Published in final edited form as:
J Neuroendocrinol. 2010 July ; 22(7): 682–691. doi:10.1111/j.1365-2826.2010.02030.x.

Gonadal and Non-Gonadal Regulation of Sex Differences in Hypothalamic *Kiss1* Neurons

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Abstract

The brains of males and females differ anatomically and physiologically, including sex differences in neuron size or number, synapse morphology, and specific patterns of gene expression. Brain sex differences may underlie critical sex differences in physiology or behavior, including several aspects of reproduction, such as the timing of sexual maturation (earlier in females than males) and the ability to generate a preovulatory gonadotropin surge (in females only). The reproductive axis is controlled by afferent pathways that converge upon forebrain gonadotropin-releasing hormone (GnRH) neurons, but GnRH neurons are not sexually dimorphic. Although most reproductive sex differences probably reflect sex differences in the upstream circuits and factors that regulate GnRH secretion, the key sexually-dimorphic factors that influence reproductive status have remained poorly defined. The recently-identified neuropeptide kisspeptin, encoded by the *Kiss1* gene, is an important regulator of GnRH secretion, and *Kiss1* neurons in rodents are sexually dimorphic in specific hypothalamic populations, including the anteroventral periventricular nucleus—periventricular nucleus continuum (AVPV/PeN) and the arcuate nucleus (ARC). In the adult AVPV/PeN, *Kiss1* neurons are more abundant in females than males, a sex difference which is regulated by estradiol signaling during critical periods of postnatal and pubertal development. In contrast, *Kiss1* neurons in the ARC are not sexually differentiated in adult rodents, but in mice, the regulation of ARC *Kiss1* cells by gonadal hormone-independent factors is sexually dimorphic during prepubertal development. These various sex differences in hypothalamic *Kiss1* neurons may relate to known sex differences in reproductive physiology, such as puberty onset and positive feedback.

Keywords

kisspeptin; *Kiss1*; GPR54; Kiss1r; sexual differentiation; sex differences; development; puberty; hypothalamus; hormone; estrogen

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In vertebrates, physiology and behavior, including aspects relating to reproduction and puberty, often vary between animals of the opposite sex. The neuroendocrine reproductive axis is governed by various hormonal and neural pathways that converge upon forebrain gonadotropin-releasing hormone (GnRH) neurons [1]. GnRH neurons direct the activation of the rest of the reproductive axis by stimulating the pituitary to synthesize and secrete gonadotropins [luteinizing hormone (LH) and follicle-stimulating hormone (FSH)] which regulate the gonads. GnRH neurons themselves do not appear to be sexually dimorphic, indicating that sex differences in the control of the reproductive axis likely reflect key sex differences in the afferent neural circuits and factors that regulate the GnRH system. However, many of the sexually-dimorphic factors that influence reproductive status have remained poorly defined. The newly-discovered kisspeptin system has recently been

implicated as an important regulator of GnRH neurons, both in development and adulthood, and sex differences in the kisspeptin system may relate to sex differences in reproductive function. This article discusses the recent evidence that supports an important role of kisspeptin signaling in the control of the reproductive axis and how this relates to sexual differentiation of reproductive physiology. The roles of kisspeptin signaling in puberty and adulthood have already been extensively reviewed [2–4] and I will therefore focus primarily on the latest findings connecting sexual differentiation and the kisspeptin system, with a specific focus on rodent animal models.

Sexual Differentiation of Reproductive Physiology and Behavior

In mammals, including humans, males and females often display differences in a variety of physiological and behavioral traits, ranging from sex differences in learning and memory to complex sociosexual and parental behaviors to differential hormonal secretion patterns. Besides sex differences in normal physiology and behavior, sex differences also exist in a variety of human health disorders and diseases, including precocious puberty (more common in girls), depression (more common in women), and autism (more common in boys) [5–10]. It is thought that many of the sex differences in physiology and behavior reflect underlying sex differences in neural mechanisms in the brain. The brain, along with other parts of the central nervous system, possesses many anatomical and physiological sex differences (reviewed in [11–15]). These neural sex differences are present in numerous brain areas, including the amygdala, hippocampus, cortex, bed nucleus of the stria terminalis (BNST), lateral septum, and several nuclei within the hypothalamus [13,16–22]. The nature of sex differences in the brain are wide-ranging and can vary from region to region (and even within a region), depending on the specific trait. For example, neural sex differences may include differences in cell morphology, neuron size, neuron number, axonal fiber projections, synapse morphology, and cellular expression levels of specific genes or proteins. In addition, sex differences in the brain can favor either males over females (male-biased) or females over males (female-biased), depending on the particular trait. For example, in rats, the BNST and the medial preoptic nucleus each contain more neurons and comprise a larger regional volume in males than females [18,22,23]. In contrast, the anteroventral periventricular nucleus (AVPV) of the hypothalamus is not only larger and possesses more cells in females than in males, but the number of AVPV neurons expressing tyrosine hydroxylase (TH) enzyme is greater in females than males [24–27].

Similar to the brain, the neuroendocrine reproductive axis has several aspects that differ between the sexes. In rodents, these reproductive sex differences include earlier sexual maturation in females than males, the presence of neural circuitry that generates preovulatory hormone surges (i.e., positive feedback) in adult females but not males, and sexually differentiated neural circuits that govern gender-specific reproductive behaviors, such as certain female proceptive behaviors (e.g., hopping and darting) and receptive mating behavior (e.g., display of the receptive lordosis posture) [6,10,28–30]. In addition to these sex differences in normal reproductive physiology and behavior, there are several reproductive health disorders and diseases which occur with a differential frequency between males and females, such as idiopathic hypogonadotropic hypogonadism (more common in men), constitutional delayed puberty (more common in boys), and precocious puberty (more common in girls [5,6,31,32].

It is likely that many of the sex differences in reproductive physiology, behavior, and health disorders reflect underlying sex differences in the brain. While studies have identified numerous sex differences in brain structures and neural phenotypes on the one hand, and reproductive physiology and behavior on the other, in most cases the actual link between a specific neural sex difference and sex differences in particular physiology/behaviors have

remained elusive. For example, in rodents (and other mammals), sex steroids, particularly estradiol (E_2), can feedback during a specific time of the estrous cycle to induce an acute surge in GnRH and LH secretion, thereby triggering ovulation. This positive feedback event is sexually differentiated, occurring only in females but not males. In fact, unlike females, male rodents given exogenous E_2 (or other sex steroid treatments) are incapable of generating an LH surge, indicating that the male brain lacks the functional neural circuitry or specific neural factors needed to produce an LH surge. However, while this fact has been known for many decades, the identity of the specific sexually-dimorphic neural population(s) that governs the LH surge has remained elusive.

Sex Differences in *Kiss1* Neurons in the AVPV/PeN

As mentioned above, the E_2-induced LH surge event (positive feedback) is sexually differentiated in rodents. GnRH neurons are the final common pathway by which the brain controls reproduction, including the LH surge, but GnRH neurons are not themselves sexually dimorphic, suggesting that other neuronal populations influence sex differences in reproductive physiology. Lesion and hormone implant studies in the 1980's implicated the AVPV region as being a critical part of the LH surge generating mechanism in rodents, findings supported by the observation that the AVPV expresses estrogen receptors, including ERα, and projects to GnRH neurons (reviewed in [33]). Moreover, several aspects of the AVPV are sexually dimorphic, with females possessing more neurons overall than males, as well as greater numbers of neurons containing tyrosine hydroxylase (TH; i.e., dopaminergic cells) and GABA/glutamate (reviewed in [11,18]). Yet, the actual involvement of any specific AVPV population in the sexually-dimorphic LH surge has, until the recent discovery of the kisspeptin system, been equivocal.

The Link Between the Kiss1 System and Reproduction

The *Kiss1* gene (*KISS1* in humans) encodes kisspeptin, a neuropeptide of 52 or 54 amino acids, depending on the species [34]. In a wide range of mammals, including mice, rats, hamsters, sheep, horses, pigs, and primates, *Kiss1* mRNA or kisspeptin protein has been detected in two discrete regions of the hypothalamus, the preoptic area [which in rodents includes the morphological continuum comprising the anteroventral periventricular nucleus and neighboring periventricular nucleus (AVPV/PeN)] and, more caudally, the arcuate nucleus (ARC; analogous to the primate infundibular nucleus) (reviewed in [2,3,35,36]). The *Kiss1* gene is also expressed in several peripheral tissues, most notably, the placenta, ovary, testis, pituitary, pancreas, and adipose tissue, but at present, little is known regarding kisspeptin's role outside the brain. Kisspeptin is a high-affinity ligand for the membrane receptor, GPR54, now renamed the kisspeptin receptor, or Kiss1r [37–39]. The *Kiss1r* gene is expressed in both peripheral tissues and the brain, most notably the hippocampus, habenula, hypothalamus, and preoptic/septal areas (including GnRH neurons) [40,41].

The *Kiss1* system was first implicated in regulating reproduction in 2003, when several groups independently reported that humans and mice with mutations in the kisspeptin receptor exhibit prominent deficits in reproductive function and puberty onset [42,43]. These initial findings were soon echoed by other similar reports in *Kiss1r* knockout (KO) mice, as well as mice lacking a functional *Kiss1* gene [44–49], suggesting that kisspeptin-Kiss1r signaling is critical for proper sexual maturation and fertility. Indeed, early experiments determined that exogenous treatment of rodents and other species, including humans, with kisspeptin robustly increases circulating LH and FSH levels (discussed in [3,50,51]). Subsequent studies from numerous species have now provided a wealth of information supporting the model that hypothalamic-derived kisspeptin directly activates GnRH neurons via Kiss1r to stimulate the reproductive axis (reviewed in [2,4,35,36,52]).

The Role of Kiss1 Neurons in Sexually-Dimorphic LH Secretion (Positive Feedback)

In adulthood, the secretion of GnRH is regulated by positive and negative feedback actions of gonadal sex steroids [i.e., testosterone (T) and estradiol (E_2)], but GnRH neurons do not express estrogen receptor α (ERα) or the androgen receptor (AR), the receptors that mediate sex steroid feedback. Thus, other sex steroid-sensitive circuits upstream of GnRH neurons likely relay sex steroid feedback signals to GnRH cells. Recent evidence suggests that hypothalamic *Kiss1* neurons are these upstream sex steroid-sensitive neurons. Specifically, it has been proposed that, in rodents, ARC *Kiss1* neurons mediate negative feedback effects of sex steroids on reproductive status, whereas AVPV/PeN *Kiss1* neurons mediate positive feedback effects of E_2 on GnRH secretion, thereby triggering the preovulatory LH surge [3,36,50]. In regards to positive feedback, which is sexually dimorphic, the evidence for a role for AVPV/PeN *Kiss1* neurons in governing the LH surge is summarized as follows:

1. Neuroanatomically, the AVPV/PeN region has been shown to mediate E_2-induced positive feedback [17,33].
2. *Kiss1* gene expression in the rodent AVPV/PeN is robustly upregulated by high levels of E_2 [53–55], correlating with the occurrence of GnRH/LH surges.
3. In the absence of adulthood gonadal steroids, such as in gonadectomized animals, *Kiss1* levels are decreased in the AVPV/PeN [53–55], correlating with an absence of GnRH/LH surges.
4. Almost all *Kiss1* neurons in the AVPV/PeN express ERα [53,54,56], the receptor subtype that mediates positive feedback.
5. Fos is induced in *Kiss1* neurons in the AVPV/PeN during the LH surge [44,54,56,57].
6. *Kiss1* neurons in the AVPV/PeN of E_2-treated female mice display circadian patterns of activation, in synchrony with the circadian timing of the LH surge [57].
7. Pharmacological or transgenic blockade of kisspeptin signaling prevents the preovulatory LH surge (positive feedback) from occurring [44,58,59], providing functional evidence for the role of kisspeptin signaling in the LH surge event.

Collectively, these findings indicate that kisspeptin signaling arising from neurons in the AVPV/PeN plays an important role in mediating positive feedback effects of estradiol in rodents. (In sheep, and perhaps other non-rodent species, positive feedback may be mediated by *Kiss1* neurons in the ARC rather than the preoptic or AVPV/PeN regions [36]).

Sexual Differentiation of AVPV/PeN Kiss1 Neurons

Given the critical role of AVPV/PeN *Kiss1* neurons in governing the LH surge, we hypothesized that the sex difference in the LH surge reflects sexual differentiation of the *Kiss1* system in the AVPV/PeN. We found that, like both TH and GABA/glutamate neurons, *Kiss1* neurons in the rat AVPV/PeN are sexually differentiated, with adult females possessing greater *Kiss1* expression in this region than males [55]. More specifically, the number of *Kiss1* mRNA-expressing neurons in the AVPV/PeN of adult female rats was 15–25 times greater than in adult males, and the relative amount of *Kiss1* mRNA per cell was also higher in this area in females than males [55]. Similar sex differences in kisspeptin protein levels in the AVPV/PeN, as determined by immunohistochemistry, as well as *Kiss1* mRNA levels, were reported in intact mice (Figure 1) [60,61]. Thus, similar to the female-biased TH and GABA/glutamate populations in the AVPV/PeN [25,27,62], the number of *Kiss1* neurons in the AVPV/PeN correlates with the ability or inability of an animal to generate an LH surge: adult females have high *Kiss1* levels and can generate an LH surge, while adult males have little AVPV *Kiss1* expression and cannot produce an LH surge, even

with E_2 treatment. However, in contrast to TH and GABA/glutamate neurons for which the functional role is still unclear, abundant evidence supports a critical involvement of kisspeptin in the LH surge event.

In adult rodents, sex steroids dramatically upregulate *Kiss1* levels in the AVPV/PeN. To assess whether sex differences in AVPV/PeN *Kiss1* neurons are attributable to sex differences in circulating levels of T or E_2 in adulthood, we measured *Kiss1* expression in adult gonadectomized male and female rats receiving identical sex steroid treatments (i.e., with or without E_2 implants). Even when sex steroid levels were similar between the sexes, females had much higher *Kiss1* expression in the AVPV/PeN than did males [55]; thus, adult females possess more *Kiss1* cells in this region than males, regardless of the circulating adult sex steroid milieu. This finding has since been replicated for *Kiss1* mRNA levels in mice, kisspeptin protein levels in mice, and both kisspeptin protein and *Kiss1* mRNA levels in rats [61,63–65]. Given the importance of kisspeptin in promoting the LH surge, the sex difference in AVPV/PeN *Kiss1* expression in adulthood likely accounts for the sex-specific ability of female rodents, but not males, to produce an LH surge. However, an important role of this AVPV/PeN *Kiss1* sex difference in other sexually-dimorphic aspects, such pubertal maturation, cannot be excluded.

The AVPV contains a sexually differentiated population of TH-positive (i.e., dopaminergic) neurons, which, like the *Kiss1* population, is greater in adult females than adult males [66,67]. Are the sexually dimorphic *Kiss1* and TH cells in the AVPV/PeN the same neuronal population or separate sexually dimorphic systems located in the same brain region? We addressed this issue by performing double-label immunohistochemistry and double-label *in situ* hybridization assays. In adult female rats, the majority of AVPV/PeN *Kiss1* neurons do not co-express *TH* mRNA, and the few *Kiss1* neurons that are co-labeled express only low levels of *TH* mRNA[55]. Similar findings were obtained with kisspeptin and TH protein co-labeling: only a small number of kisspeptin-immunoreactive cells co-express TH-immunoreactivity in the AVPV/PeN of female rats [55]. Thus, in rats, despite some overlap in the anatomical distribution of these two neuronal populations, the sexually-dimorphic *Kiss1* and TH populations in the AVPV/PeN appear to comprise two separate, sexually-differentiated populations with only a minor degree of overlap. Whether this is the case for other species remains to be determined. Unlike kisspeptin, the role of sexually dimorphic AVPV TH neurons is currently ill-defined.

Mechanisms Underlying Sex Differences in AVPV/PeN *Kiss1* Neurons

Mechanisms Governing Sexual Differentiation of the Brain

There are several ways that sex differences in the brain can develop, including gonadal sex steroid-dependent mechanisms and sex chromosome gene-dependent mechanisms. To date, most sex differences in the brain and behavior appear to reflect sex differences in the actions of gonadal sex steroid secretion during key stages of development (the “organizational hypothesis”), whereas only a few sexually-dimorphic traits, such as some aggressive and parental behaviors, nociception, and ubiquitin protease mRNA expression in the neocortex have yet to be attributed to differences in sex chromosome gene expression between males and females [68–74]. The fundamental principle of the “organizational hypothesis”, first proposed more than 50 years ago in a landmark study by Phoenix et al. [75], is that the brain is initially bipotential and develops to be male-like or female-like under the direction of gonadal sex steroids during the perinatal “critical period” of development, the duration and timing of which is species-specific (e.g., in rats, it encompasses the first 10 days of postnatal life). During the critical period, the acute secretion of gonadal T in neonatal males, but not neonatal females, induces sexually dimorphic brain regions to differentiate to be masculinized (and defeminized). This effect of perinatal T on guiding the brain’s sexual

differentiation occurs via activation of either AR, or more commonly, ER pathways (after aromatization of T to E_2 in neural target tissues) [17,76,77]. In contrast to males, females do not normally secrete significant levels of circulating gonadal sex steroids during the perinatal critical period; the absence of high circulating sex steroids in perinatal females results in their brains differentiating to be feminized (and demasculinized) [13,22,78,79]. Supporting this model, experimental manipulation of the postnatal steroid milieu alters the development of sexually dimorphic traits. Specifically, acute sex steroid treatment (T or E_2) to newborn female rodents causes the development of a male-like brain, whereas castration of newborn male rodents (thereby removing postnatal T secretion) induces the development of female-like brains. For example, postnatal T or E_2 treatment to newborn female rodents masculinizes the development of the AVPV TH population such that these females have a male-like TH phenotype in adulthood; in contrast, neonatal castration of newborn males results in a female pattern of TH expression in adulthood [25,27,67].

Like the brain, sexually-dimorphic reproductive physiology and behavior have also been demonstrated to be sexually differentiated by perinatal sex steroid signaling. For example, the ability of adult female rodents, but not adult males, to display an E_2-induced preovulatory LH surge (i.e., “positive feedback”) is sexually differentiated [29,80,81] by differential exposure to gonadal sex steroids during early postnatal life. That is, in developing females, the absence of significant circulating postnatal gonadal steroids allows the neural mechanisms necessary for generating the LH surge to develop, whereas this is prevented in males by postnatal exposure to sex steroids. In support of this model, female rats or mice treated with a single injection of T or E_2 during the postnatal critical period (to mimic male’s postnatal T secretion) fail to develop the LH surge-generating circuitry whereas newborn male rats that are castrated at the time of birth (to prevent gonadal T secretion) can generate an E_2-induced LH surge in adulthood, similar to normal adult females [29,63,80,81].

Hormonal Induction of Sex Differences in AVPV/PeN Kiss1 Neurons

What mechanisms underlie the development of sex difference in *Kiss1* neurons in the AVPV/PeN? Like TH expression in the AVPV, as well as most other sex differences in the brain, the sex difference in *Kiss1* neurons is organized early in postnatal development by the actions of gonadal sex steroids. That is, newborn female rats treated with a single injection of T (to mimic the acute T secretion in newborn males) possess very few *Kiss1* neurons in the AVPV/PeN as adults, similar to normal adult males [55]. Furthermore, newborn male rats castrated on the day of birth (to remove circulating gonadal T) have high *Kiss1* levels in the AVPV/PeN in adulthood [63], indicating that the AVPV/PeN *Kiss1* system is sexually-differentiated under the influence of postnatal gonadal sex steroids. The developmental effects of postnatal sex steroids on the sexual differentiation of *Kiss1* neurons in the AVPV/PeN are likely mediated specifically by ER rather than AR pathways, based on the following evidence. Kisspeptin immunoreactivity in the AVPV/PeN of adult females is reduced in transgenic mice that were perinatally exposed to E_2 due to knockout of the alpha-fetoprotein, which normally prevents E_2 from acting in the female brain during early development [65]. Importantly, these alpha-fetoprotein KO females were incapable of mounting an LH surge in response to sex steroid treatment in adulthood, linking the reduced kisspeptin levels to impaired positive feedback [65]. Additionally, female rats treated with a single E_2 injection during the postnatal critical period display male-like levels (i.e., low levels) of *Kiss1* mRNA and kisspeptin-immunorecativity in the AVPV/PeN in adulthood (and cannot display an LH surge) [63]. Lastly, female newborn rats exposed to E_2 or estrogen agonists display masculinized levels of kisspeptin-immunoreactivity in the AVPV/PeN in adulthood [82,83]. Thus, masculinization of the AVPV *Kiss1* system is likely

mediated via aromatization of T to E_2 during the postnatal critical period (as is also the case for the TH system).

In addition to the important role of postnatal E_2 signaling in organizing the developmental trajectory of *Kiss1* neurons in the AVPV/PeN, recent evidence suggests that E_2 may also act sometime between the postnatal period and adulthood to further regulate proper *Kiss1* development in the AVPV/PeN. This conjecture is based on recent findings that female mice lacking E_2 either permanently (i.e., aromatase KO mice or hpg mice) or during just the peripubertal period (gonadectomized from PND 22–30) express very low levels of kisspeptin-immunoreactivity in the AVPV/PeN as adults [84], even when given supplemental E_2 in adulthood [64,85]. These findings suggest that E_2 may be necessary after the postnatal critical period sometime during peripubertal life for promoting normal female-like kisspeptin levels in the AVPV/PeN. This conjecture is supported indirectly by recent observations that E_2 can act during puberty to regulate the development of certain neuronal populations[86–90]. Whether or not E_2 during puberty influences AVPV/PeN *Kiss1* development in other species besides mice requires more investigation.

While the development of the sexually dimorphic *Kiss1* system is dependent on the postnatal sex steroid milieu, it is unclear exactly how postnatal sex steroids, primarily E_2, direct the sexual differentiation of this system. That is, how does E_2 organize the sexual differentiation of the *Kiss1* population within the AVPV/PeN? Several hormone-dependent mechanisms, such as differential cell migration, neurogenesis, programmed cell death (apoptosis), and epigenetic modifications have been implicated in the sexual differentiation and development of different neural populations [18,19,91]. For example, in the developing rat hippocampus, gonadal sex steroids, which are normally higher in postnatal males than females, increase the number of new cells (i.e., neurogenesis), leading to more neurons present in this region in males than females [20]. Conversely, many sexually dimorphic populations in the brain arise via apoptotic mechanisms. In fact, sex differences in the overall size and total cell number of both the AVPV and BNST are induced by apoptosis during early development [18,21,22,24,92]. Most of these apoptotic-induced sex differences are dependent on the pro-apoptotic gene, *Bax*, which encodes Bax (Bcl-2–associated X protein), an inducer of an intracellular signaling cascade that culminates in cell death. In the developing rat AVPV, postnatal males have higher *Bax* expression than females, which correlates with the presence of fewer AVPV cells present in adult males than females [22]. Moreover, adult sex difference in the total number of AVPV neurons is eliminated in *Bax* KO mice [24], indicating that this sexually dimorphic trait is sexually differentiated via *Bax*-dependent apoptotic mechanisms. Despite these findings, the sexual differentiation of TH neurons in the AVPV does not occur via *Bax*-dependent mechanisms, because *Bax* KO mice do not have altered sexual differentiation of AVPV TH neurons [24]. Thus, different sexually dimorphic traits appear to be organized in development via a variety of mechanisms. The possibility that the sex difference in the AVPV *Kiss1* system is caused by E_2-induced changes in either neurogenesis, apoptosis, or other key mechanisms has not yet been tested.

Sex differences in ARC *Kiss1* Neurons are Species-Specific and Age-Dependent

Sex Differences Are Absent in ARC Kiss1 Neurons of Adult Rodents

Whereas sex differences in the AVPV/PeN are well-documented, the ARC has received much less attention in terms of sexual differentiation. Unlike the AVPV/PeN, BNST, and mPOA, the rodent ARC does not exhibit noticeable sex differences in overall nucleus size or total number of neurons. However, several more specific parameters of ARC cells are sexually dimorphic. Both the morphology of astroglia and the number of synapses in the

ARC differs between male and female rodents, as does growth hormone-releasing hormone gene expression and axonal projections of neurokinin B/dynorphin neurons [93–96]. Despite this, the ARC of adult rodents displays no sex differences in either the total number of *Kiss1* neurons or the amount of *Kiss1* mRNA per cell [55,61,63]. Sex steroids in adulthood are known to dramatically inhibit *Kiss1* gene expression in the ARC. However, the lack of sex difference in ARC *Kiss1* expression is not influenced by circulating sex steroids: adult male and female rats and mice display similar high levels of *Kiss1* expression or kisspeptin immunoreactivity in the ARC following gonadectomy and similar reduced *Kiss1* expression after sex steroid replacement (Figure 2) [55,61,63]. Given that *Kiss1* cells in the ARC have been proposed to mediate negative feedback regulation of GnRH secretion (a non-sexually dimorphic process) in both adult males and females (reviewed in [2,36,50]), it is not unexpected that sex differences are absent in ARC *Kiss1* neurons, at least in adult rodents.

Recent examination of *Kiss1* expression in the ARC of adult male and female sheep has yielded a different picture than in rodents. In particular, intact adult female sheep possess greater numbers of *Kiss1* neurons in the ARC than do intact males, identifying an important sex difference in the ovine *Kiss1* system [97]. Direct comparison of ARC *Kiss1* levels in adult ewes and rams treated with equivalent sex steroid levels has yet to be reported. Interestingly, prenatal androgen treatment, which is known to masculinize a number of sexually dimorphic traits in female sheep (such as dynorphin and neurokinin B expression in the ARC), does not reverse the sex difference in ARC kisspeptin cells [97], suggesting that the *Kiss1* system of the ovine ARC may not be sexually differentiated by prenatal sex steroids, at least at the specific prenatal times that were tested. The basis for the species difference in sex differences in the ARC *Kiss1* system is not entirely known, but may relate to species differences in the neural regions mediating sex steroid positive feedback. In rodents, positive feedback of E_2 is mediated by the AVPV/PeN, correlating with a sex difference in *Kiss1* in this region, whereas in sheep, the ARC has been implicated in mediating the preovulatory LH surge [36,98], correlating with a *Kiss1* sex difference in this region. Whether or not the ARC *Kiss1* system of other species, such as primates, displays adult sex differences is not currently known.

Age-dependent Sex differences in ARC Kiss1 Neurons of Mice

Interestingly, although *Kiss1* neurons in the ARC are not sexually dimorphic in adult rodents, this may not be the case in younger animals. Kauffman et al. [61] recently identified a sex difference in ARC *Kiss1* neurons in prepubertal mice that is evident when gonadal hormone feedback is removed (Figure 3). Specifically, intact prepubertal male and female mice (postnatal day [PND] 16–18) express similar low levels of *Kiss1* mRNA in the ARC. However, whereas gonadectomized PND 16–18 females display a significant increase in ARC *Kiss1* levels (reflecting loss of gonadal hormone negative feedback on *Kiss1* expression), gonadectomized males of the same age do not have elevated *Kiss1* levels in the ARC (Figure 3) [61]. The increased *Kiss1* expression in gonadectomized PND 16–18 females suggests that there is little or no gonadal hormone-independent suppression acting on ARC *Kiss1* neurons in prepubertal females at this age. Thus, the prepubertal female reproductive axis at this age appears to be kept quiescent predominantly by gonadal hormone negative feedback. In contrast, in prepubertal males, ARC *Kiss1* expression did not increase by 2 or 4 days following gonadectomy, and ARC *Kiss1* expression in gonadectomized PND 16–18 male mice was similar to that of intact males of the same age [61]. This lack of increased *Kiss1* expression after gonadectomy in prepubertal males indicates that some gonadal hormone-independent mechanism(s) suppresses ARC *Kiss1* neurons in prepubertal male mice but not females, at least at the ages tested. Adult mice of both sexes exhibit robust increases in ARC *Kiss1* expression 4 days following gonadectomy [61], indicating that the sex difference in the gonadal hormone-independent suppression of

ARC *Kiss1* neurons is present only during prepubertal development. Importantly, prepubertal LH levels followed the same sexually-dimorphic pattern as ARC *Kiss1* expression, with elevated LH in gonadectomized prepubertal females but not in gonadectomized prepubertal males [61]. Thus, the regulation of reproductive status during prepubertal development is sexually dimorphic, with PND 16–18 males, but not females, exhibiting non-gonadal suppression of ARC *Kiss1* and LH levels. This *Kiss1* sex difference may relate to known sex differences in pubertal maturation in rodents, in which males mature later than females. Specifically, later puberty onset in males may reflect sex differences in the gating of peripubertal ARC *Kiss1* neuron activation, such that there is greater (or longer-lasting) suppression of *Kiss1* circuitry in prepubertal males than prepubertal females.

Whereas gonadectomized prepubertal male mice do not demonstrate elevated LH secretion or increased ARC *Kiss1* levels after 2 or 4 days (on PND 16–18), they do exhibit increased ARC *Kiss1* and LH levels later in adulthood (PND 45) [61]. Importantly, these increases in ARC *Kiss1* expression evident in adulthood occurred in the absence of any developmental changes in gonadal hormones (since the males were castrated on PND 14). This indicates that sometime between PND 18 and PND 45 there is a key developmental change in non-gonadal regulation of male reproductive circuits, including ARC *Kiss1* neurons. However, the identity of the gonadal hormone-independent factor(s) is currently unknown, as is the specific age in development when this non-gonadal suppression dissipates. Like male mice, in primates, there is gonadal hormone-independent regulation of the developing reproductive axis, and puberty onset in primates appears to include removal of inhibitory input onto reproductive circuits, independent of changes in gonadal hormones [99,100]. However, the precise non-gonadal inhibitory factor(s) involved remains unclear.

Conclusions

Recent findings from several rodent species have provided exciting information about the role of kisspeptin neurons in the regulation of the reproductive axis, including sexually dimorphic processes such as the preovulatory LH surge. In addition, recent evidence has emerged demonstrating sex differences in various *Kiss1* populations in both rodents and sheep, perhaps identifying the specific neuroanatomical basis underlying various sexually dimorphic reproductive physiological processes. We now appreciate that *Kiss1* neurons in the rodent AVPV/PeN are sexually differentiated under the direction of sex steroid signaling, primarily E_2, during early postnatal development (and again later during pubertal development), and that these sexually-dimorphic AVPV/PeN *Kiss1* neurons comprise a key element of the neural mechanism underlying the sexually-dimorphic LH surge that occurs in adult females but not adult males. Moreover, while not sexually dimorphic in adulthood, the *Kiss1* population in the rodent ARC displays a sex difference in its regulation during prepubertal development, such that *Kiss1* neurons of prepubertal male mice experience greater non-gonadal suppression than do prepubertal female mice. This intriguing sex difference in the prepubertal ARC *Kiss1* system, or more precisely, the “upstream” systems that regulate prepubertal ARC *Kiss1* neurons, may contribute to differences in puberty onset between males and females, although this remains to be tested.

Acknowledgments

Dr. Kauffman’s research is supported by NICHD grants R00 HD056157 and U54 HD012303.

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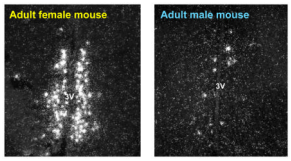

Figure 1.

Sexually dimorphic *Kiss1* gene expression in the AVPV/PeN of adult male and female mice. Females have significantly more *Kiss1* neurons and *Kiss1* mRNA/neuron in the AVPV/PeN than do males, regardless of the adulthood sex steroid milieu. (3V = 3rd ventricle) [For pictures of similar AVPV/PeN sex differences at the protein level, refer to articles by Clarkson and Herbison, 2006 and Adachi et al, 2007.]

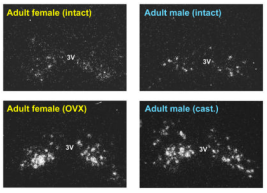

Figure 2.
Kiss1 expression in the ARC is not sexually dimorphic in adult rodents. Adult male and female mice exhibit similar numbers of *Kiss1* neurons in the ARC in both intact and gonadectomized conditions. (3V = 3rd ventricle; OVX = ovariectomized; cast= castrated)

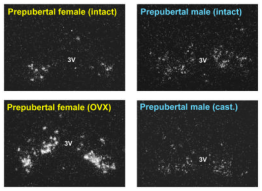

Figure 3.

Intact prepubertal male and female mice exhibit similar numbers of *Kiss1* neurons in the ARC. However, gonadectomized prepubertal females have higher ARC *Kiss1* expression than similarly-aged gonadectomized males. The absence of elevated *Kiss1* expression in gonadectomized prepubertal males suggests that some non-gonadal factor(s) acts to suppress the ARC *Kiss1* system in males at this developmental stage. (3V = 3rd ventricle; OVX = ovariectomized; cast= castrated)