smith and co-workers (94) showed that progesterone (P) may also regulate *Kiss1* expression in the ewe. The administration of P to ewes partially reverses the increase in *Kiss1* mRNA that occurs following ovariectomy, and >85% of kisspeptin neurons in the arcuate of the ewe express progesterone receptor (PR). Studies in humans also demonstrated that the expression of *Kiss1* mRNA in the arcuate increases as a function of the reduction of sex-steroid production associated with menopause (96). A similar phenomenon occurs in the female macaque following ovariectomy (96). These results suggest that *Kiss1* neurons in the arcuate of the primate, as in the case of the rodent, are targets for the inhibitory action of sex steroids and thus mediate the negative feedback effects of sex steroids on GnRH secretion.

An oscillatory network of kisspeptin neurons in the arcuate

Kisspeptin neurons in the arcuate may form an oscillatory network that drives pulsatile GnRH secretion. The majority of *Kiss1* neurons in the arcuate coexpress the neurotransmitters neurokinin B (NKB) and dynorphin, as well as the NKB receptor, NK3R (90, 97-101). The interaction of these co-transmitters may allow kisspeptin neurons to form an autofeedback loop using NKB as an excitatory signal that synchronizes their activity and dynorphin as an inhibitor that leads to their coordinated silence. This model is supported by evidence that MUA in the goat MBH coincide with LH pulses, and their frequency is increased by NKB and inhibited by dynorphin (90).

Furthermore, much like mutations in *Kiss1* and *Kiss1r*, mutations in genes that encode *NKB* and *NK3* cause gonadotropin deficiency (102, 103). This defect is thought to result from an impairment in pulsatile kisspeptin release rather than a direct effect on GnRH neurons because NK3R is either low or absent from GnRH neurons (83). Furthermore, kisspeptin release itself is episodic and coincident with GnRH pulses (88). Direct evidence that kisspeptin pulses drive GnRH pulses has yet to emerge. However, the fact that continuous infusion of kisspeptin to male monkeys fails to maintain elevated LH levels, suggests that pulsatile kisspeptin release is required to trigger GnRH/LH pulses (104).

The co-expression of NKB, NK3R, and dynorphin is specific to kisspeptin neurons in the arcuate. *Kiss1* mRNA is also expressed in a more rostral population of hypothalamic neurons, but this rostral population does not co-express any of the genes associated with the pulse-generator in any species examined to date (83). Therefore, kisspeptin neurons in the arcuate are sometimes referred to as KNDy neurons (**K**isspeptin, **N**KB, **D**ynorphin), to distinguish them from the rostral population of kisspeptin cells. Evidence suggests that KNDy neurons regulate the release of GnRH by acting on GnRH axon terminals in the ME, as supported by the juxtaposition of KNDy and GnRH fibers in this region and evidence that kisspeptin stimulates GnRH release from GnRH axon terminals from hypothalamic/ME explants (83, 84, 101, 105).

Positive Feedback of Sex Steroids on GnRH Secretion

Although E_2 inhibits GnRH and LH secretion throughout most of the ovulatory cycle, the rising tide of E_2 in the late follicular phase of the cycle triggers a surge in LH, which induces ovulation. This surge in LH is induced by a burst in GnRH (47, 106) as demonstrated by the fact that large amounts of GnRH are found in the pituitary portal circulation during the LH surge in female rats, ewes, and monkeys (32, 107, 108). Consistent with this finding, GnRH neuronal firing is altered by E_2 positive feedback, and correlates with circulating levels of LH (109).

Distinct mechanisms of positive and negative feedback on GnRH release

The mechanism that triggers the preovulatory GnRH/LH surge is separate from the one that maintains tonic GnRH/LH secretion. In a series of experiments in the late 1940s, Everett, Sawyer, Markee and Hollinshead demonstrated that a neural mechanism triggers ovulation and that in most rodents this mechanism operates by releasing neural hormones from the MBH/ME region (6, 110). However, this region alone is insufficient to induce ovulation, because cutting neural afferents from the anterior hypothalamus to the MBH impairs ovulation in the rat (46). Furthermore, electrical stimulation of the preoptic region of the anterior hypothalamus induces ovulation and electrolytic lesions of this region abolish ovulation (23, 46, 111, 112). Together, these findings led to a model arguing that, at least in the rodent, neurons in the anterior hypothalamus play an essential role in triggering ovulation, whereas those in the MBH maintain tonic GnRH/LH secretion (46). Of course, these lesion studies cannot distinguish between damage caused to GnRH neurons and damage to upstream neurons that drive their activity because GnRH cell bodies are located in the anterior hypothalamus, whereas their axons pass through the MBH. Nevertheless, electrical recordings from the MBH also support the notion that the GnRH surge is mediated by neuronal circuitry that is distinct from the GnRH "pulse generator", because a decrease in GnRH pulse generator activity occurs during both spontaneous and E_2-induced LH surges (47).

Positive feedback in rodents

In the rat and mouse, the ovulatory cycle lasts four to five days and is divided into 4 stages— diestrus 1, diestrus 2, proestrus and estrus, with each stage lasting approximately 24 hours. A "surge" of LH is released into the circulation on the late afternoon of proestrus, which triggers ovulation later that evening. On estrus, the female ovulates and becomes sexually receptive to the male (113). In rodents, this positive feedback effect of E_2 involves E_2-sensitive neurons in the anteroventral periventricular nucleus of the hypothalamus (AVPV), which act directly on GnRH neurons to stimulate the preovulatory surge of GnRH and thus LH (114-116).

ERα mediates the positive-feedback effects of E_2 on GnRH/LH secretion (117). Female mice with either global or neuronal-specific deletions in *Esr1* (the gene that encodes ERα) are incapable of producing an LH surge in response to E_2 (61). Evidence suggests that a population of ERα-responsive cells in the AVPV drives the preovulatory GnRH surge. In the rat, ERα-expressing neurons from the AVPV project to the rostral preoptic area, where GnRH neurons reside (59). In the mouse, GnRH neurons receive direct afferent input from ERα-expressing neurons whose cell bodies reside in the AVPV (61). The PR also plays an important role in the stimulation of the preovulatory LH surge. Female PR KO mice do not display E_2-induced LH surges (118), which indicates that some effects of E_2 lead to the activation of PR. Furthermore, E_2 upregulates PR expression (119). Thus, signaling through ERα-dependent mechanisms in the AVPV appears to be a prerequisite for the generation of the preovulatory GnRH/LH surge in rodents.

A role for Kiss1 neurons in the preovulatory LH surge in the rodent

Although *Kiss1* neurons in the arcuate are implicated in the negative feedback regulation of GnRH/LH secretion, *Kiss1* neurons in the AVPV are compelling candidates for the generation of the positive feedback effects of E_2 (Figure 2) (92, 93, 120-122). In the AVPV of the female, E_2 dramatically induces the expression of *Kiss1* mRNA (92, 93, 120) by acting on ERα, which is expressed in Kiss1 neurons (92). Some ERα-positive neurons in the AVPV— which are plausibly Kiss1 neurons — make direct synaptic contact with GnRH neurons (61). In fact, kisspeptin fibers that are likely to originate in the AVPV contact GnRH neurons, as shown by confocal microscopy (123). On the afternoon of proestrus, there is a robust increase in the expression of *Kiss1* mRNA as well as an increased number of Fos-expressing *Kiss1* cells in the AVPV (93, 120, 121). Although it seems clear that *Kiss1* neurons in the AVPV are activated at the time of the GnRH/LH surge in the rat, there are conflicting reports about whether *Kiss1* neurons in the arcuate also express Fos at the same time (121). Central kisspeptin activity is essential for positive feedback, because central infusion of kisspeptin antibody blocks the LH surge and estrous cyclicity (120, 121). Finally, *Kiss1* expression in the AVPV is sexually differentiated, with males having very few *Kiss1* expressing cells (124). This

observation suggests that this sex difference is at least in part responsible for the inability of male rodents to respond to positive feedback with an LH surge.

These findings suggest that kisspeptin is necessary for generating GnRH/LH surges; however, these findings do not prove that kisspeptin regulation by E_2 in the AVPV is solely responsible for driving the endogenous preovulatory GnRH/LH surge. First, kisspeptin-induced LH release in female rats is enhanced when both E_2 and P are present (*i.e.*, during proestrus, estrus, OVX + E_2 and P) compared with times when P is low or absent (*i.e.*, diestrus 1 and 2, OVX + E_2), suggesting that increased responsiveness of the Kiss1r/GnRH/LH pathway to P may also be an important component in the generation of GnRH/LH surges (125). Second, other periventricular regions that express *Kiss1* may also be involved in positive feedback. *Kiss1* is expressed in the periventricular nucleus (PeN), which lines the anterior portion of the third ventricle and is contiguous with the AVPV. *Kiss1* neurons in this region respond to sex steroids just like *Kiss1* neurons in the AVPV (92). This nucleus also contains ERα-expressing neurons that project to GnRH neurons (61), which may include *Kiss1* neurons (92). Finally, some, but not all, mice with genetically targeted deletions in *Kiss1r* retain the capacity to produce a GnRH/LH surge (91, 126, 127). Thus, although kisspeptin plays an essential role in generating the preovulatory LH surge, other neurotransmitters are also likely to contribute. One of the aims of my research was to determine how much kisspeptin is required for successful ovulation in mice.

Positive feedback in primates and sheep

In primates and ewes, the MBH contains the neural substrates necessary for both driving pulsatile GnRH (and negative feedback of sex steroids) and the GnRH surge (and positive feedback). Unlike rodents, primates have a majority of their neuroendocrine GnRH neuronal cell bodies in the MBH (30, 128, 129). The role of the anterior hypothalamus seems less important in driving GnRH secretion in the monkey, as demonstrated by the fact that monkeys with knife cuts surrounding the MBH still progress through puberty, exhibit spontaneous menstrual cycles and display discharges of LH and FSH in response to estradiol benzoate (29, 30). Moreover, lesions placed in

the anterior hypothalamus fail to delay the age of menarche or first ovulation in female rhesus monkeys (130). Likewise, E_2 acts in the MBH, not the POA, to induce the GnRH surge in ewes (131). Specifically, over one half of the cells in the arcuate express Fos during the LH surge at the end of the follicular phase, although this remains controversial (83).

Evidence suggests that kisspeptin neurons play an important role in generating the preovulatory LH surge in ewes and primates. In the ewe, administration of a Kiss1r antagonist reduces the E_2-induced LH surge (101). It is likely that kisspeptin neurons in the arcuate drive the preovulatory GnRH surge in ewes and primates. In the ewe, these neurons express Fos during the LH surge (83), and in the monkey *Kiss1* mRNA is elevated in the caudal arcuate just before the GnRH/LH surge (132). Kisspeptin neurons likely provide direct input to GnRH neurons in primates and sheep, because close appositions between kisspeptin fibers and GnRH neurons have been identified, particularly in the MBH (99, 101, 128, 132). If kisspeptin neurons are required for the LH surge in primates, the arcuate/infundibular nucleus is most likely involved because knife cuts around MBH do not block the positive feedback action of E_2 (30, 83, 131). The population of kisspeptin neurons that mediate the LH surge in primates and ewes has yet to be identified.

Puberty

Puberty in the primate

The reproductive axis awakens at puberty in response to an increase in the frequency of GnRH pulses (133, 134). In the primate, pulsatile GnRH secretion appears in early infancy, becomes quiescent in juvenile life, and reawakens at puberty (134). During juvenile (or prepubertal) life, GnRH secretion appears to be silenced by neuronal inputs, with only minimal restraint imposed by gonadal steroids. GnRH neurons in prepubertal primates have the molecular machinery necessary to produce and secrete GnRH long before the onset of puberty but for unknown reasons they are suspended in a quiescent state, only to be roused at puberty by upstream circuits, which may reduce

inhibition and/or increase excitation. Consistent with this view is the observation that pulsatile GnRH release can be stimulated in prepubertal animals by several secretagogues, including N-methyl-D-aspartic acid (NMDA), a glutamate receptor agonist, gamma aminobutyric acid (GABA) receptor antagonists, and α-1 adrenergic receptor agonists (134). The receptors targeted in such experiments are likely to play a role in regulating endogenous GnRH secretion, since at the time of its reawakening at puberty, there is an increase in glutamatergic release and a decrease in hypothalamic GABAergic release (133, 135, 136). Stimulation of Kiss1r by kisspeptins also seems to provide essential stimulation of GnRH secretion at puberty, as described below.

Kisspeptin signaling is required for puberty in primates

Kisspeptin signaling is a prerequisite for puberty in the primate, as demonstrated by the fact that humans with homozygous loss-of-function mutations in *KISS1* or *KISS1R* have hypogonadotropic hypogonadism (66, 69-71, 137). Their phenotypes vary from partial to severe hypogonadism with some having cryptorchidism, micropenis and low gonadotropins and others having only low FSH levels with normal LH (138). Although most patients lack development of normal secondary sexual characteristics, some show evidence of low levels of circulating sex steroids, including prepubertal T and partial breast development (138). In some patients (137), researchers reported small LH pulses in patients with homozygous *KISS1R* mutations. Thus, Kiss1r activation may enhance the amplitude of GnRH pulses, rather than play an obligatory role in triggering them. In fact, patients with *KISS1R* mutations achieved fertility after receiving GnRH treatment, including 2 affected females who had successful pregnancies (138). The fact that GnRH treatment is sufficient to rescue fertility in these patients suggests that KISS1R in the gonads is not required for fertility. Kisspeptin itself is required for puberty in humans as demonstrated by the fact that a homozygous point mutation in the conserved receptor-binding region causes hypogonadotropic hypogonadism in all affected sisters of a consanguinous family (71). Furthermore, heterozygous mutations in *KISS1* do not cause any obvious reproductive abnormalities, ruling out severe haploinsufficiency.

Further evidence that kisspeptin contributes to the initiation of puberty in humans comes from the fact that gain-of-function mutations in *KISS1R* or *KISS1* are linked to central precocious puberty (CPP) (139, 140). A heterozygous mutation in the intracellular tail of Kiss1r was identified in an 8-year-old girl with early breast development, accelerated bone growth and other markers of CPP (138, 140). This mutation causes prolonged activation of the Kiss1r in response to kisspeptin treatment and lower rates of internalization from the cell surface, suggesting that the mutation causes a reduction in the rate of desensitization of the receptor (138, 140). It is conceivable that this reduced desensitization could increase the amplitude of GnRH pulses. A heterozygous missense mutation in the *KISS1* gene was also found in a one-year-old boy with precocious puberty (139). This mutation occurred in the N-terminal region of kisspeptin-54, which is important for protein stabilization. It's conceivable that this mutation reduced the degradation rate of kisspeptin. Although this evidence supports the idea that kisspeptin drives puberty onset, only a small fraction of CPP cases are explained by mutations in *KISS1* or *KISS1R* (138).

Evidence suggests that an increase in pulsatile kisspeptin release drives the increase in GnRH pulses at puberty in the primate. Kisspeptin is released in a pulsatile manner from the MBH/ME of female monkeys and the amount of released kisspeptin increases at puberty (83, 88). Furthermore, exogenous kisspeptin leads to precocious release of GnRH and LH in the juvenile male monkey (141). Intermittent kisspeptin administration is more effective at maintaining GnRH secretion in these animals than continuous kisspeptin infusion (104, 142), which produces desensitization of Kiss1r. This suggests that the pulsatile release of kisspeptin is required to sustain GnRH secretion. Furthermore, hypothalamic levels of *Kiss1* mRNA increase across pubertal development in both the male and female monkey (141). The increase in kisspeptin release and synthesis at puberty may be relevant in humans because serum kisspeptin levels correlate with gonadotropin and sex steroid levels in children (143, 144). Hypothalamic expression of *Kiss1r* also increases in the female monkey at puberty (although apparently not in agonadal males) (141), which suggests that the GnRH

neuronal network becomes more sensitive to kisspeptin's effects as a function of development.

Puberty in the rodent

The neuroendocrine mechanisms that control the onset of puberty in rodent species may be fundamentally different from those operating in the primate; however, even these distantly related mammals share important common features of puberty (133, 134). Pulsatile GnRH secretion increases at puberty in the rat, as is the case in the monkey and human (133). Furthermore, as the female rat matures, she gradually gains the ability to show an LH surge in response to E_2, which cannot be triggered before the third week of life (133). Changes in afferent inputs to GnRH neurons may contribute to the increase in GnRH secretion at puberty. GnRH neurons appear to receive an increasing number of excitatory inputs as puberty approaches and metamorphose from cells with smooth outlines to irregular spiny neurons, perhaps reflecting an increase in the number of synaptic inputs (133). GnRH release can be stimulated in prepubertal rats with NMDA, norepinephrine, or NPY (133). Treatment with NMDA or NPY advances puberty, whereas treatment with an NMDA antagonist or an NPY antibody delays the onset of puberty. Since hypothalamic content of both glutamate and NPY and the turnover rate of norepinephrine increase as a function of pubertal development, it seems reasonable to infer that these factors contribute to the activation of GnRH secretion at puberty. Finally, peripheral factors, such as leptin and insulin, that signal adequate nutritional state play a permissive role in the initiation of puberty in both primate and rodent species (133, 134). Despite the evidence that certain neurotransmitters and peripheral signals influence the timing of pubertal maturation, none of the factors or pathways identified so far appear to be *the* critical signal— with one possible exception.

Kisspeptin signaling in rodent puberty

Kisspeptin signaling may be the pivotal event governing the onset of puberty in the rodent, as is the case in primate species. Mice with genetically targeted deletions in *Kiss1r* or *Kiss1* exhibit a similar phenotype to humans with disabling mutations— they do not progress through normal sexual maturation (65, 67-69, 145). A Kiss1r antagonist has been shown to delay vaginal opening (VO) in rats (146). Kisspeptin induces LH and FSH secretion in prepubertal rats and mice (80, 147). However, kisspeptin may be less potent in stimulating LH in the prepubertal animal compared with the adult, which suggests that this pathway may not be fully engaged before puberty (80, 148, 149). Nevertheless, chronic intracerebroventricular (ICV) kisspeptin administration from post-natal day (PND) 26 to PND 31 induces precocious puberty in the female rat, which is reflected by increased sex hormone production, increased uterine weight, and early VO (147).

Kisspeptin/Kiss1r signaling likely increases at puberty. In the rat, maximal expression of hypothalamic *Kiss1* and *Kiss1r* mRNA occurs at puberty (148). *Kiss1* mRNA also increases between the juvenile and adult state in the AVPV/PeN of the male mouse— but not in the arcuate (80). The number of kisspeptin cells [as determined by immunohistochemistry (IHC)] in the AVPV/PeN of the male and female mouse also increases across pubertal development, and in the female, is accompanied by an increase in the number of kisspeptin-containing fibers (114). Upregulation of kisspeptin may enhance GnRH secretion, since the number of kisspeptin-containing fibers in close apposition to GnRH neurons increases across postnatal development (114). In the female rat, upregulation of *Kiss1* expression in the AVPV/PeN may explain the increased ability of the animal to respond to the positive feedback effects of E_2 on gonadotropin secretion as the animal matures. However, whether the increase in *Kiss1* expression in the AVPV/PeN that occurs at the onset of puberty is steroid-dependent or -independent remains uncertain. If the increase in *Kiss1* expression in the AVPV/PeN is steroid-dependent, this phenomenon would be unlikely to represent the proximate cause of pubertal maturation but rather may only reflect the physiological consequences of puberty.

In addition to the putative increase in kisspeptin input to GnRH neurons that may occur as a function of pubertal development, GnRH neurons may also become more sensitive to kisspeptin stimulation. Perforated patch-clamp recordings from GnRH neurons in slice preparations show an increased responsiveness to kisspeptin as a function of pubertal development (80). GnRH neurons from juvenile animals are initially depolarized by kisspeptin, but their response is transient, lasting only 2-3 min. In contrast, GnRH neurons from the adult mouse exhibit a prolonged depolarization, typically lasting as long as the recording (~30 min). *In vivo* results corroborate these *in vitro* observations. In experiments with ICV injections of kisspeptin, only the highest dose of kisspeptin tested (0.1 nmol) induced an LH response in juvenile mice, whereas even the smallest dose tested (10 fmol) increased serum LH levels in adult mice (80). Furthermore, kisspeptin's effect on LH secretion in the adult male rat is more prolonged than in the prepubertal male rat (148). The apparent increase in sensitivity of GnRH neurons to kisspeptin is not likely to result from an increase in *Kiss1r* mRNA in GnRH neurons of the mouse (80). The enhanced sensitivity to kisspeptin may be attributable to post-transcriptional regulation of *Kiss1r* or enhancement of the Kiss1r signaling cascade.

Evidence for kisspeptin-independent sexual development

Some degree of kisspeptin-independent sexual maturation exists in the rodent. Some lines of mice with disrupted kisspeptin signaling achieve VO, a secondary sexual characteristic that depends on estradiol secretion (68). This suggests that synthesis of low levels of estradiol does not require kisspeptin. It is conceivable that this estradiol synthesis results from small, kisspeptin-independent GnRH pulses, as both *Kiss1* and *Kiss1r* KO mice have low gonadotropins that are suppressed by a GnRH antagonist (145) and humans with *KISS1R* mutations have small LH pulses (69). Finally, if the majority of kisspeptin or Kiss1r-expressing neurons is ablated in female mice during development, these females become sexually mature and fertile (150). As a result, some contend that developmental compensation for a lack of kisspeptin neurons occurs if neurons are absent (as opposed to the absence of the neurotransmitter). However,

the experiments upon which these conclusions were drawn left a few kisspeptin neurons intact, suggesting that reproductive development in these animals could be sustained by just a few kisspeptin neurons. To address this controversy, one of the key questions of my research was to determine whether low kisspeptin levels are sufficient to sustain sexual maturity.

Which population of kisspeptin neurons initiates puberty?

There is some debate about whether the rostral or caudal population of Kiss1 neurons is responsible for initiating puberty. Clarkson and Herbison argue that the rostral population in the AVPV and PeN in the rodent (which they collectively call the R3PV) is responsible because kisspeptin expression increases over pubertal development in the AVPV/PeN but not in the arcuate (151). It is conceivable that kisspeptin release from these neurons could drive a positive feed-forward loop that increases synthesis of gonadal steroids and kisspeptin release, based on the fact that gonadal steroids stimulate *Kiss1* expression in this region (92, 122). However, increases of *Kiss1* expression in the AVPV/PeN at puberty could be a consequence of increasing sex steroid exposure rather than a cause (84, 89, 152, 153).

Other evidence argues against a primary role of the rostral population in puberty initiation. Males have limited *Kiss1* expression in the AVPV during development and in adulthood, but they still initiate puberty (124, 154). Furthermore, unleashing arcuate kisspeptin neurons from negative feedback inhibition through deletion of ERα leads to early VO and an increase in *Kiss1* expression in the arcuate, while *Kiss1* expression in the AVPV/PeN remains low (155). These results suggest that activity of arcuate neurons correlates with the initiation of sex-steroid synthesis (83). However, the increased activity of arcuate neurons is not sufficient to fully initiate puberty, since these females fail to ovulate, most likely because they cannot activate *Kiss1* neurons in the AVPV. Finally, the control of *Kiss1* expression in the arcuate mirrors the mechanism of puberty in monkeys and rodents. E_2 inhibits kisspeptin expression and LH secretion in juvenile rats, but the increase in kisspeptin during puberty is steroid-independent in male monkeys (83, 141). In the primate, kisspeptin neurons located in the infundibular

nucleus (homologue of the arcuate nucleus in the rodent) are thought to drive puberty onset. Brain lesions in the anterior hypothalamus or knife cuts that interrupt inputs from the anterior hypothalamus do not delay age at menarche or first ovulation (83, 130, 156, 157), suggesting that kisspeptin neurons in the anterior hypothalamus are not required for puberty.

Sexual Differentiation

Activational effects of sex steroids

Steroid hormones produced by the gonads have activational effects on the brain in adult animals and organizational effects during development that program adult responses to sex steroids (158). The first experiment demonstrating the activational effects of sex steroids on the brain was conducted in 1849 by Berthold (3, 159), who is considered by many to be the founder of endocrinology. He found that roosters with removed testes stopped mating with hens, crowing, and fighting aggressively with other males. Remarkably, he found that if he transplanted testes back into the castrated roosters, their male sexual and aggressive behaviors were restored. Based on the finding that the transplanted testes regenerated blood vessels, Berthold deduced that the testes release substances into the bloodstream that impact the behavior of the whole organism, including the brain. We now know that those substances are T and other sex steroids.

Activational effects of sex steroids on Kiss1 expression

Sex steroids inhibit *Kiss1* expression in the arcuate and stimulate its expression in the AVPV/PeN (92, 122). In the male, T inhibits *Kiss1* expression in the arcuate through both ERα and AR, as demonstrated by the inhibitory actions of E_2 and dihydrotestosterone (DHT), the 5-α-reduced metabolite of T, respectively (122). T stimulates *Kiss1* expression in the AVPV/PeN of the male only through ERα after its conversion to estradiol. In the female, both the inhibition of *Kiss1* in the arcuate and its stimulation in the AVPV/PeN require ERα, but not ERβ (92). The opposite effects of E_2

in the AVPV/PeN *vis-à-vis* the arcuate can be explained by the differential role of classical and non-classical ERα signaling in these regions (160). In the AVPV, ERα binds to the estrogen-response element to stimulate *Kiss1* expression, whereas in the arcuate E_2 works through a non-classical pathway, whose molecular details have yet to be elucidated. Furthermore, in the AVPV E_2 increases ERα binding and histone H3 acetylation at the *Kiss1* promoter but in the arcuate E_2 decreases both of the latter events (161). In either case, increased histone H3 acetylation is thought to facilitate *Kiss1* transcription (161).

Organizational effects of sex steroids

Sex steroids organize the brain during critical periods of development. The first critical period determines gonadal sex by the expression of the *SRY/Sry* gene on the Y chromosome. *SRY/Sry* is expressed in the first weeks or days of gestation in humans or rodents, respectively (162), when it initiates formation of the testes, which secrete T. Formation of the testes promotes survival of the male urogenital tract and the active suppression of the female tract. In contrast, in the absence of *SRY/Sry*, the gonad becomes an ovary and the male system degenerates due to a lack of androgen (162). Kisspeptin may stimulate T production during fetal sexual development, as several male patients with *KISS1R* mutations were born with cryptorchidism and micropenis (70, 137, 138, 140, 163).

Sexual differentiation of the brain occurs after sex determination, in response to gonadal hormones. Animals with long gestational periods, such as primates, experience this critical period *in utero*, whereas those with short gestations, such as rats and mice, experience a neonatal critical period that ends after the first week of life (164). During this period, T or its aromatized product, E_2, masculinize hypothalamic nuclei essential to the preovulatory surge in GnRH and LH (in rodents but not primates). Male rats that are castrated at birth display E_2-induced LH surges (165), whereas females treated with T during this same period are unable to surge and remain permanently infertile (165-167). Barraclough and Gorski demonstrated that this organization takes place at the level of the medial preoptic area, rather than that of the anterior pituitary (167) by showing that

androgenized females lose the ability to ovulate in response to stimulation of this region. Since then, the AVPV has been specifically identified as the sexually differentiated nucleus essential for the preovulatory LH surge (106).

The organizational effects of T during critical periods occur directly through T or DHT binding to the androgen receptor (AR) or through aromatized products, such as E_2 and activation of the ERα (162, 164). These steroid hormones can affect neurogenesis, apoptosis, the degree of dendritic arborization, and synaptic density between the sexes (162, 168). In addition, specific genes can be epigenetically modified such that their ability to be expressed becomes imprinted in adult animals (162, 169, 170).

Following the initial critical period of sexual differentiation, the nervous system retains some degree of plasticity in response to sex steroids during puberty (158). Male hamsters that are deprived of testicular hormones during puberty have reduced sexual behavior responses to T in adulthood (171). In contrast, female hamsters that are deprived of ovarian steroids during puberty display female-typical sex behavior sooner after exposure to a male than controls. Organizational effects of sex steroid hormones during puberty extend beyond sexual behavior, since sex differences in many brain regions develop during adolescence in primates and rodents (158).

Species differences in the mechanisms of sexual differentiation

Species differ in the mechanisms of sexual differentiation. In rodents, neonatal exposure to E_2 (as a result of aromatization of T) prevents animals from responding to high E_2 levels with a GnRH/LH surge in adulthood (164, 172, 173). Newborn males have 2-3 times more E_2 in the hypothalamus relative to females, but this does not apply to brain regions outside the hypothalamus (162), suggesting that T is locally aromatized to E_2. In contrast, the ability to respond to E_2 with a GnRH/LH surge is not sexually differentiated in the primate (164, 174). Treatment of males with a 5-α-reductase inhibitor during gestation alters male genitalia, but not the ability to have an LH surge (164). Furthermore, androgens are the primary determinants of brain masculinization in the primate, although brain aromatization does play a role (175, 176). Sexual behavior

is also highly dependent on sexual differentiation of the brain during critical periods in both rodents and primates. If female rhesus macaques are exposed to high levels of androgens during the critical period of sexual differentiation *in utero*, they later display an increase in male-typical behavior and a decrease in female-typical behavior (164, 176).

Kisspeptin expression in the AVPV is organized by sex steroids

Evidence suggests that sexual differentiation of the LH surge in rodents results from organizational effects of sex steroids on *Kiss1* expression in the AVPV/PeN. Female rodents have ~10 times more kisspeptin-expressing cells in the AVPV/PeN than males (83, 124, 177). This sex difference persists even when adult sex steroids are controlled for, ruling out activational effects as an explanation. In fact, there is evidence that the sexual differentiation of kisspeptin neurons in the AVPV/PeN results from organizational effects of T during the neonatal critical period. If female rats are neonatally treated with T, the number of *Kiss1*-expressing cells is reduced, even if they are treated with E_2 as adults (124, 177). Furthermore, neonatal castration of male rats increases the number of *Kiss1* mRNA and kisspeptin-expressing cells in the AVPV/PeN (177) and enables males to have an E_2-induced LH surge. Finally, Kiss1r KO males have a feminized AVPV, which could result from a lack of sex-steroid exposure during the neonatal critical period (178).

The organizational effects of T are likely to be mediated by the aromatization of T to E_2, based on the fact that treatment of neonatal females with E_2 also suppresses *Kiss1* expression and the capacity to have an LH surge (177). Furthermore, male mice with mutations in *Cyp19a1* (the gene that encodes aromatase), which cannot convert T to E_2, have more kisspeptin in the AVPV than their WT counterparts, supporting a role for neonatal E_2 in masculinization (179, 180). Thus, neonatal T, likely acting through aromatization to E_2, masculinizes *Kiss1* expression in the AVPV and the ability to generate an LH surge. In addition, E_2 may feminize *Kiss1* expression in this region during puberty. *Cyp19a1* KO females have fewer kisspeptin cells in the AVPV than WT females, in spite of E_2 treatment in adulthood. Furthermore, treating the KO females

with E_2 during puberty rescues kisspeptin expression (179, 180). These results suggest that E_2 exposure is required during puberty to allow full kisspeptin expression in adult females.

Potential mechanisms of masculinization in the AVPV/PeN

Several hypotheses have emerged about the mechanism of sexual differentiation of *Kiss1* expression in the AVPV/PeN. First, E_2 (converted from T) could cause apoptotic cell death of *Kiss1* neurons. This hypothesis is based on the fact that the AVPV has fewer total cells in males than in females, and that this sex difference requires Bax-mediated apoptosis (181). However, sexual differentiation of Kiss1 neurons persists in *Bax* KO animals (154), demonstrating that Bax-dependent apoptosis is not responsible. Nevertheless, this finding does not rule out Bax-independent apoptosis. Second, it is conceivable that neonatal E_2 prevents neurogenesis of Kiss1 neurons in males that normally occurs in females. Although there is some evidence of post-natal neurogenesis in the olfactory bulb and hippocampus (169), AVPV neurons are born during fetal life, making differential neurogenesis a less convincing possibility. Third, *Kiss1* gene expression could be epigenetically suppressed by T or E_2 in males or enhanced in females. Semaan *et al*. recently investigated several epigenetic mechanisms for sexual differentiation of *Kiss1* expression in the AVPV. They did not find evidence for sexual differentiation by a histone-acetylation mechanism, which usually enhances transcription by relaxing chromatin (182). However, they found sex differences in CpG methylation, which is thought to prevent binding of transcription factors or RNA polymerase to regulatory regions (182). Male mice had less methylation than females at CpG sites in the *Kiss1* promoter and first intron of all cell types from the AVPV/PeN, suggesting that males have more transcriptional repressors binding than females (169, 182). One of the aims of my research was to determine whether the sex difference in *Kiss1* expression in the AVPV reflects a difference in cell number (due to apoptosis in males or neurogenesis in females) or a difference in gene expression.

Comparison of Kiss1 *expression between the sexes in the arcuate*

Although some sex differences have been reported for *Kiss1* expression in the

arcuate, they are less clear and more difficult to interpret than those in the AVPV/PeN. *Kiss1* mRNA does not differ between adult male and female rodents and the total number of *Kiss1*-expressing neurons is not affected by neonatal treatment with T (124). However, during post-natal development, females have more kisspeptin fibers and cells in the arcuate than males (180) and part of this difference depends on the presence of E_2. Female *Cyp19a1* KO mice have less kisspeptin immunoreactivity in the arcuate than WT females during post-natal development (180). Furthermore, prepubertal female mice have a gonadal restraint on *Kiss1* expression in the arcuate, whereas males have a central restraint, as demonstrated by the fact that gonadectomy increases *Kiss1* mRNA expression and LH release in juvenile females but not males (183). The higher expression of kisspeptin in the juvenile arcuate (and possibly the AVPV/PeN) of females relative to males could be an underlying cause for earlier puberty onset in female mammals.

Relevance of sexual differentiation to humans

There are many disorders of sexual development that result from abnormal sexual differentiation. For example, girls with congenital adrenal hyperplasia produce excessive androgens (143) and can develop precocious puberty (which is also seen in rodents exposed to androgens). This could result from abnormal stimulation of the reproductive axis. Girls with mutations that cause aromatase deficiency exhibit boy-typical behavior and male gender identity from an early age (143). A similar phenotype is found in female mice with congenital aromatase insufficiency. Interestingly, the likelihood of developing some reproductive disorders is also sexually differentiated. Precocious puberty is 10 times more common in girls than in boys (184) whereas hypogonadotropic hypogonadism is 5 times more common in boys than in girls (143, 185).

Sexual differentiation of the kisspeptin system in humans

Evidence suggests that kisspeptin expression is sexually differentiated in humans. Females have more kisspeptin in the infundibulum (equivalent of the arcuate) than males (128) and healthy pubertal girls have elevated serum kisspeptin when

compared to developmentally-matched healthy boys (186). Although direct evidence for this notion has not emerged, it is conceivable that sex differences in *Kiss1* expression could contribute to earlier puberty onset in girls than boys as well as to the discrepancy in the incidence of precocious puberty and idiopathic hypogonadotropic hypogonadism between boys and girls.

Expression of Kisspeptin Outside the Hypothalamus

Although *Kiss1* expression has been identified in several extra-hypothalamic brain regions (and outside the brain), its function in these regions is not yet known. *Kiss1* mRNA is expressed in the medial amygdala (76, 187), the Bed Nucleus of the Stria Terminalis (BNST) (76, 132), the dentate gyrus of the hippocampus (Goodman & Lehman review), and layers 2/3 and 6 of the cerebral cortex (188). Expression in the medial amygdala and BNST raises the possibility that kisspeptin has a role in the pheromonal control of sex behavior and LH secretion (83). Other functions of kisspeptin expression outside the hypothalamus do not, however, seem to be essential, as no known abnormalities have been reported in either humans or mice with *KISS1/Kiss1* or *KISS1R/Kiss1r* mutations besides those related to reproduction. Nevertheless, close investigation of behavior in animals with these mutations has not been examined to date.

Statement of the Problem

Characterization of Kiss1-CreGFP mice

Animals that express Cre recombinase under the control of a particular promoter are designed to gain specificity in activation or disruption of genes containing loxP DNA sequences (189). This tool has revolutionized our understanding of the functions of genes in specific cells. However, recent studies demonstrate that caution must be taken in interpreting the results of studies using Cre-expressing animals because Cre recombination is carried forward through developmental cell lineages, sometimes in cells with opposite physiological functions (190, 191). Furthermore, Cre recombination may be more widespread than anticipated as a result of low-level promoter activity that

is not detectable by methods such as *in situ* hybridization or IHC. To specifically manipulate gene expression in Kiss1 neurons, Dr. Robert Steiner's laboratory generated a *Kiss1-CreGFP* knock-in mouse in collaboration with Dr. Richard Palmiter (192). This mouse expresses Cre recombinase and GFP under the endogenous *Kiss1* promoter, making it possible to see GFP only when *Kiss1* is expressed. My first set of goals were to evaluate the predictions made about GFP expression and to characterize the extent of Cre recombination in *Kiss1-CreGFP* knock-in mice. This information was essential for planning future experiments requiring manipulation of genes through Cre recombination.

Analysis of the quantitative requirement for Kiss1 *expression for reproductive competence*

Over the past decade evidence has accumulated to support the idea that GnRH neurons require direct stimulation by kisspeptin to drive reproduction—both to initiate puberty and coordinate reproductive function in the adult (65, 66, 68, 69, 71, 76, 77, 80, 145). However, a recent study cast doubt on whether Kiss1 neurons are absolutely necessary for reproductive function (150). In that study, *Kiss1*-expressing cells in the brain (and elsewhere in the body) were congenitally ablated in *Kiss1-Cre* mice using a diphtheria toxin-mediated strategy— yet, female mice that bore this lesion exhibited normal puberty onset and fertility. These results suggest that Kiss1 neuron-dependent signaling in the brain is either dispensable for reproduction or that some residual action of Kiss1-GnRH signaling persists in those animals. I disputed the idea that compensation is sufficient to explain the phenotype of mice with putative ablations of their Kiss1 neurons and argued that expression of *Kiss1* by a few remaining neurons explains their reproductive phenotype.

In light of the controversy about the requirement for kisspeptin in reproduction, I hypothesized that kisspeptin signaling is safeguarded by production of kisspeptin far in excess of what is required to support reproductive function. There is precedence for the notion of superfluity in other cellular and molecular features of the neuroendocrine reproductive axis. For example, ~10% of the normal population of GnRH neurons is

sufficient for reproduction (150, 193-195), so it seems plausible that an over-production of kisspeptin represents yet another fail-safe to guarantee success. To test this hypothesis, I examined the reproductive phenotype of mice with a 95% reduction in *Kiss1* transcript levels.

Investigation of the mechanism of sexual differentiation of the AVPV/PeN

Although it is well established that female rodents express more *Kiss1* in the AVPV/PeN than males as a result of neonatal exposure to T, the mechanism by which T permanently diminishes *Kiss1* levels in males remains to be elucidated. It is not known whether the difference in *Kiss1* – expressing cells results from different numbers of Kiss1 neurons (as a result of apoptosis in the male or neurogenesis in the female) or from different expression levels in equivalent numbers of neurons. I tested the hypothesis that *Kiss1* expression in the male is diminished as a result of reduced *Kiss1* expression rather than apoptosis or neurogenesis. I used two approaches to test this hypothesis. First, I compared the number of cells that ever expressed *Kiss1* between males and females in the AVPV/PeN by crossing the *Kiss1-CreGFP* mouse to a *tdTomato* reporter mouse, thus marking cells red forever after *Kiss1* mRNA is expressed. Second, I used a more sensitive *in situ* hybridization approach to determine whether male mice expressed low levels of *Kiss1* mRNA in a large number of cells in the AVPV/PeN. Since the results of these first experiments were consistent with the hypothesis, I set out to hone in on the timing of *Kiss1* expression in the male AVPV by examining tdTomato (cumulative *Kiss1*) expression in prepubertal males.

Chapter II. Materials and Methods

All animal procedures were approved by the Institution of Animal Care and Use Committee at the University of Washington, in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Analysis of the Quantitative Requirement of *Kiss1* Expression for Reproduction

Kiss1-CreGFP mice

The *Kiss1-Cre* knock-in mouse has a *CreGFP* cassette inserted upstream of the translation start site for *Kiss1*, which retains an in-frame coding sequence (Figure 3). Sequences that facilitate endonuclear cleavage of the primary transcript and promote polyadenylation are inserted on the 5' end of the *Kiss1* gene (but 3' of the *CreGFP gene*), resulting in low *Kiss1* transcript levels. Heterozygous *Kiss1-CreGFP* mice ($Kiss1^{Cre/+}$) were backcrossed onto the C57Bl/6J background two or five generations and bred to homozygosity to produce $Kiss1^{Cre/Cre}$ mice. WT littermates were always used as controls to reduce effects of genetic background. Animals were housed in a 14:10 light:dark cycle. *Kiss1-CreGFP* knock-in mice are described in detail in (192).

Quantitative RT-PCR

Concentrations of total RNA were measured with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Asheville, NC), and samples were diluted to equal concentrations. *Kiss1* reactions were amplified with the use of Taqman Gene Expression Assays (Applied Biosystems/Life Technologies, Foster City, CA), with a primer/probe set designed to span the intron between the 2 coding exons of the *Kiss1* gene and one step Brilliant II QRT-PCR Master Mix (Agilent Technologies, Santa Clara, CA). *Tac2, Tacr3, Pdyn* and *Actb* were amplified by using one step SYBR Brilliant II QRT-PCR Master Mix and the primer sets listed in Table 1. All qRT-PCR reactions were conducted with a MX3000P PCR machine (Stratagene, La Jolla, CA). For PCR protocols, see Table 2. Each control, standard and unknown was amplified in duplicate. Negative controls included: 1) reactions without reverse transcriptase; 2) reactions

without RNA; 3) RNA from complete *Kiss1*-KO animals (68). The standard curve was made by serial dilution of RNA from WT gonadectomized (GDX) animals and assigning arbitrary values based on known concentrations of total RNA. The efficiency of all standard curves was between 85% and 115%. For each unknown, values were derived from the standard curve. Then, each unknown value was normalized to values from amplification of *Actb* to control for small variations in RNA concentration among samples. Finally, these normalized values were reported as a percent of the mean WT value. *Actb* expression did not change in response to treatments or genotypes.

IHC for kisspeptin

Brains were post-fixed overnight in paraformaldehyde (PFA), cryopreserved in 30% sucrose in PBS, and sectioned at 35 µm. Free-floating sections were incubated at a 1:3000 concentration of AC053 antibody from Alain Caraty for 5 days at 4°C. The secondary antibody was an Alexa-555 donkey anti-sheep antibody (1:250) (Invitrogen, Carlsbad, CA). Sections were imaged with a Zeiss LSM5 Pascal confocal microscope.

Peptide extraction for kisspeptin radioimmunoassay

The MBH or an equivalently sized piece of cerebellum (to use as a negative or recovery control) was cut using a mouse brain matrix (Zivic Labs, Pittsburgh, PA), stored in 2 ml Eppendorf tubes, frozen on Dry Ice, and stored at -80°C until the tissue was processed.

Peptide purification

Tissue was transferred into 2 ml of 0.5 M boiling acetic acid in a glass 7 ml Dounce homogenizer. One half of the cerebellar tissue samples received 1 or 5 µg kisspeptin-52 as a positive control for the recovery of kisspeptin. The tissue was boiled for 7 min, homogenized in the acetic acid, transferred to Corex glass tubes and centrifuged at 3,000 g for 30 min at 4°C. The top layer was collected with a 2.5 ml syringe and frozen at -20°C for later use. The homogenate was thawed and applied to activated Sep-Pak R Plus C18 Columns (Waters). The columns had been activated with 10 ml methanol followed by 20 ml of distilled water, applied slowly using syringes. The

acetic acid mixture containing the peptides was loaded onto the equilibrated column, the column was washed with 10 ml 4% acetic acid and the peptides were eluted with 2 ml methanol into a glass tube. Each step was performed very slowly but also sequentially so that the column never dried. The eluate was dried with a centrifugal concentrator. The tubes with the residue were stored at -20°C with desiccant until the next day.

Delipidation

To delipidate the extracts, each was dissolved in 1 ml of water with 0.1% trifluoroacetic acid and transferred to a clean Corex tube. Three ml of dichloromethane were added and the mixture was vortexed 3 times for 10 sec each time. The mixture was centrifuged at 3,000 g for 30 min at 4°C to separate into 2 phases. The bottom phase, containing the dichloromethane and lipids was removed with a long needle. The delipidation steps were repeated for a total of 4 times and the last upper phase was dried with a centrifugal concentrator. The tubes were sent to Dr. Caraty in France, where he performed the RIA as described in (196).

ICV injections

ICV injections were conducted as previously described (197, 198). On the day prior to beginning vaginal smears (at least 8 days prior to the injection), a hole was made in the skull while mice were under isofluorane anesthesia. The skull was manually perforated at a point that was 1.5 mm lateral and 0.5 mm posterior to Bregma, with a 27-gauge needle covered with polyethylene tubing, leaving 3.5 mm of the needle's tip exposed. On the day of the injections, 2 µl of either senktide or vehicle solution were injected over a period of 30 sec through the hole. For ICV injections of the kisspeptin antagonist, peptide-234, female mice were OVX and a hole was poked in the skull 7 days prior to the injections. After the injection, the needle was kept in place for 1 min to limit backflow through the needle's track.

Hormone assays

Serum measurements for LH, FSH and T were conducted in duplicate at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core. For specific assay information, reportable ranges and intra-assay coefficients of variation, see Table 3.

Statistical analyses

GraphPad Prism software was used for all analyses, except for the Fisher's exact test, for which look-up tables were used (199). All data are represented as the mean ± SEM. For normally distributed data, an ANOVA was used for comparisons among more than 2 groups with Tukey's post-hoc test. For hormone assays wherein some samples fell outside the reportable range, a Kruskal Wallis non-parametric test was used. A two-way ANOVA (group vs. treatment) with Bonferroni correction was used to analyze LH levels in Experiments 6 and 10. In Experiment 7, differences between genotypes were assessed by survival analysis with the Log-rank Mantel-Cox test for VO and PPS and two-way repeated-measures ANOVA for body weight. In Experiment 8, comparison of the fraction of mice within each group that had pups was made with the Fisher's exact test. For comparison of the number of pups per litter, data were only included if animals had one or more pups. In contrast, the average number of corpora lutea (CL) did include animals with zero CL. A comparison of litter size among genotypes was made with an unpaired t-test.

Investigation of the Mechanism of Sexual Differentiation of the AVPV/PeN

tdTomato reporter mice

The *tdTomato* gene encodes a bright red fluorescent protein that diffuses throughout the cytoplasm and is detectable in neuronal processes. The reporter mice express this gene under the control of a constitutively active promoter only if Cre recombinase has excised a transcriptional stop cassette upstream of the tdTomato gene (Figure 4). Once Cre has excised the stop cassette, tdTomato will be expressed forever in that cell and all of its daughter cells. tdTomato reporter mice are described in detail in (200). Animals with the Ai14 reporter were housed in a 14:10 light:dark cycle.

Comparison of tdTomato expression between adult males and females

Kiss1$^{Cre/+}$;*tdTomato*$^{+/-}$ animals were perfused with 4% PFA. Brains were post-fixed overnight in PFA, cryopreserved in 30% sucrose in PBS, sectioned at 35 µm, mounted with Prolong Gold anti-fade reagent (Invitrogen), and imaged using a Nikon Upright microscope. For counting, the regions were outlined using the "selection" feature in Fiji based on the Paxinos and Franklin Mouse Brain Atlas. The same selections were used for all images, and the selection placement was adjusted using the ventral edge and the third ventricle for reference. Cells were counted manually by two researchers who were unaware of the animals' sex using Fiji Cell Counter and their counts were averaged.

Kiss1 in situ hybridization

Wild type (WT) or *Kiss1* KO males (65) (n = 3-4 per group) were sacrificed and their brains were immediately frozen on Dry Ice and sectioned at 20 µm. Sections were stored at -80°C until processed for *in situ* hybridization with 0.7 pmol/ml *Kiss1* cRNA probe described in (76). After post-hybridization washes, sections were dipped in photographic emulsion and stored for 7 days until they were developed and coverslipped. Images were taken of WT and KO sections at the same settings on a Leica microscope.

Developmental comparison of tdTomato expression between males and females

Kiss1$^{Cre/+}$ mice were mated to *tdTomato*$^{+/-}$ mice. Offspring were genotyped and trans-cardially perfused at post-natal day 12 with 4% PFA. Tissue was stained with an anti-GFP antibody as in (192) to distinguish between cells that currently express *Kiss1* (via GFP) and those that have expressed *Kiss1* prior to the collection time point (via tdTomato). Briefly, sections were stained overnight with a rabbit anti-GFP antibody (Invitrogen) at a 1:1000 concentration at 4°C, followed by an Alexa-488 anti-rabbit antibody (Invitrogen) at 1:500 for 2 hours at room temperature. Sections were mounted and imaged on a Nikon upright microscope.

Chapter III. Experimental Design and Results

Characterization of *Kiss1-CreGFP* Mice

Experiment 1: Validation that CreGFP expression is regulated by the Kiss1 promoter

Sex steroids inhibit *Kiss1* mRNA expression in the arcuate and stimulate it in the AVPV (92, 122). The gene for a CreGFP fusion protein was knocked in to the *Kiss1* locus upstream of the *Kiss1* open reading frame (192). Thus, if *CreGFP* is regulated by the *Kiss1* promoter, then GFP expression should also be inhibited by sex steroids in the arcuate and stimulated in the AVPV. The purpose of this experiment was to verify that GFP is regulated by sex steroids in the same way as *Kiss1*.

Methods: In collaboration with Michelle Gottsch and Elisenda Sanz, female mice were either GDX or GDX and treated with E_2 as described in (192). One week later, animals were perfused, brains were collected, post-fixed and sectioned at 35 µm. Sections were imaged with a Leica confocal microscope for endogenous or antibody-stained GFP in the arcuate or AVPV, respectively.

Results: As expected, GDX females had higher GFP expression in the arcuate than GDX + E_2 treated females (Figure 5A & B). In contrast, GDX + E_2 treated females had higher expression of GFP immunoreactivity in the AVPV than GDX females, where GFP was barely detectable (Figure 5C & D). GFP fluorescence was nuclear, reflecting localization of Cre, which is targeted to the nucleus. These results indicate that the expression of the CreGFP fusion protein in the *Kiss1-CreGFP* mouse is regulated by sex steroids similar to *Kiss1* mRNA.

Experiment 2: Mapping Cre recombination in adult Kiss1-CreGFP mice

The purpose of this experiment was to identify the extent of Cre recombination in the brain to inform experimental design of genetic manipulations in Kiss1 neurons.

Methods: *Kiss1*$^{Cre/+}$ animals were mated to *tdTomato*$^{+/-}$ animals. Adult offspring were genotyped (n=8 males and 8 females) and the brains of mice that were heterozygous for both the Cre and the tdTomato alleles (*Kiss1*$^{Cre/+}$*; tdTomato*$^{+/-}$) or control animals that lacked the Cre allele (*Kiss1*$^{+/+}$*; tdTomato*$^{+/-}$) were collected from perfused animals. Examples of whole brains were imaged on a dissecting microscope. The brains were sectioned at 35 µm and sections were examined on a Nikon fluorescent microscope with a 1X objective.

Results: Control *Kiss1*$^{+/+}$*; tdTomato*$^{+/-}$ mice lacked tdTomato labeling in the brain, demonstrating the requirement of Cre for *tdTomato* expression. The extent of tdTomato labeling in *Kiss1*$^{Cre/+}$*; tdTomato*$^{+/-}$ mice was bimodal. About one half of the animals (4/8 males and 5/8 females) had pervasive tdTomato labeling in the brain (Figure 6A middle and 6B) and periphery (not shown). In coronal sections of the cerebrum from these animals, individual cells were not clearly visible due to pervasive labeling in cells and blood vessels (Figure 6B). However, the cerebellum of these animals had more restricted labeling making distinct neurons visible (not shown). The remaining animals had more restricted expression in the brain and periphery. These animals had clear, discernible cells in sub-regions of the hypothalamus, including the arcuate and AVPV/PeN (Figure 6C). However, even in these animals, tdTomato was present in many cells and fibers outside the hypothalamus, including the cerebral cortex, hippocampus, BNST, corpus callosum, anterior commissure and others (Figure 6C). No obvious sex differences appeared in the distribution of tdTomato labeling, with the surprising exception of more lateral labeling in the male AVPV and medial portion of the medial preoptic nucleus (MPOM) described in Experiment 12.

Experiment 3: Mapping Cre recombination in prepubertal Kiss1-CreGFP *mice.*

The purpose of this experiment was to determine whether tdTomato labeling outside the hypothalamus represented developmental *Kiss1* expression.

Methods: Four pairs of *Kiss1*$^{Cre/+}$ animals and *tdTomato*$^{+/-}$ animals were mated as above and offspring were genotyped and perfused at PND 12, brains were post-fixed,

sectioned, and observed under a Nikon fluorescent microscope as above. Of the 7 animals that were *Kiss1*$^{Cre/+}$*; tdTomato*$^{+/-}$, 4 were females and 3 were males.

Results: At PND 12, the extent of tdTomato labeling in the brain fell on a continuum (Table 4 and Figure 7), with one animal having pervasive labeling and indistinguishable cells (like group 1 in the adults), two having distinct but abundant cells in extra-hypothalamic regions, and the rest having restricted labeling, primarily in the hypothalamus with only a few dispersed cells in extra-hypothalamic regions including the cerebral cortex and hippocampus.

Analysis of the Quantitative Requirement of *Kiss1* Expression for Reproduction

Experiment 4: Quantification of Kiss1 transcript in Kiss1$^{Cre/Cre}$ animals

The purpose of this experiment was to assess levels of *Kiss1* mRNA and gonadotropins in *Kiss1*$^{Cre/Cre}$ and littermate control mice. To quantify the extent to which *Kiss1* transcript was suppressed in *Kiss1*$^{Cre/+}$ and *Kiss1*$^{Cre/Cre}$ mice, I performed qRT-PCR on the MBH of intact males, GDX males and females and on the AVPV/preoptic area of GDX + E_2 treated females.

Methods: To maximize *Kiss1* expression in the MBH, mice of each genotype were GDX (n = 6-8 females and 4-5 males/group). To measure *Kiss1* expression in the AVPV, a different subset of female mice (n = 6/group) were GDX and received an implant containing 20 µl of 1 mg/ml 17 β-estradiol dissolved in sesame oil. Implants were made from silastic tubing having 1.47 mm inner diameter and 1.96 mm outer diameter, cut to 15 mm in length (Dow Corning, Elizabethtown, KY) and sealed with silicone adhesive sealant 2.5 mm away from each end. One week after surgery, blood was collected from the orbital sinus for gonadotropin measurements. Brains were removed, after which the MBH or AVPV/preoptic area were cut in a mouse brain matrix (Zivic Labs, Pittsburgh, PA), frozen on Dry Ice, and stored at -80 C until RNA was extracted with a Qiagen RNeasy Lipid Tissue Kit (Qiagen, Valencia, CA). QRT-PCR was performed as described in the qRT-PCR section.

Results: In GDX males, *Kiss1*$^{Cre/+}$ mice had 43.1 ± 4.5 % of the *Kiss1* transcript found in WT mice, and *Kiss1*$^{Cre/Cre}$ males had only 5.7 ± 0.4% of the WT transcript in the MBH ($p< 0.0001$ one way ANOVA; $p < 0.05$ for all comparisons Tukey's post-hoc test) (Figure 8A). The same pattern was observed in the MBH of intact males (Figure 8B) and GDX females (Figure 8C) (intact males $p < 0.005$; GDX females $p < 0.0001$ one way ANOVA). The level of *Kiss1* transcript in the MBH was approximately 10 times higher in GDX vs. intact animals for each of the 3 genotypes (Table 5), consistent with the known response of *Kiss1* expression to gonadal steroids (92, 122). In the preoptic area/AVPV region, GDX + E_2-treated *Kiss1*$^{Cre/Cre}$ females had 17.0 ± 10.2% of the *Kiss1* transcript

found in their WT littermates treated in the same manner (Figure 8D; $p < 0.05$ with two-tailed t-test). Neither the samples from *Kiss1* KO animals (68) nor those lacking reverse transcriptase had any amplification after 45 cycles, confirming that the amplification product was derived from *Kiss1* mRNA. LH levels confirmed the sex steroid treatment of the animals (e.g. GDX males had elevated LH levels relative to intact males and GDX + E_2 treated females had LH levels below the limit of detection). For LH levels in intact and GDX males, see the section on adult phenotype (Figure 11B and Table 5). These findings demonstrate that the *Kiss1* transcript is dramatically reduced in *Kiss1*$^{Cre/Cre}$ animals, albeit present at very low levels.

Experiment 5: Detection of kisspeptin by IHC

To determine whether kisspeptin peptide levels were reduced in parallel to the *Kiss1* transcript in *Kiss1*$^{Cre/Cre}$ mice, I performed IHC on tissue containing the arcuate from GDX WT, *Kiss1*$^{Cre/Cre}$ and *Kiss1* KO mice with an anti-kisspeptin antibody.

Methods: This antibody was raised in sheep against a 14-amino-acid-peptide fragment close to the N terminus of mouse Kp-52 (201) by Alain Caraty. Mice (n = 4/group) were castrated and perfused one week later with 4% PFA.

Results: Cell bodies and fibers were visible through the rostral to caudal extent of the arcuate in WT mice (shown in red, Figure 8E). Sections of the arcuate of *Kiss1*$^{Cre/Cre}$ mice had a few faint kisspeptin-labeled cells, indicating reduced peptide levels. These kisspeptin-labeled cells (cytoplasmic staining) were positive for nuclear GFP (Figure 8E, center), the expression of which was driven by the *Kiss1* promoter (Figure 3). No staining was visible in the arcuate of *Kiss1* KO animals (Figure 8E, right), indicating the specificity of the antibody. These results indicate that kisspeptin peptide expression was profoundly reduced (as were the number of identifiable cells) but not eliminated in the *Kiss1*$^{Cre/Cre}$ mice.

Experiment 6: Bioassay for endogenous kisspeptin signaling in Kiss1$^{Cre/Cre}$ mice

The purpose of this experiment was to determine whether the low levels of kisspeptin activity in *Kiss1*$^{Cre/Cre}$ mice could stimulate GnRH/LH release. To assess this possibility, I challenged WT and *Kiss1*$^{Cre/Cre}$ mice with senktide, an agonist of the NKR3 receptor, which is expressed by Kiss1 neurons. Since senktide stimulates GnRH/LH release only if kisspeptin signaling is intact (202, 203), I could deduce that if I observed an increase in LH levels in response to senktide, there must be residual kisspeptin signaling capacity in the *Kiss1*$^{Cre/Cre}$ mice.

Methods: To test whether senktide could stimulate LH in *Kiss1*$^{Cre/Cre}$ mice, I injected either senktide (600 pmol in 2 µl from Tocris Bioscience, Ellisville, MO) or vehicle (sterile saline) into the lateral cerebral ventricle of WT and *Kiss1*$^{Cre/Cre}$ female mice (n = 7-10/group) (202). Prior to the injection, female mice were cycled for at least 2 consecutive estrous cycles and injected on diestrus. Blood was collected from the orbital sinus 30 min after the injection between 1130 and 1500 h.

Results: Senktide increased serum levels of LH in both WT and *Kiss1*$^{Cre/Cre}$ females at diestrus (Figure 9). Two-way ANOVA revealed a significant effect of treatment, but not genotype or interaction ($p<0.01$ two-way ANOVA). These results demonstrate that *Kiss1*$^{Cre/Cre}$ animals produce sufficient amounts of kisspeptin to induce GnRH/LH release.

Experiment 7: Development of secondary sexual characteristics in Kiss1$^{Cre/Cre}$ *mice.*

Kiss1 KO female mice have delayed VO, and most KO males lack preputial separation (PPS), indicating a defect in pubertal maturation, which reflects insufficient levels of sex steroids (68). In contrast, mice with > 97% ablation of their Kiss1 neurons exhibited normal VO (150), suggesting that minute amounts of kisspeptin suffice for development of secondary sexual characteristics at puberty. Therefore, I sought to determine whether the residual kisspeptin remaining in *Kiss1*$^{Cre/Cre}$ mice would be sufficient to drive sexual maturation. The purpose of this experiment was to determine whether *Kiss1*$^{Cre/Cre}$ mice had delayed development of secondary sexual characteristics,

which depend on T or E_2. Circulating E_2 can influence body weight through its effects on appetite (204, 205). Therefore, I also weighed animals daily during pubertal maturation.

Methods: Starting on PND 21, *Kiss1*$^{Cre/Cre}$, *Kiss1*$^{Cre/+}$ and WT mice (n = 6-26) were checked daily for VO or PPS, while I was unaware of the animal's genotype. VO or PPS were considered to be the first of 3 consecutive days that the vagina was open or the foreskin separated from the penis. Body weight was also measured daily between days 21 and 35, then once weekly until the animals were 8 weeks old.

Results: *Kiss1*$^{Cre/Cre}$ males had a 1.5-day delay in the mean day of PPS in comparison to their WT and *Kiss1*$^{Cre/+}$ littermates (Figure 10A; Log-rank Mantel-Cox test, $p<0.05$). In spite of this delay, all males achieved PPS. These findings suggest that *Kiss1*$^{Cre/Cre}$ males have a slight deficit in T secretion but have sufficient redundancy or compensatory mechanisms to exhibit a relatively normal phenotype. All female *Kiss1*$^{Cre/Cre}$ mice attained VO with the same time-course as their WT and *Kiss1*$^{Cre/+}$ littermates (Figure 10B).

Kiss1$^{Cre/Cre}$ female mice were slightly, but consistently, heavier than either their WT or *Kiss1*$^{Cre/+}$ counterparts, from PND 21 to PND 47 (~0.8 g/day difference in mean weights; two-way repeated measures ANOVA revealed significant effects of genotype $p<0.05$; data not shown). No significant differences in body weight were found between genotypes in males or adult females weighed at a single time point. These results demonstrate that drastic reductions in *Kiss1* expression cause only very subtle delays in the development of secondary sexual characteristics.

Experiment 8: Reproductive phenotype in adult Kiss1$^{Cre/Cre}$ *mice*

The finding that *Kiss1*$^{Cre/Cre}$ mice exhibit secondary sexual characteristics suggests that they produce physiologically adequate levels of gonadotropins to sustain sex steroid synthesis, despite having greatly reduced *Kiss1* expression. I sought to determine whether residual expression of kisspeptin is sufficient to sustain adult reproductive function.

Methods: Underweight gonads, compromised gonadotropin and sex steroid secretion and infertility are hallmarks of hypogonadotropic hypogonadism, including that of mice having mutations in the *Kiss1/KISS1* gene (65, 68). Thus, I measured gonadotropins and weighed gonads from animals collected in Experiment 4. I also tested whether *Kiss1*$^{Cre/Cre}$ mice were fertile over the course of one typical estrous cycle. For the fertility experiment, *Kiss1*$^{Cre/Cre}$ or WT littermates were paired for 5 consecutive days with a WT mate of proven fertility. Beginning 18 days later, the cages were checked daily for pups for the subsequent 10 days, and the number of pups was counted.

Male Results: The testes of *Kiss1*$^{Cre/Cre}$ animals had modestly reduced weights (by ~ 25%) relative to their WT and *Kiss1*$^{Cre/+}$ littermates ($p < 0.0001$ one way ANOVA) (Figure 11A) — in marked contrast to the dramatic reduction of testicular weight in *Kiss1* KO animals (68). Despite reduced testicular size, *Kiss1*$^{Cre/Cre}$ testicular morphology appeared normal when examined histologically and contained abundant spermatozoa, like those of WT animals (data not shown).

WT, *Kiss1*$^{Cre/+}$, and *Kiss1*$^{Cre/Cre}$ males responded to GDX by increasing LH levels ($p<0.0005$ for two-way ANOVA effect of treatment and $p<0.05$ Mann Whitney U-test comparing intact to GDX animals; Table 5). Although plasma LH was slightly lower in GDX *Kiss1*$^{Cre/Cre}$ males relative to WT and *Kiss1*$^{Cre/+}$ littermates, this reduction was not statistically significant (Figure 11B; n = 4-5 per group). A second experiment comparing LH levels between WT and *Kiss1*$^{Cre/Cre}$ GDX males also did not reveal a statistically significant difference (WT = 4.19 ± 0.82 ng/ml; *Kiss1*$^{Cre/Cre}$ = 2.54 ± 0.4; n = 8-9 per group). Levels of LH in intact animals were also normal in *Kiss1*$^{Cre/Cre}$ males compared to WT controls (data not shown). Although LH values for intact animals were above the limit of sensitivity for the assay, they were close to the limit of detection, which may not provide sufficient resolution to discriminate among genotypes. In contrast, intact *Kiss1*$^{Cre/Cre}$ males had significantly lower serum levels of FSH compared to their WT and *Kiss1*$^{Cre/+}$ littermates (Fig. 11D; $p < 0.005$ one way ANOVA; n = 7-19/group). Although T in *Kiss1*$^{Cre/Cre}$ males appeared to be reduced relative to WT and *Kiss1*$^{Cre/+}$ littermates (Figure 11C), this difference was not statistically significant due to variability in WT and *Kiss1*$^{Cre/+}$ animals (Kruskal-Wallis non-parametric test; n = 9-22/group).

Given that *Kiss1*$^{Cre/Cre}$ males displayed only mild signs of hypogonadism, I suspected that they would be fertile. When paired with WT females, *Kiss1*$^{Cre/Cre}$ males succeeded in impregnating their mates, which delivered normal-sized litters (Table 6; n = 9-14/group). There were no significant differences in either the rate of fertility or litter size between *Kiss1*$^{Cre/Cre}$ and WT males. These results indicate that adult *Kiss1*$^{Cre/Cre}$ males maintain virtually all of their reproductive capacity in spite of having only a small fraction of available kisspeptin.

Female Results: Similar to males, *Kiss1*$^{Cre/Cre}$ females had significantly reduced gonadal weight relative to WT and *Kiss1*$^{Cre/+}$ littermates (Figure 12A; $p<0.005$ one-way ANOVA; n = 6-8 per group; *Kiss1*$^{Cre/Cre}$ ovarian weight was 63% of WT). *Kiss1*$^{Cre/Cre}$ females had normal LH levels at diestrus (Figure 9), consistent with intact LH levels of *Kiss1* KO mice (68) and progressed through the estrous cycle, although they occasionally had extended periods of estrus. GDX *Kiss1*$^{Cre/Cre}$ females had significantly lower levels of LH compared to their WT and *Kiss1*$^{Cre/+}$ littermates (Figure 12B; $p < 0.05$ one-way ANOVA; n = 6-8 per group), which had normal LH levels.

In addition to being smaller, ovaries of *Kiss1*$^{Cre/Cre}$ mice showed markedly reduced numbers of CL relative to WT and *Kiss1*$^{Cre/+}$ littermates, indicating significantly reduced ovulation ($p < 0.0001$ one-way ANOVA; n = 6-7 per group; Figure 12C and D). Only 3 of 6 *Kiss1*$^{Cre/Cre}$ mice had any CL compared to the plethora of CL present in all WT animals. *Kiss1*$^{Cre/+}$ mice were also compromised in their ability to ovulate, showing an intermediate number of CL relative to WT and *Kiss1*$^{Cre/Cre}$ mice, suggesting that *Kiss1* gene dose correlates with ovulation. However, *Kiss1*$^{Cre/Cre}$ ovaries contained antral and preovulatory follicles (Figure 12C), suggesting that they receive sufficient gonadotropin drive to support follicular maturation.

Predictably, compromised ovulation correlated with compromised fertility in *Kiss1*$^{Cre/Cre}$ females. When paired with a WT male, ~ 80% of WT females became pregnant and delivered pups. In contrast, only ~ 30% of *Kiss1*$^{Cre/Cre}$ mice sustained pregnancy ($p<0.05$ Fisher's exact test; n = 11 per group; Figure 12E). Furthermore, the *Kiss1*$^{Cre/Cre}$ mice that had pups had significantly reduced litter sizes compared to WT littermates (unpaired t-test $p<0.05$, Table 6, Figure 12F). These results indicate that

although *Kiss1*$^{Cre/Cre}$ females maintain some degree of fertility, a dramatic loss of kisspeptin compromises their reproductive success.

Experiment 9: Search for compensation in Kiss1$^{Cre/Cre}$ *mice*

My mentor and I reasoned that as a result of the profound reduction of kisspeptin expression in *Kiss1*$^{Cre/Cre}$ mice, Kiss1 neurons might compensate by up-regulating the expression of the stimulatory co-transmitter NKB and its receptor, NK3R, which is also expressed by Kiss1 neurons, or down-regulating the expression of the indirect auto-inhibitory co-transmitter dynorphin (206).

Methods: To this end, I measured the expression of the genes that encode NKB, NK3R and dynorphin (*Tac2*, *Tacr3* and *Pdyn*, respectively) by qRT-PCR in the MBH from of WT and *Kiss1*$^{Cre/Cre}$ males collected in Experiment 4. Since these genes are regulated by sex steroids (100, 207), I also compared their expression in the intact and GDX state as an internal control.

Results: As expected, I observed an increase in expression of all 3 genes with GDX ($p<0.01$ two-way ANOVA effect of treatment). There was no significant difference in the expression of *Tac2*, *Tacr3* or *Pdyn* between WT and *Kiss1*$^{Cre/Cre}$ animals in the MBH, in either the intact or GDX states (Figure 13).

Experiment 10*: Test for the ability of kisspeptin to sustain endogenous GnRH/LH release*

The purpose of this experiment was to test the hypothesis that kisspeptin in *Kiss1*$^{Cre/Cre}$ mice sustains endogenous GnRH/LH release (as opposed to a kisspeptin-independent compensatory mechanism).

Methods: WT and *Kiss1*$^{Cre/Cre}$ female mice were GDX to maximize LH and injected ICV with 5 nmol of a drug marketed as a Kiss1r antagonist (peptide 234) or a control solution (89). Blood was collected 30 min after the injections.

Results: As expected, peptide 234 suppressed LH levels in WT animals (t-test; $p<0.05$). Surprisingly, this drug had the opposite effect in *Kiss1*$^{Cre/Cre}$ animals, in which it *stimulated* LH (t-test $p<0.05$) (Figure 14). ANOVA showed no effect of treatment or genotype, but had a significant interaction ($p < 0.01$). These results suggest that peptide 234 suppresses LH release by acting as a weak agonist that competes with kisspeptin. Thus, peptide 234 is not a true antagonist and cannot be used to block Kiss1r signaling or to test the hypothesis.

Experiment 11: Regulation of kisspeptin levels by testicular hormones in WT mice

In an effort to quantify kisspeptin levels in *Kiss1*$^{Cre/Cre}$ animals, I collaborated with Dr. Alain Caraty to measure kisspeptin from the MBH using radioimmunoassay (RIA). Because we know that GDX increases *Kiss1* mRNA expression ~10 fold, I reasoned that using GDX animals would increase the likelihood of detecting very low peptide levels in *Kiss1*$^{Cre/Cre}$. I tested this hypothesis in a pilot experiment, in which I compared kisspeptin peptide levels between intact and GDX males.

Methods: I extracted peptides from the MBH of WT males that had been GDX a week earlier or left intact (n = 5/group) and shipped the peptides to Dr. Caraty, who performed the kisspeptin RIA.

Results: In contrast to *Kiss1* mRNA, which increases in the MBH in GDX animals, kisspeptin peptide levels *decrease* in the MBH of GDX animals (Figure 15). I suspect this decrease results from kisspeptin being released and degraded in larger quantities in the GDX state (208, 209) than in the intact state.

To quantify the amount of kisspeptin in *Kiss1*$^{Cre/Cre}$ mice, I extracted peptides from intact WT and *Kiss1*$^{Cre/Cre}$ MBH. Unfortunately, the RIA results were erratic and revealed extremely high readings in some samples (data not shown). After reflecting on the procedure with Dr. Caraty, we concluded that samples were contaminated due to the reuse of Dounce homogenizers and glass tubes. I was unable to repeat the experiment because additional animals were not available.

Investigation of the Mechanism of Sexual Differentiation of the AVPV/PeN

Experiment 12: Comparison of tdTomato expression between adult males and females

The purpose of this experiment was to test the hypothesis that males have fewer Kiss1 neurons in the AVPV/PeN due to decreased *Kiss1* expression. To test this hypothesis, *Kiss1* neurons were permanently marked after *Kiss1* expression by crossing *Kiss1*$^{Cre/+}$ mice with *tdTomato*$^{+/-}$ mice. In mice containing both the *Cre* and *tdTomato* alleles, Cre turns on *tdTomato* if *Kiss1* is expressed and *tdTomato* is transcribed forever in those cells and their daughters (Figure 4). Therefore, if Kiss1 neurons died after *Kiss1* was expressed (or were never born), then male mice would have fewer tdTomato labeled cells in the AVPV/PeN than females. However, if the *Kiss1* gene were turned on transiently and then silenced (e.g. through epigenetic mechanisms), then males would have similar numbers of tdTomato-labeled neurons to females.

Methods: To compare the number of tdTomato-labeled cells between males and females in the AVPV, PeN, and the medial preoptic nucleus (MPOM), *Kiss1*$^{Cre/+}$ animals were mated to *tdTomato*$^{+/-}$ animals. Adult offspring were genotyped (n=6 males & 6 females) and the brains of Kiss1$^{Cre/+}$*; tdTomato*$^{+/-}$ mice were collected from perfused animals. Upon examination of the tissue under the microscope, one half of the animals had widespread expression of *tdTomato* throughout the brain (Figure 6), making the identification of individual cells impossible. These animals were removed from the study. For the remaining animals (n=3 males and 3 females), sections from the AVPV/PeN/MPOM were matched at the same anatomical level using images at 4X magnification. One image from each animal containing the AVPV/PeN and MPOM was selected. Images taken at 10X magnification were then used to count cells, using one side of the respective region. Cells were counted in the AVPV, PeN, and MPOM by 2 experimenters while they were unaware of the animals' sex. Statistical comparison was made using students' t-test in GraphPad Prism software.

Results: All males had an abundance of tdTomato cells in the AVPV/PeN/MPOM regions. In fact, the number of tdTomato-labeled cells is not statistically different

between the sexes in any of the analyzed regions (Figure 16B, mean ± SEM for AVPV: 43 ± 4 in male and 43 ± 3 in female; PeN: 9 ± 3 in male and 10 ± 2 in female; and MPOM: 209 ± 45 in male and 181 ± 16 in female). The distribution of tdTomato cells appeared to extend further laterally into the MPOM of males than of females (Figure 16A). These results suggest that sex differences in *Kiss1* expression in the AVPV/PeN arise from reduced gene expression in males rather than from fewer neurons.

Experiment 13: Sensitive detection of Kiss1 mRNA in the male AVPV/PeN/MPOM

Given that there were abundant tdTomato-labeled cells in the AVPV/PeN/MPOM of male mice, I reasoned that either *Kiss1* is expressed at low levels in these regions in adult males, but was not easily detected by *in situ* hybridization or IHC, or that *Kiss1* was transiently expressed there during development and subsequently silenced. The purpose of this experiment was to test the hypothesis that males express low levels of *Kiss1* mRNA in the AVPV/PeN/MPOM.

Methods: I compared *Kiss1* mRNA expression between WT (n=4) and *Kiss1* KO (n=3) males using a higher *Kiss1* probe concentration than had been used previously to increase the chances of detecting low levels of *Kiss1* mRNA. I used KO males to differentiate non-specific binding of the probe from low-level *Kiss1* mRNA expression.

Results: Adult WT males had higher levels of silver grains representing *Kiss1* mRNA in the adult AVPV/PeN/MPOM relative to *Kiss1* KO males (Figure 17). These results suggest that Kiss1 neurons in the adult male express *Kiss1* mRNA, albeit at much lower levels than females.

Experiment 14: Developmental comparison of tdTomato expression between males and females

Based on the fact that T stimulates expression of *Kiss1* mRNA in the adult male AVPV (122) and that adult males have expressed *Kiss1* in many cells in this region (Figure 16), I hypothesized that T transiently stimulates *Kiss1* expression in the AVPV/PeN/MPOM during the neonatal critical period while also inducing epigenetic

changes that limit the ability of the gene to be induced by T in adulthood. The neonatal critical period ends during the first week of life. The earliest time that *Kiss1* expression has been reported is PND 10, when it is barely detectable (154). At PND 12, *Kiss1* expression is still low (154), but it begins to be sexually differentiated with females expressing more than males. My mentor and I reasoned that if T (or E_2) transiently activated *Kiss1* expression during the neonatal critical period, there would be more tdTomato-labeled cells in the AVPV/PeN of males than females at PND 12 even though females express more *Kiss1* transcript at this age.

Methods: I compared the extent of tdTomato labeling between males and females at PND 12. I also compared the current expression level of *Kiss1* transcript between the sexes by staining for GFP, which is under the control of the *Kiss1* promoter. Male (n=2) and female (n=2) offspring were perfused on PND 12 and their brains were processed as described in the materials and methods.

Results: Males and the females at PND 12 had abundant tdTomato cells in the AVPV/PeN/MPOM (Figure 18), in marked contrast to the sparse cell numbers previously identified by *in situ* hybridization (154). Furthermore, GFP-labeled nuclei were also present in both males and females, although males had fewer, consistent with the previously reported sex difference at PND 12 (154).

Chapter IV. Discussion

Characterization of *Kiss1-CreGFP* Mice

GFP expression reflects activity of the Kiss1 promoter

In collaboration with Michelle Gottsch and Elisenda Sanz (192), I demonstrated that GFP expression in *Kiss1-CreGFP* knock-in mice is regulated by E_2, just like *Kiss1* mRNA, validating that expression of the CreGFP fusion protein is regulated by the *Kiss1* promoter (Figures 3 and 5). (The nuclear localization of GFP is consistent with the design of the *CreGFP* construct to generate a fusion protein.) This validation is important because it supports the idea that any Cre recombination that is visible upon crossing to tdTomato (or other) reporter mice accurately reflects the activity of the endogenous *Kiss1* promoter (either during development or in adulthood).

Widespread Cre recombination may reflect developmental and adult Kiss1 *expression outside the hypothalamus*

Upon crossing *Kiss1-CreGFP* mice to tdTomato animals, I found that tdTomato expression extended to many extra-hypothalamic regions and that the degree of expression varied between animals (Figure 6). Because tdTomato reflects the cumulative developmental history of *Kiss1* expression, I can draw three conclusions from these findings.

First, the fact that approximately one half of the animals have pervasive Cre recombination in the brain and periphery suggests that *Kiss1* mRNA is expressed early in development, perhaps in a set of precursor cells. This conclusion is further supported by the finding that some animals have brains that are virtually completely pink as early as PND12 (Figure 7) and even at embryonic day 17.5 (data not shown). Second, the early developmental expression of *Kiss1* appears to be stochastic, as demonstrated by the variability among mice, even from the same litter. *Kiss1* may be expressed at low levels during development, therefore leading to the transcription and translation of only a few Cre recombinase molecules, which randomly encounter the loxP sites to turn on tdTomato expression in some animals.

Second, my observation that animals with more restricted tdTomato expression have fewer tdTomato cells outside the hypothalamus at PND 12 (Figure 7) than adults suggests that *Kiss1* expression in regions such as the cortex and hippocampus increases during post-natal development and is present a low levels in adults. In fact, *Kiss1* mRNA has been described in the cortex (188) and hippocampus of adult animals (210) and Kiss1r is also present in many brain regions outside of GnRH neurons, including the hippocampus. Although the function of kisspeptin in these regions remains to be determined, kisspeptin does not seem to be required for vital functions because mice and humans with loss-of-function mutations in *KISS1/Kiss1* and *KISS1R/Kiss1r* appear to be normal with the exception of being hypogonadal and infertile.

Third, these results indicate that caution must be exercised when interpreting studies that manipulate genes by crossing *Kiss1-Cre* mice to mice with loxP-flanked alleles. If “floxed” genes of interest are widely expressed in the brain, then some *Kiss1-Cre* KO mice may not be much more specific than global knockouts of those genes. In fact, one could argue that the variability of Cre recombination would make the *Kiss1-Cre* approach less practical than the use of a global KO. One possible remedy for this stochasticity problem would be to assess Cre recombination in each experimental mouse through PCR on genomic DNA (spanning loxP-flanked regions). In contrast, if the “floxed” gene of interest has restricted expression overlap with *Kiss1* expression, then *Kiss1-Cre* mice would be more specific in genetic manipulations than global KOs. Furthermore, manipulations of Kiss1 neurons that are region-specific, such as those using viral injections into specific brain regions where Cre is expressed in adult animals are likely to yield interpretable results because they are not subject to the developmental history of Cre recombination.

Redundancy in *Kiss1* Expression Safeguards Reproduction in the Mouse

The results of experiments 4-9 demonstrate that *Kiss1*$^{Cre/Cre}$ mice sustain some level of reproductive function despite having a 95% reduction of *Kiss1* expression, corroborating the results, but not the conclusion of Mayer & Boehm (150). Male *Kiss1*$^{Cre/Cre}$ mice with 5% of the normal *Kiss1* transcript and meager amounts of kisspeptin protein in their MBH can sire normal-sized litters. These results argue that WT male mice produce substantially more kisspeptin than is required for normal reproduction. Notwithstanding, experimental disruption of *Kiss1* expression impairs some aspects of reproduction, most notably in females. Although *Kiss1*$^{Cre/Cre}$ females become pregnant and produce viable litters, they have fewer offspring. Thus, I conclude that WT females possess an abundance of *Kiss1* expression — a failsafe to guard reproductive success, just like males. However, they are more sensitive to disruptions of Kiss1 signaling than males — perhaps reflecting the additional complexity required for ovulation.

Redundancy in the reproductive axis

How is fertility maintained despite a major impairment of Kiss1 signaling in the brain? First, kisspeptin stimulates sustained firing of GnRH neurons at vanishingly low concentrations (76, 80), testifying to the remarkable sensitivity of the Kiss1r signaling cascade. Second, the GnRH system itself is highly redundant. Classic experiments by Krieger and her colleagues demonstrated that transplants of only a few GnRH neurons into GnRH deficient mice are sufficient to increase gonadotropin levels, gonadal development, and spermatogenesis in males (195). Similar results were obtained from studies in mice with compromised GnRH neuronal migration (194). Males with only 12% of the normal constituency of GnRH neurons are just as fertile as their WT counterparts. Although tiny amounts of GnRH can sustain normal levels of LH, males with compromised production of either GnRH or kisspeptin have impaired FSH secretion, which is consistent with the reduction in gonadal weight found in *Kiss1*$^{Cre/Cre}$ males (194, 211) (Figure 11A and D). Reduced FSH could decrease epididymal sperm count, but a

lack of FSH does not compromise male fertility (211), which is consistent with my results in *Kiss1*$^{Cre/Cre}$ males.

Similar to males, WT females appear to have redundancy in their Kiss1 signaling. *Kiss1*$^{Cre/Cre}$ females bearing only 5% of the normal *Kiss1* transcript in the arcuate and 17% in the AVPV attain VO at the normal age (Figure 10B), indicating their ability to produce timely and normal amplification of estradiol secretion at puberty. *Kiss1*$^{Cre/Cre}$ females exhibit normal cyclicity in vaginal cytology, and at diestrus, their LH levels are indistinguishable from WT animals (Figure 9). These results are consistent with those of Mayer and Boehm, who found that female mice with 95-98% reduction in *Kiss1* mRNA had VO, estrous cycling, and normal LH levels (150). The intact LH results are also consistent with those of *Kiss1* KO animals, which have normal LH in the intact state, pointing to the presence of kisspeptin-independent GnRH/LH release (145). However, when *Kiss1*$^{Cre/Cre}$ females are challenged to increase LH secretion following ovariectomy, they have reduced LH levels relative to WT animals (Figure 12B), suggesting that kisspeptin plays an important role in driving maximal GnRH/LH output. These observations suggest that low levels of kisspeptin are sufficient to sustain basal pulsatile LH release and steroidogenesis in females — as is the case with GnRH.

Females require more Kiss1 expression than males for fertility

Why are *Kiss1*$^{Cre/Cre}$ females sub-fertile? Several studies have shown that kisspeptin plays a crucial role in generating the preovulatory LH surge (92, 121, 126). My results also suggest that the neural mechanisms that trigger the LH surge and ovulation require a considerable boost from kisspeptin. Although *Kiss1*$^{Cre/Cre}$ females exhibit follicular development, they produce few CL (Fig. 12C and D), indicating a disruption of ovulation. Furthermore, the *Kiss1* mRNA content in hypothalami correlates with the number of CL in the ovaries (Fig. 8C and 12D), suggesting a gene-dose effect of *Kiss1* on ovulation. That is, the magnitude of the kisspeptin surge plausibly correlates with the magnitude of the GnRH/LH surge and thus the number of follicles that ovulate. Consistent with this idea, female mice with ~12% of the normal allotment of GnRH neurons lack an E_2/P-induced LH surge, whereas those with ~34 % of GnRH neurons

have an intermediate phenotype and a blunted LH surge (194). Taken together, these observations are consistent with the idea that a surge in kisspeptin drives the preovulatory surge in GnRH and that normal WT females require most of their available kisspeptin to generate a robust and successful GnRH/LH surge.

Phenotypic differences between scarce Kiss1 transcript and sparse neurons

If it were true that females required > 50 % of the natural endowment of *Kiss1* to drive normal ovulation and fertility, as my results suggest (Figure 12D), why were the animals described by Mayer & Boehm, which lacked ± 97% of their Kiss1 neurons, fertile (150)? I provide three possible explanations. First, it is possible that developmental compensation occurs (e.g., through rewiring of inputs to GnRH neurons) when Kiss1 neurons are congenitally ablated (in the Mayer & Boehm study), but not when *Kiss1* transcript is dramatically reduced (in my study). I find this explanation to be unlikely. Although compensation has been reported to occur when AgRP neurons are congenitally ablated (212), congenital disruption of the NPY or AgRP genes only cause a mild phenotype, unlike the dramatic infertility reported to occur in *Kiss1* KO animals (65, 68). Furthermore, it is difficult to imagine how the greater insult caused by cell death (including a reduction in NKB and dynorphin in the arcuate, and dopamine in the AVPV) could have a more mild effect on reproduction than the partial insult caused by transcriptional suppression, unless it were easier for the nervous system to replace whole circuits than to replace the function of genes within preexisting neurons. Second, differences in the approach to assessing fertility might explain the discrepancy in results. I noticed that most *Kiss1*$^{Cre/Cre}$ females did not become pregnant when mated for only 5 days, but some did after 2 months (data not shown). Therefore, if the mice in the Mayer and Bohem study had reduced *frequency* of ovulation, as suggested by their prolonged estrous cycles, this effect could be masked by a prolonged mating period. Although it was not revealed whether those mice had reduced ovulation efficiency, they were reported to have normal-sized litters, suggesting that those animals had normal numbers of ovulations per cycle and that kisspeptin levels were sufficiently high to trigger ovulation in that mouse strain. Third, variation in genetic background strains of mice used in the two studies could account for differences in absolute levels of

kisspeptin or in how much *Kiss1*/kisspeptin is required to generate the GnRH/LH surge. Reproductive capacity differs remarkably among strains of mice (213), with strains that are particularly sensitive to disruptions in fertility having 10 times less *Kiss1* mRNA in the hypothalamus relative to C57Bl6/J animals (214). The *Kiss1*$^{Cre/Cre}$ mice were on a mixed genetic background, which was ~75% C57Bl/6J and ~25% 129/Sv. The genetic background of the mice used in the Mayer & Boehm study was not reported, so I cannot make a direct comparison. Therefore, differences in genetic background could well contribute to the discrepancies between my results and those of Mayer & Boehm.

One limitation of my study is that I do not know exactly how much kisspeptin peptide is released in *Kiss1*$^{Cre/Cre}$ animals. However, I demonstrated that sufficient kisspeptin is released to trigger an LH response to the NK3R agonist senktide (Figure 9) – which requires Kiss1 signaling to stimulate LH (202, 203). I also showed that kisspeptin is reduced in *Kiss1*$^{Cre/Cre}$ mice with IHC (Figure 8E) and that some kisspeptin is still present in neurons that normally express *Kiss1*, as demonstrated by faint kisspeptin immunolabeling surrounding GFP-expressing nuclei (Figure 8E). Taken together, my results are consistent with the idea that dramatically reduced levels of kisspeptin are sufficient to sustain endogenous gonadotropin release. However, having an oversupply of kisspeptin would not rule out other mechanisms of *compensation* for the reduction in kisspeptin production.

Compensation for kisspeptin deficiency

I reasoned that if gonadotropin secretion were sustained by kisspeptin signaling, and not by compensatory pathways, I could block gonadotropin release with a Kiss1r antagonist in *Kiss1*$^{Cre/Cre}$ animals. In WT GDX females, the Kiss1r antagonist successfully blocked LH release (Figure 14) but in *Kiss1*$^{Cre/Cre}$ GDX females, the antagonist *increased* LH release. These findings suggest that the antagonist, peptide 234, acts as a weak agonist. In WT animals, which normally have a large proportion of Kiss1 receptors occupied by kisspeptin, peptide 234 displaces kisspeptin and weakens Kiss1r signaling on GnRH neurons and thus GnRH/LH release. In contrast, *Kiss1*$^{Cre/Cre}$ animals make little kisspeptin and have few Kiss1 receptors occupied. Thus, peptide

234 could stimulate the unoccupied receptors on GnRH neurons and increase GnRH/LH release. Although this approach did not provide an answer to the question of compensation, it provides valuable information about a drug that is widely used in the kisspeptin field.

My mentor and I reasoned that animals might compensate for reduced *Kiss1* expression by increasing the activity of Kiss1 neurons — perhaps by allowing one or both of the Kiss1 co-transmitters to remediate the situation as part of a backup system. There are other examples of compensation in the hypothalamus following insults (215-218), so I sought specific evidence for adaptation. I envisioned that one possible remedy for the reduction of kisspeptin might be either to increase auto-stimulation by amplifying NKB/NK3R signaling or decrease auto-inhibition by reducing dynorphin synthesis (90), either of which might maximize release of residual kisspeptin. I did not find evidence for changes in the expression of genes that encode NKB, NK3R or dynorphin. Based on these observations, I cannot rule out a role for NKB or dynorphin signaling in compensation. These genes are expressed in other neurons of the MBH (100, 219) and without careful parsing of these different populations, I cannot say for certain that some degree of compensation within Kiss1 neurons did not occur.

How else might *Kiss1*$^{Cre/Cre}$ animals compensate for diminished *Kiss1* expression? First, GnRH neurons could become more sensitive to kisspeptin with its chronic loss. This seems plausible, since some (but not all) *Kiss1* KO animals have an augmented LH response to kisspeptin administration (68, 220). Second, the pituitary could become sensitized to GnRH. Indeed, increased sensitivity to GnRH has been shown to occur in some patients with *KISS1R* mutations (69). Third, some kisspeptin-independent mechanisms may contribute to GnRH secretion. A patient with loss of function mutations in *KISS1R* had small LH pulses (69) and residual gonadotropin secretion persists in both *Kiss1* and *Kiss1r* KO mice. This gonadotropin secretion is blocked by a GnRH antagonist (145); thus, some GnRH secretion remains even in the face of complete ablation of Kiss1 signaling, which could be driven by other stimulatory inputs to GnRH neurons, such as glutamate. Indeed, *Kiss1* KO mice exhibit increased

sensitivity to NMDA (220), suggesting that in the absence of kisspeptin input, GnRH neurons become more sensitive to some excitatory inputs. Furthermore, a subpopulation of GnRH neurons responds to metabotropic glutamate receptor agonists but not kisspeptin and vice-versa (221), suggesting that some GnRH neurons have kisspeptin-independent activity. Fourth, it is conceivable that mating-induced ovulation occurs through the noradrenergic system, as this occurs in certain conditions in animals that are not normally reflex ovulators (222). Finally, Kiss1 signaling in peripheral tissues could play a role in fertility and compensation may occur outside the brain. Nevertheless, any such mechanism is insufficient to compensate fully for the absence of kisspeptin signaling, as demonstrated by infertility in animals lacking functional *Kiss1* and *Kiss1r* genes (65, 68, 69, 145). Collectively, these observations demonstrate that animals require kisspeptin for reproduction and synthesize this peptide in excess to ensure reproductive success. Despite the evidence that kisspeptin signaling plays a critical role in the onset of puberty, kisspeptin may be one of many factors that are necessary but not sufficient to trigger puberty onset. Most likely, kisspeptins do not act alone to initiate puberty but rather are elements in a complex network of transmitters and hormones that guide the trajectory of pubertal maturation.

Castration decreases kisspeptin levels in the MBH

In collaboration with Dr. Caraty, I discovered that kisspeptin peptide levels in the MBH are regulated in the opposite direction by sex steroids relative to *Kiss1* mRNA (Figure 15) (92, 122). These findings suggest that upon removal of negative feedback, kisspeptin release from arcuate neurons increases and the released kisspeptin is likely to become quickly degraded in the synapse. *Kiss1* transcription is likely upregulated to replenish the diminished supply of kisspeptin. In fact, Dr. Caraty discovered that GnRH neurons themselves have a similar response to castration. Castration decreases hypothalamic content of GnRH (208) but increases GnRH release (209).

The finding that kisspeptin peptide levels decrease in the MBH in response to castration is consistent with that described by Brock and Bakker (180), who demonstrated that gonadectomy decreases total kisspeptin immunoreactivity in the

arcuate and sex-steroid treatment reverses this effect. However, most of the immunoreactivity in their study consisted of fibers and it was not clear whether those fibers were afferents from neurons in the AVPV/PeN or the arcuate. Although it is conceivable that my study presents a similar problem, there is a large population of Kiss1 cell bodies in the arcuate, making it more likely that the majority of the kisspeptin detected in the RIA came from those cell bodies.

Sexual Differentiation of *Kiss1* in the AVPV/PeN Reflects Differences in Gene Expression rather than Cell Number

It was previously shown that males express less *Kiss1* mRNA in the AVPV/PeN than females as a function of neonatal T exposure (124, 177). Based on the fact that neonatal T induces apoptosis in the AVPV, we and others speculated that apoptosis occurs specifically in Kiss1 neurons. If this were the case, the number of cells that ever expressed the *Kiss1* gene (marked by tdTomato) would be smaller in males than in females. In contrast, I showed that this number does not differ significantly between the sexes, suggesting that sexual differentiation of the AVPV/PeN arises through mechanisms other than cell death in males (or neurogenesis in females). These results are consistent with those of Semaan *et al.*, who found that a sex difference in the number of Kiss1 neurons persists in animals lacking the apoptosis-inducing gene *Bax* (154) and suggest that males and females differ in their *Kiss1* gene expression levels.

Having established that Kiss1 neurons in the AVPV/PeN of adult males are alive, I confirmed that *Kiss1* mRNA is expressed in a significant number of neurons in these regions, albeit at low levels (Figure 17). These results are consistent with those previously reported by our group in the male (76, 122). In fact, Smith et al. showed that when males are treated with T or E_2, they have an average of ~40 *Kiss1* mRNA-expressing neurons in the AVPV (122). Nevertheless, even when treated with sex steroids, males cannot attain the level of *Kiss1* expression of females in the AVPV/PeN (124).

My results together with others suggest that the *Kiss1* gene is epigenetically suppressed in males (or activated in females) in the AVPV/PeN during development. Semaan *et al.* found decreased CpG methylation in the *Kiss1* locus of males in the AVPV/PeN region (182), perhaps allowing more transcriptional repressors to bind to the *Kiss1* promoter than in females. Differences in epigenetic modifications could result from masculinizing actions of T and/or E_2 during the neonatal critical period (124, 177) or from the feminizing actions of E_2 during puberty in females. Recent studies from

Broke and Bakker suggest that E_2 is required during pubertal development for full feminization of *Kiss1* expression in the AVPV/PeN (179, 180).

If it were true that neonatal T (or E_2 following aromatization) caused epigenetic suppression of the *Kiss1* gene, these hormones might also transiently activate *Kiss1* transcription in males prior to epigenetic silencing. If this were the case, then a large number of tdTomato-expressing cells would be visible in the AVPV/PeN of males after the first week of life but before puberty, when T activates *Kiss1* expression. To test this possibility, I examined tdTomato expression in the AVPV/PeN of males at PND 12, a time when very few Kiss1 neurons are detectable by *in situ* hybridization (154). Indeed, males had an abundant number of tdTomato cells in the AVPV at PND 12 (Figure 18). Although the presence of so many cells is consistent with the hypothesis that neonatal T (or E_2) transiently induces *Kiss1* expression, their presence may simply reflect the fact that tdTomato labeling is more sensitive than previous *in situ* hybridization and IHC detection methods at this age. The fact that females also have more tdTomato cells in the AVPV/PeN than GFP-expressing cells supports the latter interpretation. Therefore, I will examine tdTomato expression at PND 5, five days before the earliest age for detectable *Kiss1* expression in either sex. If my hypothesis is correct, I would anticipate that males would have more tdTomato cells in the AVPV than females at PND 5.

Possible function of Kiss1 neurons in the male AVPV/PeN/MPOM

Although evidence implicates Kiss1 neurons in the AVPV/PeN in generating the preovulatory LH surge in females, less is known about their function in males. The MPOM plays an important role in male sex behavior (223) and my results demonstrate that *Kiss1* transcript is expressed in this region (Figures 16 & 17). However, kisspeptin signaling is unlikely to play a direct role in sexual behavior, as demonstrated by the fact that male mice lacking the Kiss1r have normal sex behavior if they receive T replacement (178). These neurons could be involved in sexual partner preference because males lacking Kiss1r lose female-specific partner preference in an olfactory test (178) and female urine activates Kiss1 neurons in the male AVPV (179).

Chapter V. Conclusions and Future Directions

Uses and Limitations of *Kiss1-Cre* Mice

In the process of characterizing *Kiss1-CreGFP* mice, I found that GFP expression faithfully recapitulates expression of the *Kiss1* gene and its regulation by sex steroids. This indicates that GFP expression in these animals is an excellent tool to identify cells for electrophysiological recordings. Furthermore, the animals are useful for local genetic manipulations using viral gene delivery. I also found that *Kiss1* is likely to be expressed stochastically during development and that *Kiss1* is widely expressed outside the hypothalamus in many brain regions of adult animals, making crosses to animals with "floxed" alleles difficult to interpret. To make Cre-expression more specific, future generations of *Kiss1-Cre* mice could be designed to require multiple promoters to drive Cre expression (224). For example, the coding sequence for the N-terminus of Cre recombinase could be driven by one promoter and the C-terminus sequence by another. Assembly of Cre recombinase would be facilitated by inserting the sequence for a leucine-zipper domain on each half of the Cre sequence. Thus, function of Cre recombinase and recombination could occur specifically in cells that express two different genes (e.g. *Kiss1* and *Tac2*).

The Requirement of *Kiss1* Expression for Puberty Onset

By dramatically reducing *Kiss1* transcript, I showed that only 5% of normal *Kiss1* expression is sufficient for puberty in both sexes, but that this reduction reduces fertility in females, strengthening the evidence that Kiss1 neurons are required for normal reproduction. There are many unanswered questions about the mechanisms by which kisspeptin initiates puberty. First, the specific roles of kisspeptin neurons in the AVPV/PeN, arcuate and perhaps even extra-hypothalamic regions in driving puberty onset remain to be tested. One of the controversial questions centers on whether kisspeptin neurons in the AVPV/PeN initiate puberty or simply respond to increasing sex steroid levels at puberty. To address this question, one would need ways to selectively manipulate Kiss1 neurons in the arcuate or AVPV/PeN, perhaps through requiring activity of two promoters for Cre recombinase assembly (224) and/or injection of

viruses/toxins into specific brain nuclei. As is demonstrated by the findings of Mayer and Boehm (150) taken together with my own, experiments using ablation of Kiss1 neurons or *Kiss1* expression will be challenging because the lack of an effect after incomplete ablation would not necessarily rule out a role for a specific group of Kiss1 neurons in puberty onset.

One of the arguments against a role for Kiss1 neurons in the AVPV/PeN in driving puberty onset (rather than simply responding to increasing sex steroid levels) is that males have very low levels of *Kiss1* expression in this region, yet they still progress through puberty. My finding that males require very little *Kiss1* expression for puberty weakens this argument. Furthermore, the appearance of many Kiss1 neurons in the male AVPV (revealed by tdTomato) at PND 12, suggests that *Kiss1* expression in males has been turned on before T increases substantially at puberty. Whether this reflects past sex-steroid induction of the gene during the neonatal critical period or initiation by a central mechanism remains to be determined. Examination of tdTomato expression in the AVPV/PeN at PND 5 should shed some light on this question. If males have more tdTomato cells than females at PND 5, this would support neonatal induction because females do not experience the neonatal exposure to sex steroids. In contrast, if both sexes have abundant tdTomato cells at PND 5, then it would be more likely that *Kiss1* expression in the AVPV/PeN is turned on by central mechanisms.

Another unanswered question is how kisspeptin interacts with other neurotransmitter systems (including GABA, glutamate, NKB and dynorphin) to regulate the timing of puberty onset and the activity of GnRH neurons. Kiss1 neurons in the arcuate co-express vesicular glutamate transporter 2 (vGlut2) and those in the AVPV synthesize GABA, supported by the expression of the enzyme GAD67 (188). These neurotransmitters play important roles in puberty onset (133, 135, 136, 225-227). Glutamate or GABA from Kiss1 neurons could modulate the activity of GnRH neurons or of Kiss1 neurons themselves (through auto-receptors or interneurons). Such signals could facilitate in timing bursts of neuronal activity associated with GnRH pulses or the GnRH surge. If new tools become available to increase specificity of genetic manipulations in Kiss1 neurons, it would be interesting to determine whether animals lacking glutamate or GABA in Kiss1 neurons could reproduce.

Epigenetic Factors May Explain Sexual Differentiation of *Kiss1* Expression

I found that both males and females have abundant Kiss1 neurons in the AVPV/PeN/MPOM, but these neurons express less *Kiss1* mRNA in males than in females (124). These results are consistent with the idea that the sex difference in *Kiss1* expression is not attributable to apoptosis or neurogenesis but to epigenetic modifications that occur during development. However, the molecular mechanisms that silence the *Kiss1* gene in males or activate it in females remain to be fully elucidated.

Recent studies are consistent with a role for epigenetic differences in *Kiss1* regulatory regions in the AVPV of males and females. First, several CpG methylation sites differ between males and females in the promoter and first intronic regions of the *Kiss1* locus in the AVPV, but not the arcuate (182). To determine whether these sites are causal, sexual differentiation of *Kiss1* expression could be examined in animals with point mutations or deletions of these CpG regions in Kiss1 neurons. Second, there is evidence that E_2 increases *Kiss1* expression in the AVPV of females by increasing histone H3 acetylation in the promoter region and facilitating the interaction between the promoter and the 3' intragenic region (161). It would be interesting to determine whether this chromatin loop forms in males and if not, whether neonatal treatment of females with T would prevent this loop from forming. Alternatively, treatment of males with E_2 during puberty might feminize the AVPV by facilitating loop formation. Finally, it remains to be determined whether females lacking this 3' intergenic region are masculinized in their ability to have a preovulatory LH surge.

Relevance of Kiss1 sexual differentiation to primates

The GnRH surge mechanism of primates is located in the MBH and is not sexually differentiated, in contrast to rodents. Nevertheless, women express more *Kiss1* than males in the hypothalamus (128) and may require these higher kisspeptin levels for the GnRH/LH surge, as my results suggest (201). Therefore, if sexual differentiation of *Kiss1* expression involves feminization through E_2 exposure during puberty, as is suggested by studies in *Cyp19a1* KOs (179, 180), then investigating sexual differentiation of *Kiss1* in primates could be relevant to understanding fertility in women.

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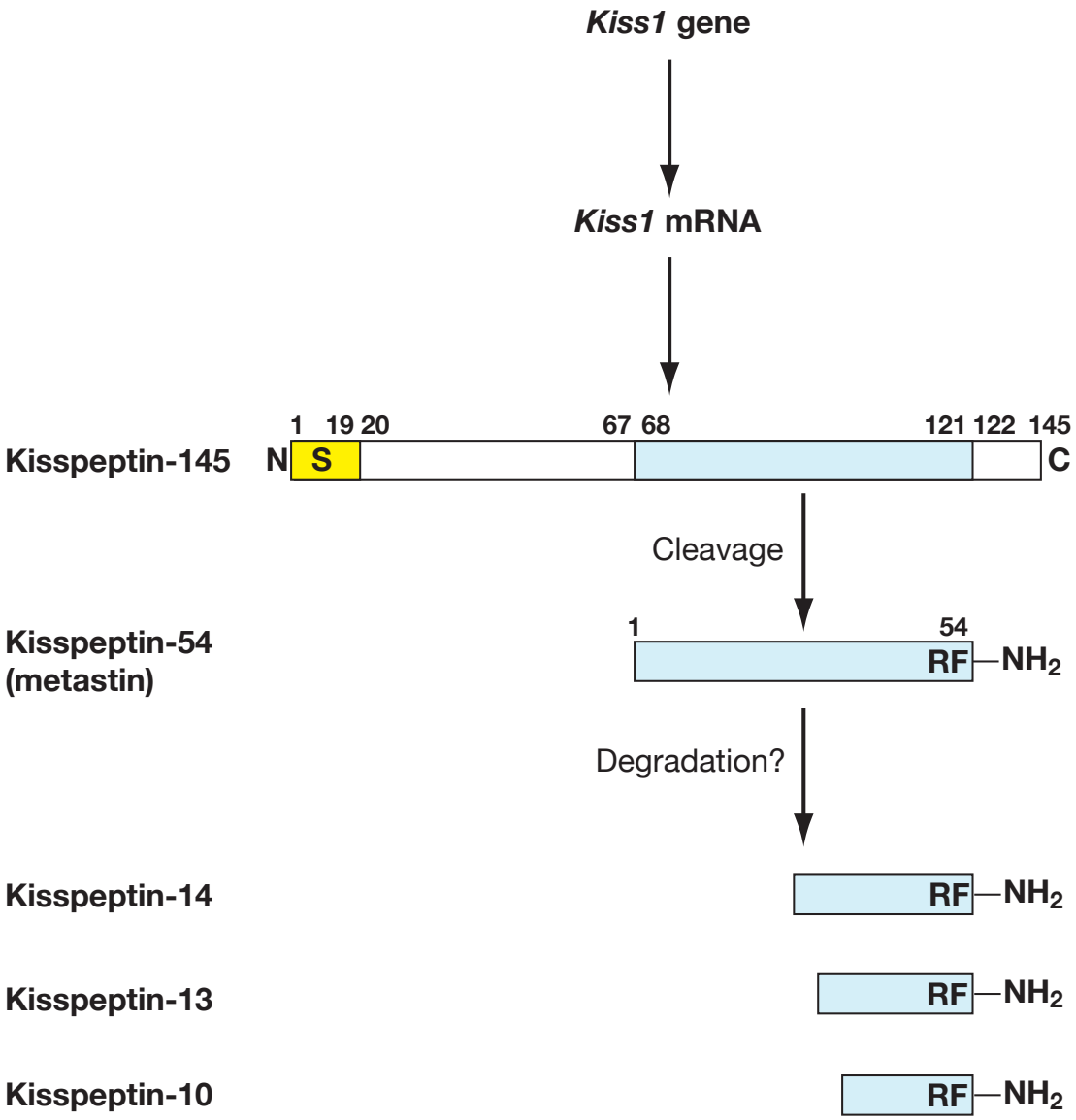

Figure 1. Products of the *Kiss1* gene. *Kiss1* mRNA is transcribed from the *Kiss1* gene and translated to form a 145-amino-acid propeptide called kisspeptin-145. Shown are the signal peptide (S) and cleavage sites on the propeptide that lead to the production of the RF-amidated kisspeptin-54, also known as metastin. Shorter peptides (such as kisspeptin-10, -13, and -14) were identified by mass spectrometry. These peptides share a common C-terminus and RF-amidated motif with kisspeptin-54. Because no putative cleavage sites have been identified on the propeptide that would lead to synthesis of the shorter peptides, such peptides may be degradation products of kisspeptin-54. Modified from (63).

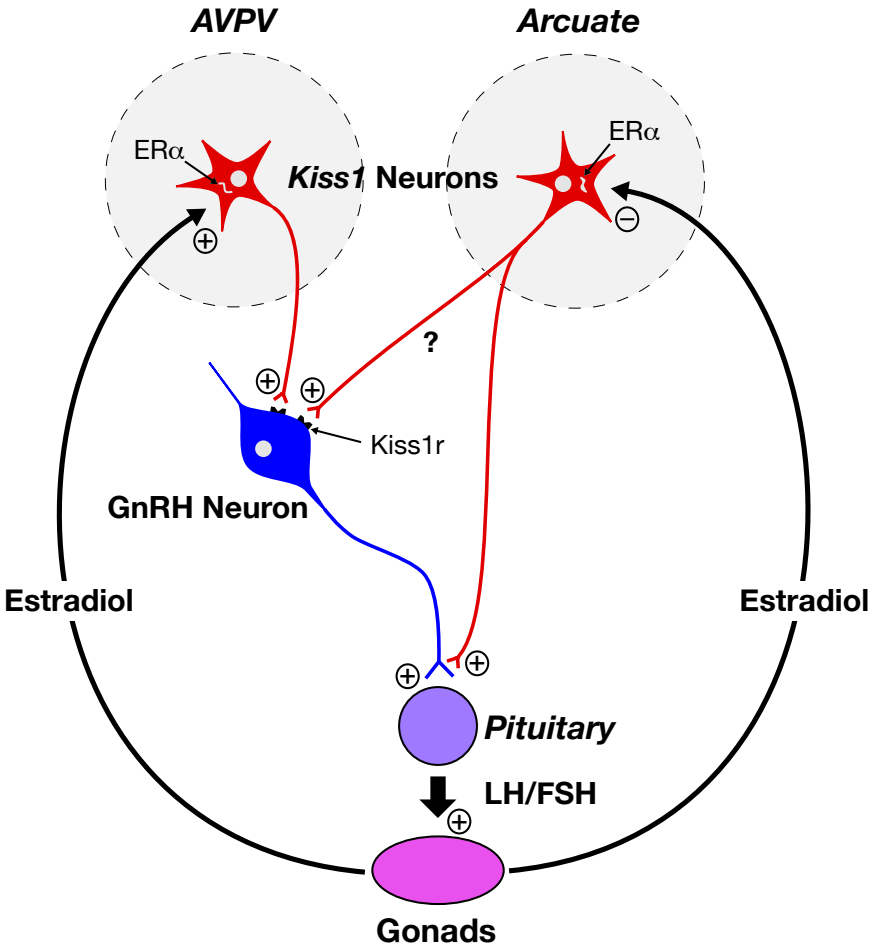

Figure 2. *Kiss1*-expressing neurons are thought to relay negative- and positive- feedback effects of sex steroids on gonadotropin-releasing hormone (GnRH) secretion. Kisspeptin excites GnRH neurons and stimulates GnRH release by acting on the G protein-coupled receptor Kiss1r. GnRH release results in increased gonadotropin secretion from the pituitary gland, which stimulates sex-steroid synthesis and secretion from the gonads. Sex steroids (*e.g.*, estradiol) regulate *Kiss1* expression such that inhibition of *Kiss1* in the arcuate nucleus of the hypothalamus may reduce kisspeptin input to GnRH neurons and reduce GnRH and gonadotropin release in both sexes. In females, stimulation of *Kiss1* expression by estrogen in the anteroventral periventricular nucleus (AVPV) may increase kisspeptin input to GnRH neurons as well as GnRH and gonadotropin release. ERα, estrogen receptor α; LH, luteinizing hormone; FSH, follicle-stimulating hormone. Adapted from (63).

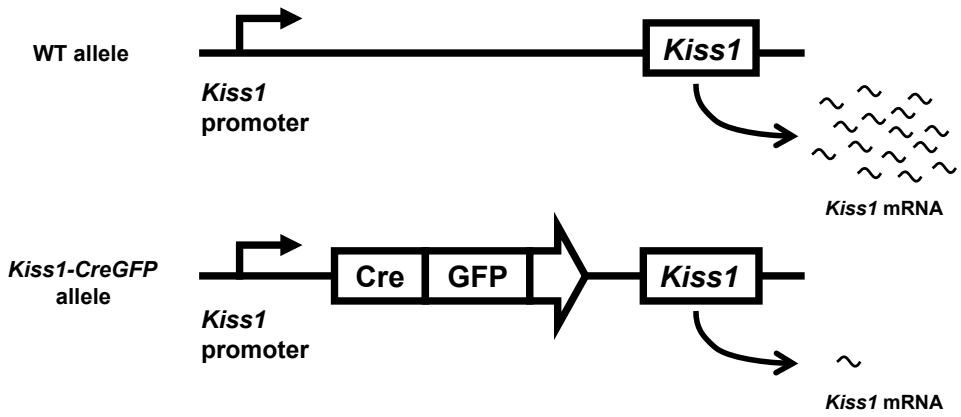

Figure 3. *Kiss1-CreGFP* construct knocked into the *Kiss1* locus to reduce *Kiss1* transcription. **Top**: Schematic of wild type *Kiss1* allele. **Bottom**: Schematic of *Kiss1-CreGFP* allele described in detail in (192). The arrow depicts sequences that promote cleavage of primary transcript and polyA tail addition, which dramatically reduces expression of the *Kiss1* gene. Modified from (201).

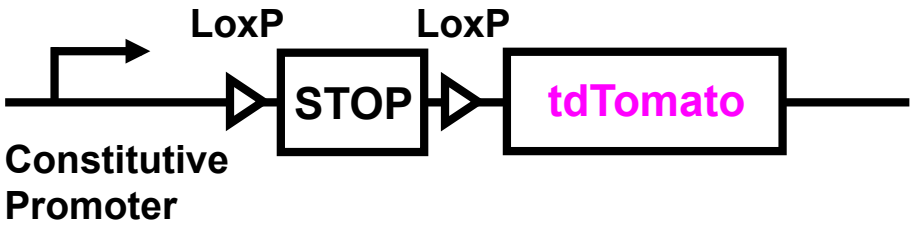

Figure 4. Schematic of the tdTomato reporter mouse construct. In the presence of Cre, the transcriptional stop cassette is excised and tdTomato is constitutively expressed under the CAG-modified Rosa 26 promoter (200).

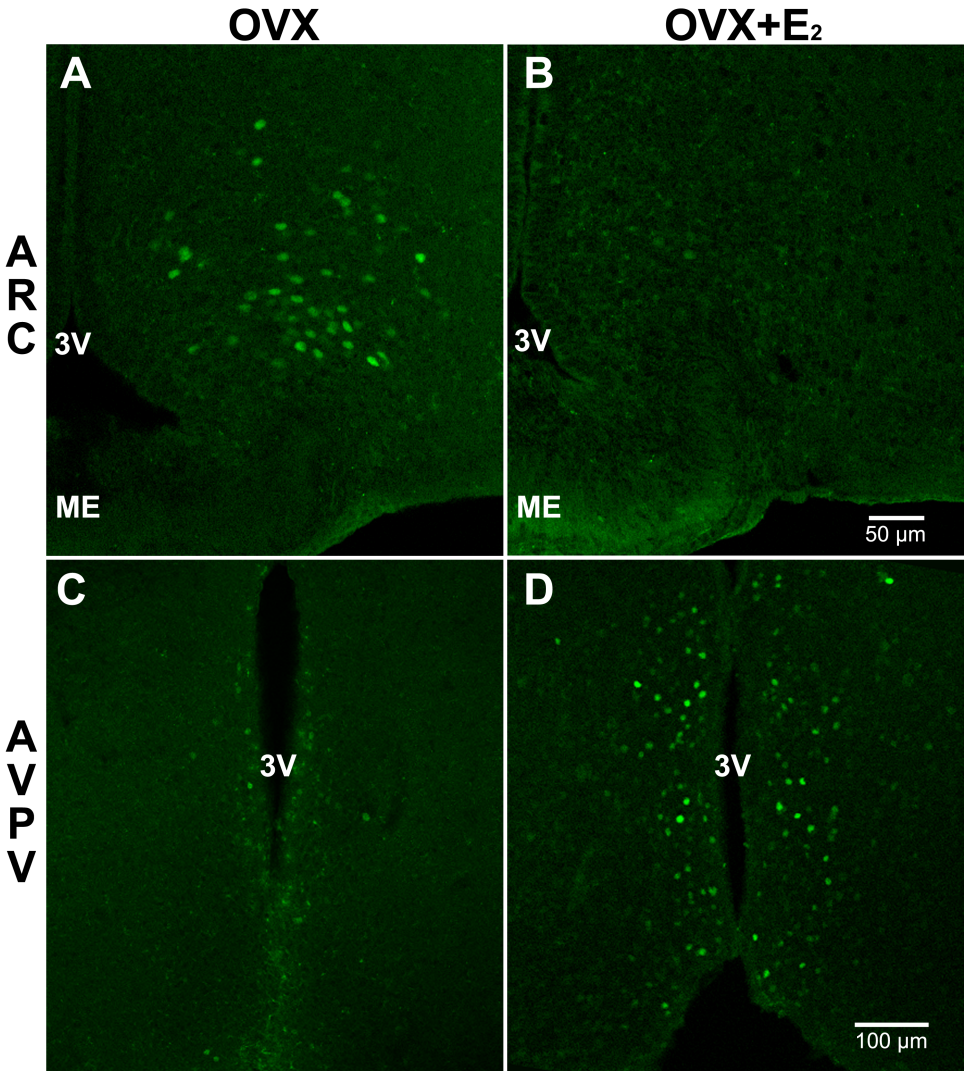

Figure 5. CreGFP expression in the *Kiss1-CreGFP* knock-in mouse is regulated by sex steroids like the *Kiss1* gene. A. Endogenous GFP expression in the ARC of a GDX female. B. Endogenous GFP expression decreases in a GDX + E female. C. GFP immunofluorescence in the AVPV of a GDX female. D. GFP immunofluorescence increases in the AVPV of an E_2-treated female. Reprinted from (192).

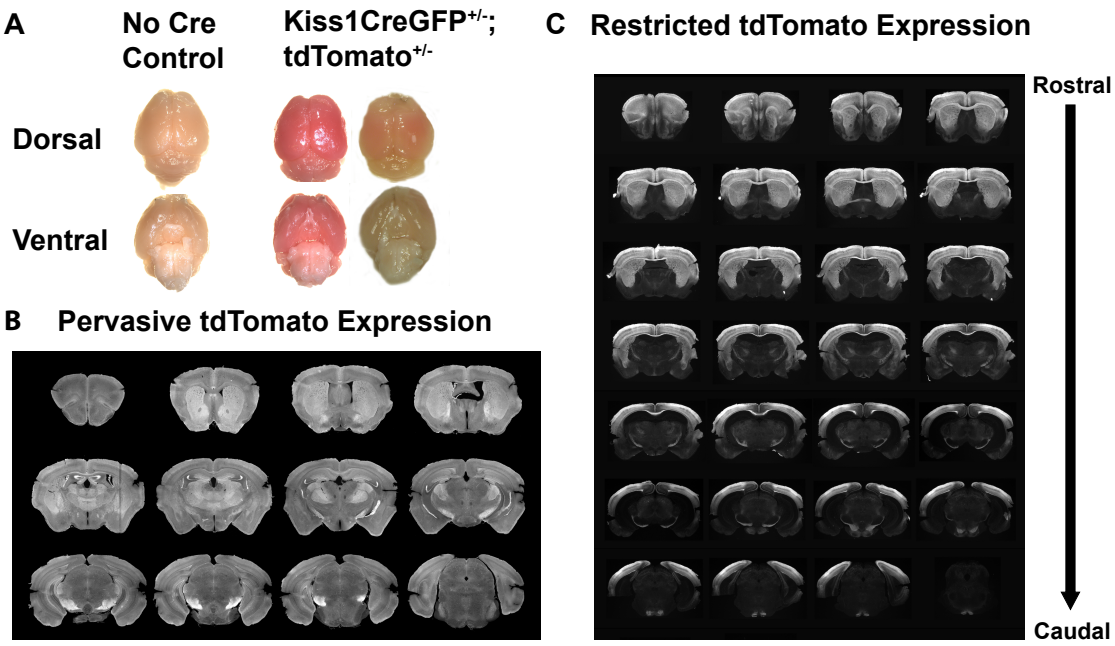

Figure 6. Cumulative history of *Kiss1* expression in the adult brain is bimodal. A. Representative images of *Kiss1-CreGFP*$^{+/-}$*; tdTomato*$^{+/-}$ brains, half of which express tdTomato in most cells (middle) and half of which have restricted expression (right). B and C. Representative coronal sections from brains in A at 1X magnification showing pervasive (B) (n = 9) or restricted (C) (n = 7) tdTomato labeling in the bright regions. Labeling includes cell bodies and fibers.

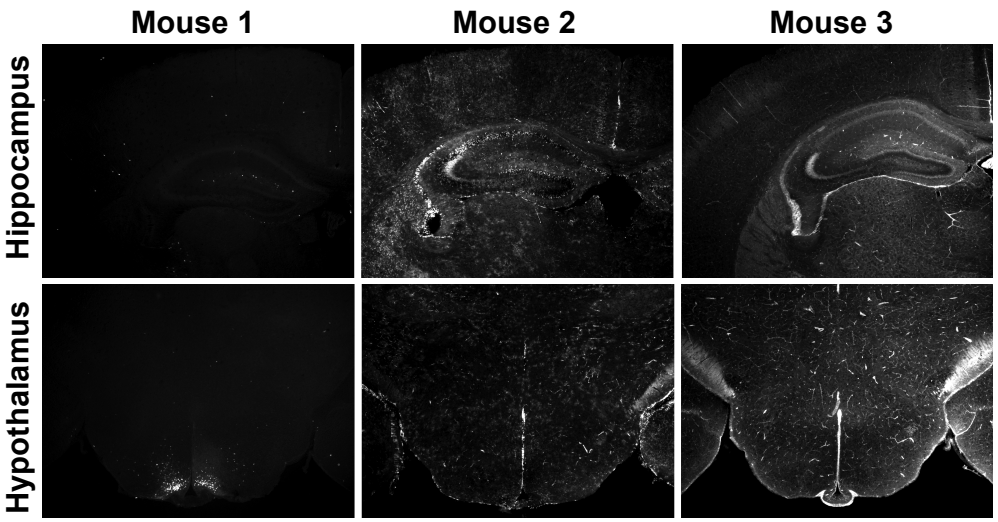

Figure 7. Cumulative *Kiss1* expression in the developing brain lies on a continuum. Expression of tdTomato in *Kiss1CreGFP*$^{+/-}$*; tdTomato*$^{+/-}$ mice at post-natal day 12 in the hippocampus/ cortex (top) and the hypothalamus (bottom). [n = 4 restricted: 2 males & 2 females (left), 2 intermediate: 1 male & 1 female (middle), 1 pervasive female (right)].

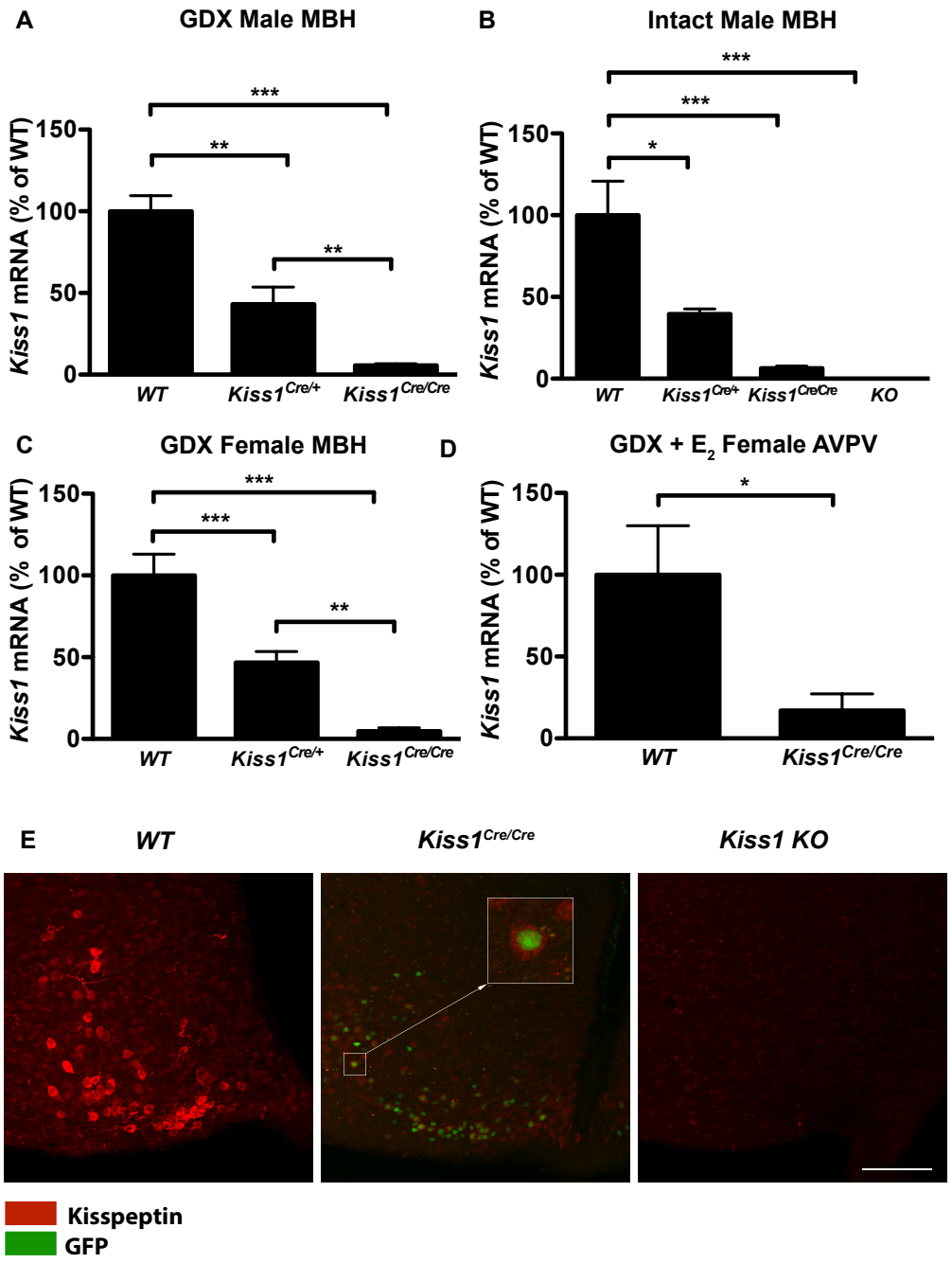

Figure 8. *Kiss1* gene expression is dramatically reduced in *Kiss1*$^{Cre/Cre}$ mice. **A-D**. Quantitative RT-PCR of *Kiss1* mRNA normalized first to *Actb* mRNA, then expressed as a % of WT levels (n = 4-6 males and 4-8 females). **E.** Immunohistochemistry for kisspeptin (red) in the arcuate nucleus of the hypothalamus of GDX male WT, *Kiss1*$^{Cre/Cre}$ and complete *Kiss1* KO animals (from left to right) (n = 4). In center panel, nuclei express GFP driven by the *Kiss1* promoter (see Figure 3). Scale bar = 100 μm. *p<0.05; **p<0.005; ***p<0.0005. Reprinted from (201).

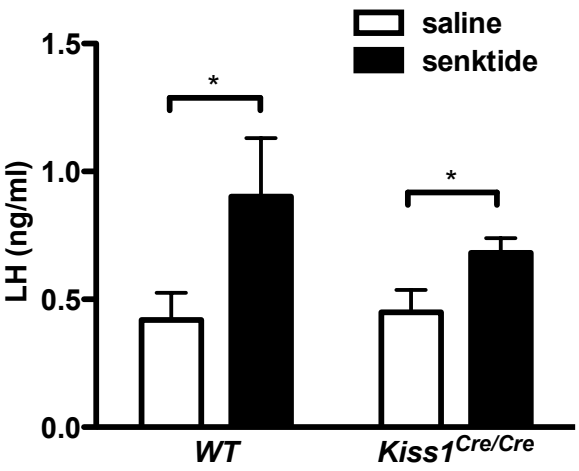

Figure 9. Kisspeptin is physiologically active in *Kiss1*$^{Cre/Cre}$ female mice. Senktide stimulates LH release only when kisspeptin signaling is intact (n = 7-10). *p<0.05 one-tailed t-test within each genotype because we specifically sought to determine whether senktide would cause an increase in LH and p<0.01 for effect of treatment for two-way ANOVA. Reprinted from (201).

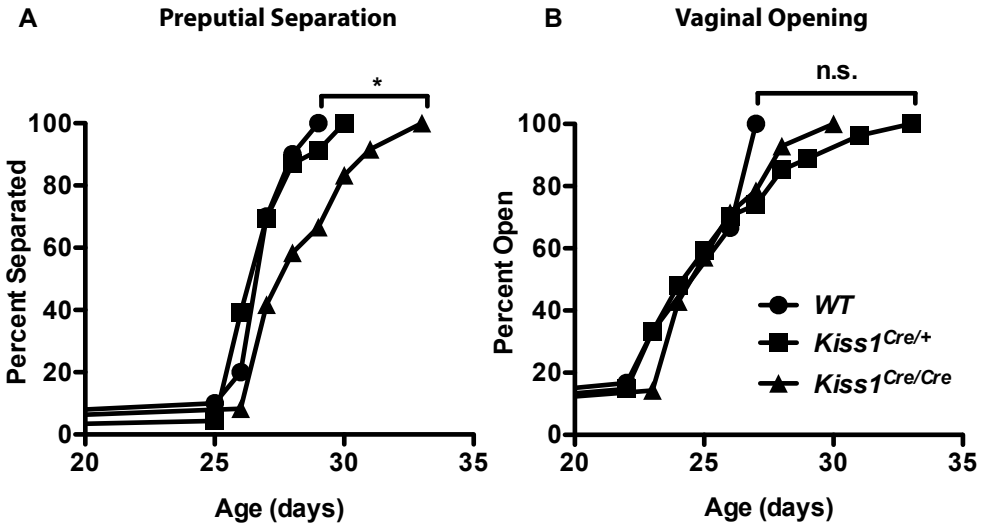

Figure 10. Sexual maturation in *Kiss1*$^{Cre/Cre}$ mice. Preputial separation (**A**) and vaginal opening (**B**) were checked daily in WT, *Kiss1*$^{Cre/+}$, and *Kiss1*$^{Cre/Cre}$ males and females, respectively, as markers for circulating gonadal steroids (n = 6-26). Circles = WT; squares = *Kiss1*$^{Cre/+;}$ triangles = *Kiss1*$^{Cre/Cre}$ mice. *p<0.05 Log-rank Mantel-Cox test. n.s. = not statistically significant. Reprinted from (201).

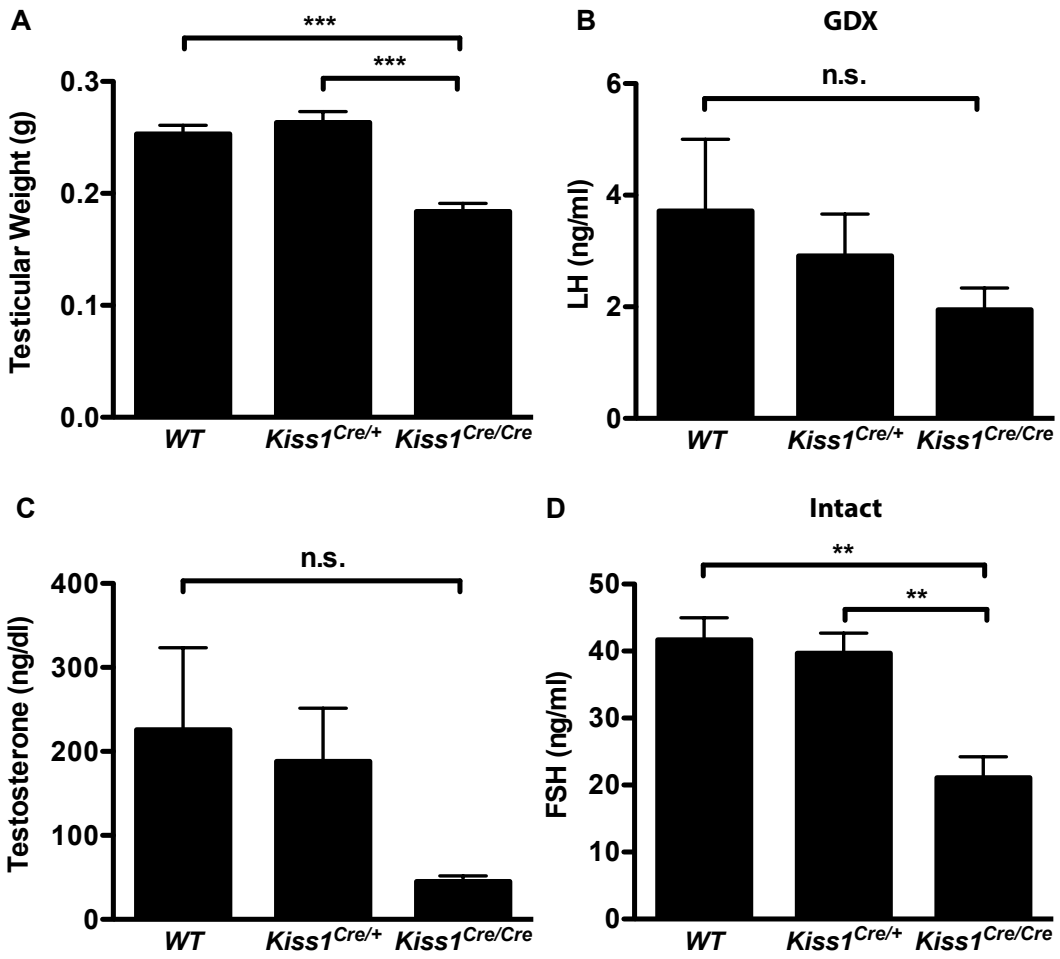

Figure 11. Phenotype of adult *Kiss1*$^{Cre/Cre}$ males. **A.** Testicular weight (n = 10-12). **B.** LH in gonadectomized (GDX) mice (n = 4-5). **C.** Testosterone (n = 9-22). **D.** FSH in intact mice (n = 7-19). **p<0.005; ***p<0.0001; A and D were analyzed with one-way ANOVA with Tukey's multiple comparison test. **B.** and **C.** were analyzed with Kruskal Wallace non-parametric test because some samples were out of the range of the assay. Reprinted from (201).

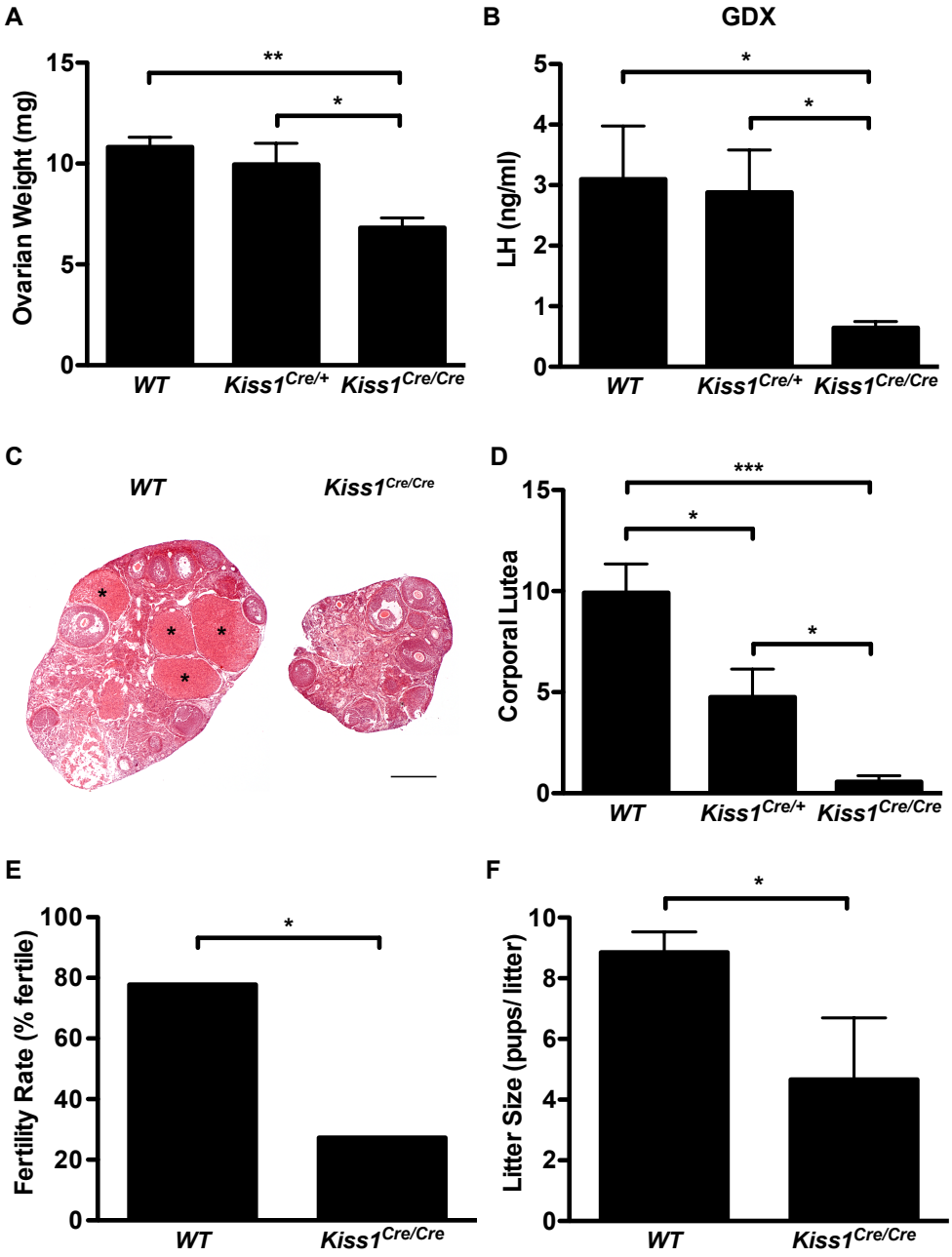

Figure 12. Phenotype of adult *Kiss1*$^{Cre/Cre}$ females. **A.** Combined ovarian weight (n = 6-8). **B.** LH in OVX mice (n = 6-8). **C.** Photomicrographs from WT (left) and *Kiss1*$^{Cre/Cre}$ (right) ovaries. Scale bar = 500 µm. * = corpus luteum. **D.** Mean number of corpora lutea in both ovaries obtained by averaging 2 sets of sections from each ovary analyzed by 2 experimenters blind to genotype (n = 6-7). **E.** Percent of females fertile of all females paired with WT males (n = 9-11). (It is not possible to calculate error bars from a percentage). **F.** Mean number of pups per litter only includes data from females that had pups (n = 3-7). *p<0.05; **p<0.005; ***p<0.0001. **A, B** and **D** One-way ANOVA with Tukey's Multiple Comparison Test. **E.** Fisher's exact test. **F.** Two-tailed t-test. Reprinted from (201).

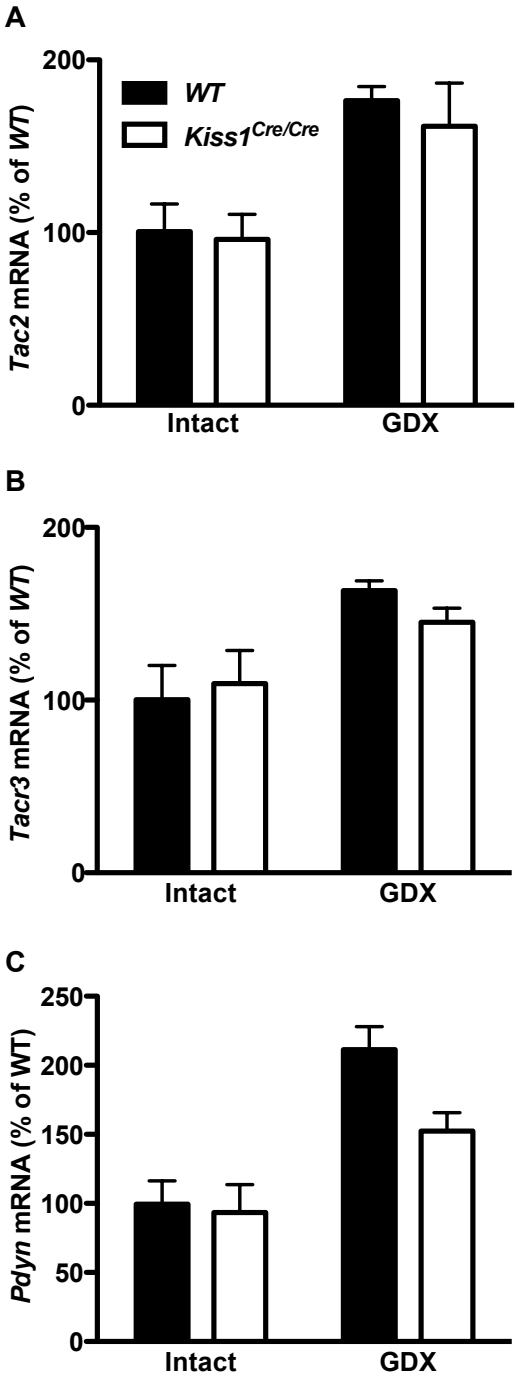

Figure 13. Co-neurotransmitter and receptor expression in the MBH of *Kiss1*$^{Cre/Cre}$ male mice (n = 4-6). Two-way ANOVA revealed a significant effect of GDX on the expression of **A.** *Tac2* (which encodes NKB, $p < 0.005$), **B.** *Tacr3* (which encodes the NKB receptor, $p < 0.01$), and **C.** *Pdyn* (which encodes dynorphin, $p < 0.001$). There was no effect of genotype and no interaction between treatment and genotype for any of the 3 genes. Reprinted from (201).

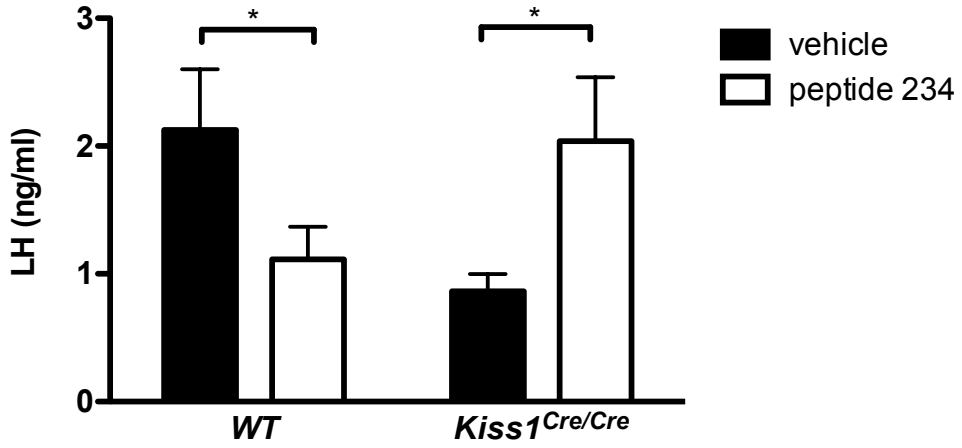

Figure 14. Kiss1 receptor "antagonist", peptide 234, acts as a weak agonist in female mice (n = 7-9). To determine whether endogenous LH release was sustained by Kiss1r signaling in *Kiss1*$^{Cre/Cre}$ mice, peptide 234 was administered ICV to WT control and *Kiss1*$^{Cre/Cre}$ mice. (two-way ANOVA interaction $p < 0.05$; * t-test for vehicle vs. peptide 234 $p<0.05$).

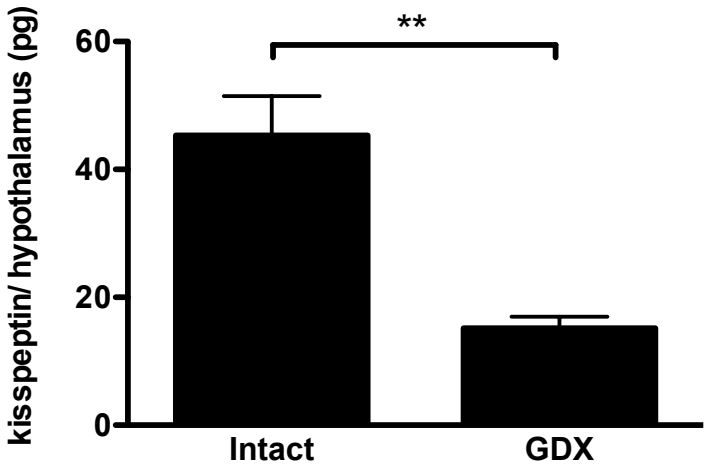

Figure 15. Removing negative feedback inhibition from the testes by GDX decreases kisspeptin 10 content in the MBH. Peptide levels were measured by radioimmunoassay (n = 5 / group).

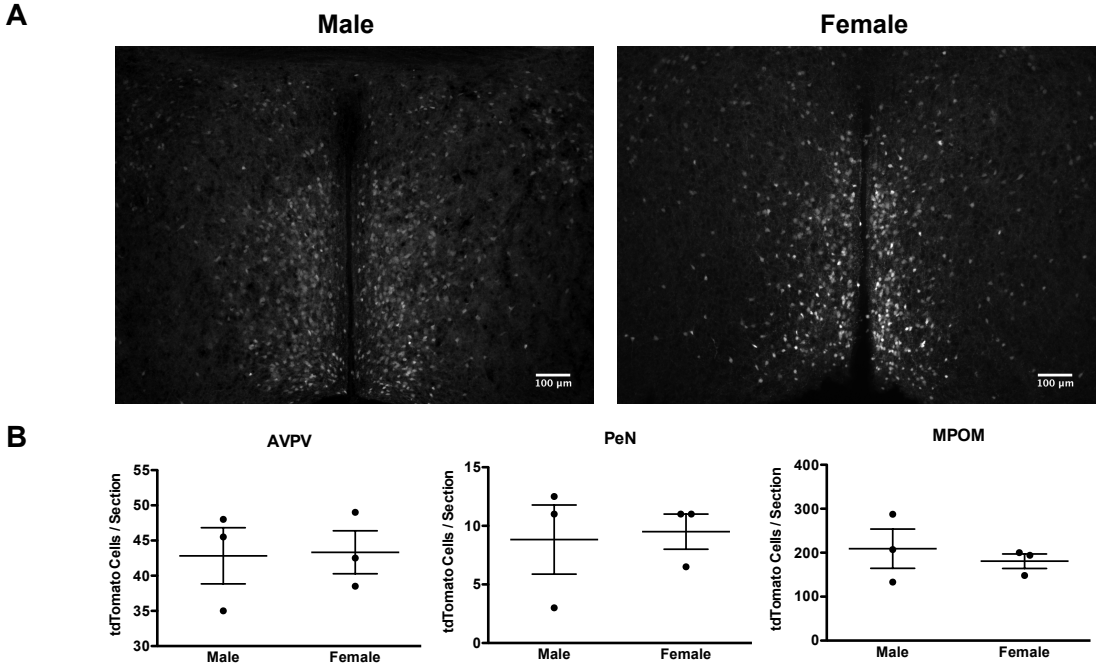

Figure 16. The number of *Kiss1* neurons does not differ significantly between males and females in the AVPV/ PeN/ MPOM regions. A. Representative images of male and female tdTomato-labeled cells. B. Comparison of the number of cells per section in the AVPV, PeN and MPOM between male and female mice (n = 3 / group; Mean ± SEM).

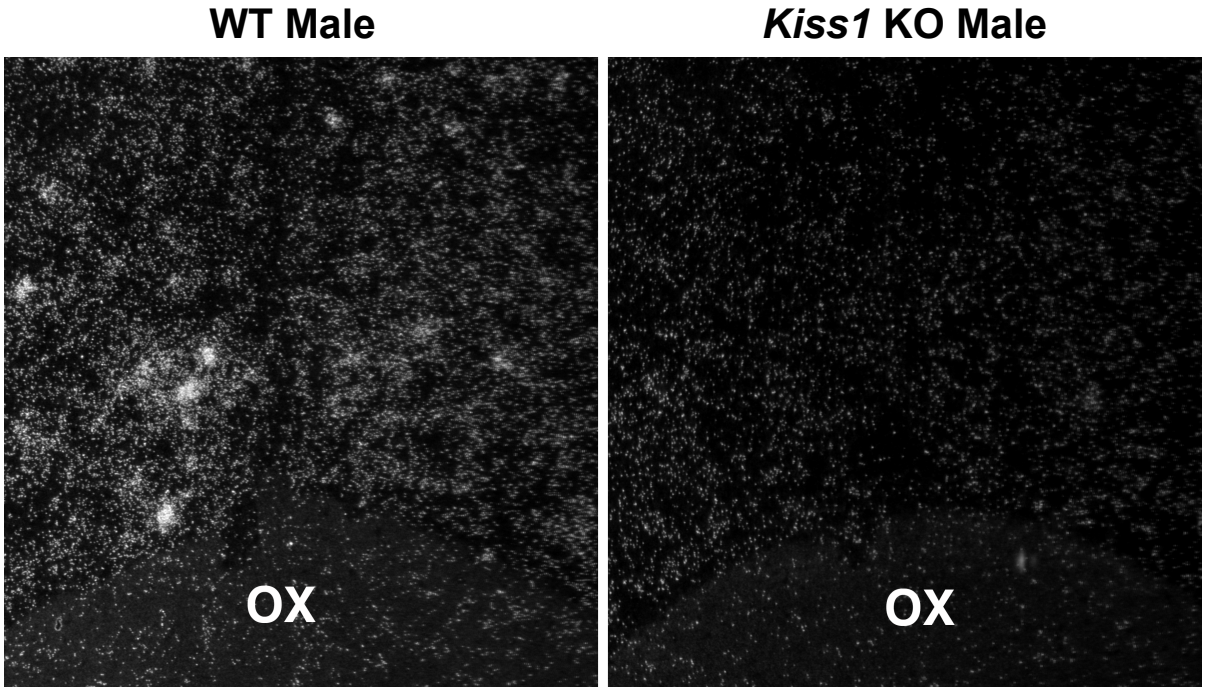

Figure 17. Males express low levels of *Kiss1* mRNA in many cells of the AVPV/ PeN/ MPOM. Intact WT males (left) have clusters of white silver grains over cells expressing *Kiss1* mRNA (n = 3-4). These grain clusters are not visible in Kiss1 KO males (right).

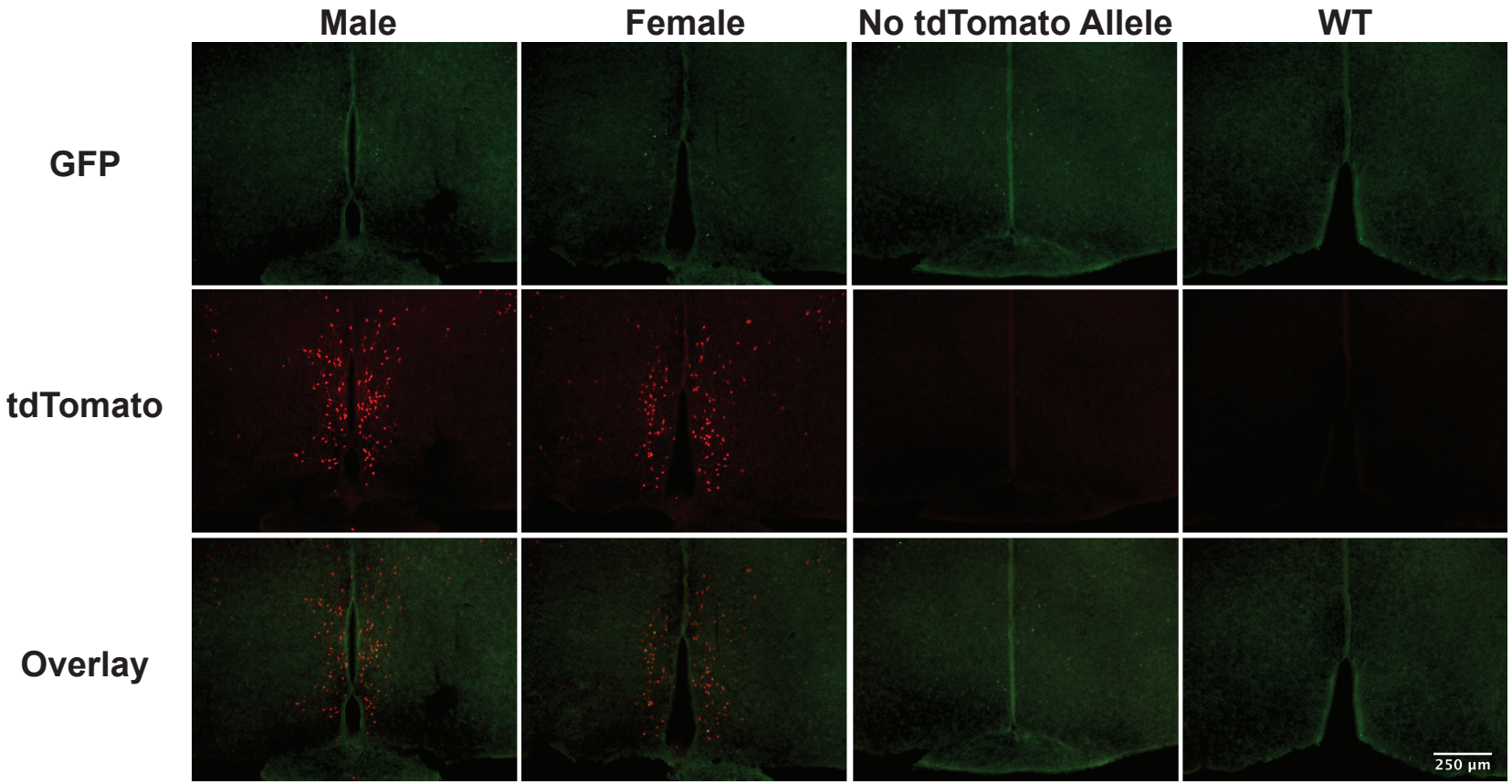

Figure 18. Males and females express *Kiss1* in many AVPV neurons before puberty (post-natal day 12, n = 2 / sex). Nuclear GFP (top), under the control of the endogenous *Kiss1* promoter, represents current levels of *Kiss1* expression in *Kiss1Cre*$^{+/-}$*; tdTomato*$^{+/-}$ mice. tdTomato (middle row) reveals cumulative *Kiss1* mRNA expression *Kiss1Cre*$^{+/-}$*; tdTomato*$^{+/-}$ mice. Bottom: overlay of GFP (current) and tdTomato (cumulative) expression.

Gene – Primer	Oligo Sequence
Tac2-FOR	TTC CAC AGA AAC GTG ACA TGC
Tac2-REV	GGG GGT GTT CTC TTC AAC CAC
Tacr3-FOR	GCC ACA GCC ACT AAG ATT GTC
Tacr3-REV	CGG CCT GGC ATG ACT TTT ATT TT
PDyn-FOR	GAG GTT GCT TTG GAA GAA GGC
PDyn-REV	TTT CCT CTG GGA CGC TGG TAA
Actb-FOR	AGTGTGACGTTGACATCCGTA
Actb-REV	GCCAGAGCAGTAATCTCCTTCT
Kiss1 (Taqman)	Applied Biosystems (proprietary) Mm03058560_m1

Table 1. Primers for SYBR and Taqman qRTPCR.

Cycle	Time	Temperature	Step
1	30 min	50 C	Reverse Transcription
2	10 min	95 C	Polymerase Activation
3	15 - 30 sec	95C	Denaturation
4	1 min	60C	Annealing/ Elongation
5	Repeat Steps 3-4 for 45 cycles		
6	1 min	95C	Denaturation
7	30 sec	55C	Annealing
8	55C to 95C temperature ramp with fluorescence read every second	55C-95C	Melting curve to determine number of PCR products
9	30 sec	95C	Final step of melting curve

Table 2. QRTPCR steps. The melting curve in steps 6-9 was only used for SYBR reactions.

Assay	Treatment & Sex	Reportable Range	Intra-assay % CV
LH multiplex	GDX + E females	0.24-30.0 ng/ml	9%
LH multiplex	GDX males	0.24-30.0 ng/ml	7%
FSH multiplex	GDX males	2.4-300.0 ng/ml	4%
Testosterone RIA	Intact males	5.9-1100 ng/dl	4%
LH IRMA	GDX females	0.04-37.4 ng/ml	16%
LH IRMA	Intact females (senktide)	0.04-37.4 ng/ml	7%
FSH IRMA	Intact males	3.1-75.0 ng/ml	6%
Kisspeptin RIA	Intact & Castrated Males	Low end 4 pmol/ml	12%

Table 3. Reportable ranges and intra-assay coefficients of variation for hormone assays. % CV = coefficient of variation.

Labeling Pattern in the Brain	Number of Animals
Pervasive (no distinguishable cells)	1/7 (female)
Intermediate (distinct but abundant cells outside the hypothalamus)	2/7 (1 female, 1 male)
Restricted (labeling in hypothalamus and only very few cells in cortex, hippocampus and other hypothalamic regions)	4/7 (2 females, 2 males)

Table 4. Continuum of tdTomato labeling in the brain of *Kiss1*$^{Cre/+}$*;tdTomato*$^{+/-}$ mice at PND 12.

	WT	*Kiss1*$^{Cre/+}$	*Kiss1*$^{Cre/Cre}$
***Kiss1* Intact**	11.93 ± 2.48	4.71 ± 0.33	0.75 ± 0.16
***Kiss1* GDX**	100.00 ± 9.56***	43.11 ± 10.47***	5.65 ± 1.01
LH Intact	0.26 ± 0.17	0.84 ± 0.73	0.39 ± 0.10
LH GDX	3.72 ± 1.28**	2.91 ± 0.75	1.95 ± 0.39

Table 5. *Kiss1* transcript and LH increase in response to castration. *Kiss1* mRNA levels are expressed as a percent of castrated WT animals. Quantitative RTPCR with Taqman primers/probe data are normalized to *Actb*. **p<0.01 and ***p<0.001 for two-way ANOVA.

	Percent Fertile	Mean Litter Size
WT males	79 % (11/14)	8.0 ± 0.6
***Kiss*$^{Cre/Cre}$ males**	56 % (5/9)	7.6 ± 0.9
WT females	78 % (7/9)	8.3 ± 0.6
***Kiss*$^{Cre/Cre}$ females**	27 % (3/11)*	4.7 ± 2.0*

Table 6. Fertility Results. *Kiss1*$^{Cre/Cre}$ animals or their WT littermates were paired with fertile WT mates of the opposite sex for 5 consecutive days, after which the mates were removed. Center column: The number of fertile animals is expressed as a percentage of the total number of animals mated per group. Right column: Litter size does not include data from animals without offspring. *$p<0.05$ Fisher's exact test for Percent Fertile and two-tailed t-test for Mean Litter Size.