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Organizational and activational effects of sex steroids on kisspeptin neuron development

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Abstract

Kisspeptin, encoded by the *Kiss1* gene, is a neuropeptide required for puberty and adult reproductive function. Understanding the regulation and development of the kisspeptin system provides valuable knowledge about the physiology of puberty and adult fertility, and may provide insights into human pubertal or reproductive disorders. Recent studies, particularly in rodent models, have assessed how kisspeptin neurons develop and how hormonal and non-hormonal factors regulate this developmental process. Exposure to sex steroids (testosterone and estradiol) during critical periods of development can induce organizational (permanent) effects on kisspeptin neuron development, with respect to both sexually dimorphic and non-sexually dimorphic aspects of kisspeptin biology. In addition, sex steroids can also impart activational (temporary) effects on kisspeptin neurons and *Kiss1* gene expression at various times during neonatal and peripubertal development, as they do in adulthood. Here, we discuss the current knowledge—and in some cases, lack thereof—of the influence of hormones and other factors on kisspeptin neuronal development.

Keywords

kisspeptin; Kiss1; Kiss1r; GPR54; GnRH; reproduction; development; sexual differentiation; puberty; estradiol; hormones

1. Introduction

Reproductive success is dependent on many neuropeptide and hormonal systems working in concert to regulate gonadal function and sexual behavior. Within the brain, an interconnected web of neurons regulates the pulsatile release of gonadotropin-releasing hormone (GnRH), which itself regulates reproductive status by governing the secretion of pituitary gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH). However, many of the key mechanisms and factors involved in the regulation of GnRH release, including the activation of increased GnRH pulsatility and secretion during puberty,

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remain poorly-defined. Within the past decade, kisspeptin, the product of the *Kiss1* gene, has been shown to have potent stimulatory actions on GnRH release in mammals, including humans, and both kisspeptin and its receptor, Kiss1r (formerly known as Gpr54), are necessary for normal reproductive function and sexual maturation.

One aspect of kisspeptin biology that is of great interest is the bi-directional relationship between the development of the *Kiss1* system and the role of kisspeptin in governing reproductive development. Many aspects of reproductive development and physiology, including those pertaining to kisspeptin, are affected by gonadal sex steroids, testosterone (T) and estradiol (E2), which can feedback to the brain to influence the development, maturation, and functioning of reproductive and pubertal circuits. “Organizational” effects of sex steroids are those that are permanent and irreversible, and which essentially regulate the developmental trajectory of neural circuits and other physiological systems. These permanent organizing effects of T or E2 are typically only observed at discrete and specific developmental periods, so-called “critical periods,” before or after which sex steroids no longer exert long-term developmental effects. In contrast, “activational” effects of sex steroids are acute and transient, dissipating after the sex steroid signal is gone, and can occur at any age in development or adulthood. Activational effects of sex steroids can influence gene expression and neuronal activation, and are inherent to the mechanisms underlying positive and negative feedback by sex steroids on the reproductive axis.

In this article, we will briefly review the function and regulation of kisspeptin in reproductive biology to provide a framework for discussing recent data regarding the organizational and activational effects of sex steroids on the neuronal kisspeptin system throughout postnatal and pubertal development. Our discussion will be limited to data collected in mammals, primarily rodents, as these animal models have been most studied in terms of kisspeptin development. Although our understanding of the development and regulation of the kisspeptin system is still incomplete, as we shall see, considerable advances have been made in recent years to appreciate how this important neuropeptide system develops.

2. Kisspeptin and the HPG axis

The discovery of kisspeptin can be credited to cancer biologists, who first identified it as a peptide that inhibited metastasis in several cancer cell lines [82; 83; 84]. Kisspeptin is produced from a 145 amino acid pro-peptide encoded by the *Kiss1* gene (*KISS1* in humans). The pro-peptide is proteolytically cleaved to produce a 52 to 54 amino acid peptide (depending on the species) with an arginine-phenylalanine-amide (RFamide) motif at the C-terminus. Kisspeptin may be further processed to produce shorter peptides, such as kisspeptin-14, kisspeptin-13, kisspeptin-10, all of which have biological activity, but whose biological relevance remains unclear [77]. The RFamide C-terminal motif is present in a variety of neuropeptides, such as prolactin-releasing peptide, RFamide-related peptides (RFRP) 1 and 3, and neuropeptides FF and AF, suggesting that *Kiss1* and the genes that encode these other peptides may have evolved from a common ancestral gene [150]. Given the similar structural motif of these peptides, it has been difficult to generate monoclonal antibodies that are specific for kisspeptin and do not interact with other RFamide peptides, such as RFRP-1 [11; 59], although some antibodies currently available appear to have better specificity in rodents, sheep, and monkeys [19; 37; 46; 51; 102; 116].

The receptor for kisspeptin, originally called G-protein coupled receptor 54 (Gpr54), was characterized in mice and humans several years after kisspeptin [77; 81; 94] and has recently been renamed Kiss1r (KISS1R in humans)[49; 76]. This naming is appropriate, as peptides related to kisspeptin have very low affinity for Kiss1r, demonstrating an extremely specific

receptor-ligand relationship between Kiss1r and kisspeptin [104; 105]. However, kisspeptin may have some agonist activity for other G-protein coupled receptors, which should be considered when interpreting the effects of kisspeptin [87; 104]. To date, there are no well-validated antibodies available for detecting Kiss1r protein in rodents and most other mammalian species, making protein distribution and quantification impossible, and leaving *in-situ* hybridization, reverse transcriptase PCR (RT-PCR), or quantitative PCR (qPCR) detection of the transcript as the primary methods of analysis.

The essential role of kisspeptin and Kiss1r in reproductive endocrinology was first reported in 2003 from the characterization of two consanguineous families suffering from then idiopathic hypogonadotropic hypogonadism [29; 126]. These families each carried recessive, inactivating mutations for KISS1R. Subsequently, various *Kiss1r* and *Kiss1* knockout (KO) mice were generated [18; 26; 38; 80; 92; 126]. All of these mice lines phenocopied the absent puberty and hypogonadotropic hypogonadism observed in patients with impaired KISS1R: extremely low serum gonadotropins, nearly undetectable sex steroids, underdeveloped gonads, a failure of sexual development, and infertility. Moreover, female transgenic mice in which all of the *Kiss1* neurons have been ablated in adulthood via Cre-lox technology have impaired estrous cyclicity and are infertile, further supporting the importance of kisspeptin signaling in reproduction [90]. It should be noted, however, that ablation of kisspeptin neurons induced early in development did not impair estrous cyclicity and fertility, suggesting that developmental compensation may occur in mice experiencing *Kiss1* neuron ablation before puberty. Yet, in this case, a small subset of *Kiss1* neurons reportedly survived [90] and it is unknown if these few kisspeptin neurons were able to successfully drive fertility.

Further experimentation in rodents, and subsequently, other species including humans, demonstrated that exogenous kisspeptin is able to induce a robust secretion of LH and FSH [67; 75; 92; 97; 98] and that this kisspeptin-induced stimulation of gonadotropins involves a GnRH-dependent mechanism [48; 60]. Additional evidence from rodents demonstrated that kisspeptin activates GnRH neurons, as determined by detection of c-fos induction in GnRH cells (a marker of neuronal activation) [60; 67], dramatic stimulation of electrical firing of GnRH neurons in brain explants [54; 111], and increased GnRH release *in situ* [27; 92]. Complementary work in sheep showed that kisspeptin increases GnRH secretion into the portal vasculature [140]. These effects of kisspeptin on GnRH neurons are thought to be direct, as kisspeptin neuronal fibers have been shown to appose GnRH neuron somas and/or axons [17; 85; 157] and the majority of GnRH neurons highly express *Kiss1r* [54; 60; 92]. The high expression of *Kiss1r* in the majority of GnRH neurons is noteworthy as other G-protein coupled receptors for GnRH regulating RFamide peptides, such as RFRP-3's receptor, *Gpr147*, are only expressed in a small subset of GnRH neurons in mice [114]. Thus, the available evidence indicates that kisspeptin exerts its reproductive effects directly and specifically on GnRH neurons. Despite this, some data suggests that kisspeptin may also act at the level of the pituitary, though this issue still remains controversial [53; 63; 140; 143].

3. Distribution and Regulation of *Kiss1* mRNA in the brain

3.1 Brain expression of kisspeptin in rodents

Within the rodent central nervous system, kisspeptin and *Kiss1* mRNA soma are found primarily in two regions: the hypothalamic continuum of the anteroventral periventricular nucleus and neighboring rostral periventricular nucleus (AVPV/PeN, sometimes referred to rostral portion of the third ventricle [RP3V]), and the arcuate nucleus (ARC) [17; 48] (Figure 1). In addition, a smaller *Kiss1*-expressing population has also been identified in the medial amygdala (MeA) in adult rodents of both sexes [74]. Moreover, sparse kisspeptin

immunoreactivity or weak *Kiss1* hybridization has sometimes been detected in the bed nucleus of the stria terminalis (BNST), dorsal-medial hypothalamus, ventromedial hypothalamus, and brain stem, though whether this staining represents true kisspeptin populations is contentious and remains to be validated [11; 17; 20; 156]. While kisspeptin is generally thought of as a neuropeptide, it is also found in a variety of non-neural tissues, including the placenta, pancreas, gonads, pituitary, and adipose tissue [14; 15; 77; 103; 146; 148], but very little is known about the function or regulation of these non-neural populations.

In rodents, the AVPV/PeN and ARC kisspeptin populations differ noticeably in their efferent projections and co-expression of other neuropeptides and neurotransmitters. It appears that kisspeptin fibers from the AVPV/PeN project to regions where GnRH cell bodies are located [64; 157] and kisspeptin immunoreactive fibers are in apposition with GnRH cell bodies [17; 22]. In contrast, fewer ARC kisspeptin neurons project to brain regions with GnRH cell bodies [157], though some ARC kisspeptin cells do appear to target GnRH soma [64]. In addition, ARC kisspeptin cells may project to and make appositions with GnRH axons or terminals in the median eminence [27; 151; 156], though whether or not this occurs in the external or internal zone of the median eminence is controversial and may be species specific. Thus, the two kisspeptin populations likely act to stimulate GnRH neurons at separate cellular regions; the AVPV/PeN kisspeptin neurons are proposed to stimulate mainly at GnRH cell bodies, while ARC kisspeptin neurons stimulate mainly at GnRH axons and terminals, although this requires more supportive evidence. Secondly, the phenotype of the two kisspeptin populations differs with respect to which other genes are co-expressed. Specifically, the majority (>80%) of AVPV/PeN kisspeptin neurons are dopaminergic (express tyrosine hydroxylase) in mice (but less so in rats) [66; 124]. In addition, a subset of kisspeptin neurons in the rodent AVPV/PeN express either galanin (~87%) or met-enkephalin (~33%) [64; 115]. In contrast, in the ARC, kisspeptin neurons do not readily co-express tyrosine hydroxylase, met-enkephalin, or galanin, but do highly co-express both neurokinin B (NKB, encoded by the *Tac2* gene) and dynorphin, as well as the NKB receptor, *Tacr3* [99]. Although the exact roles of many of these kisspeptin co-transmitters is still not entirely clear, it is likely that the phenotypic differences between the AVPV/PeN and ARC kisspeptin populations underlie important differences in their biological functions. For example, the finding of high kisspeptin-NKB-dynorphin co-expression in the ARC has led to a proposed model in which NKB and dynorphin work in a cyclic and autocrine/paracrine fashion to regulate pulsatile neuronal firing within the ARC which may govern GnRH pulsatility [99].

3.2 Sex steroid regulation of *Kiss1* in the adult rodent AVPV/PeN

The AVPV/PeN is known to be an essential hypothalamic region for mediating E2 positive feedback for the preovulatory LH surge in rodents, and kisspeptin neurons in this region appear to be a major factor in this process [21; 57]. In adults, *Kiss1* mRNA in the AVPV/PeN is robustly increased by E2 in female rodents, as well as in males [66; 67; 134; 135], and almost all *Kiss1* neurons in the AVPV/PeN express estrogen receptor- α (ER α), providing evidence of direct route by which E2 could act to stimulate these neurons [1; 134; 135]. Although AVPV/PeN *Kiss1* cells also moderately co-express estrogen receptor- β (ER β), at least in females [134], ER α is believed to be the primary estrogen receptor for upregulating *Kiss1* expression because E2 treatment does not stimulate AVPV/PeN *Kiss1* expression in ER α KO mice but still does so in ER β KO mice [134]. The stimulatory action of E2 on AVPV/PeN *Kiss1* neurons is thought to occur through “classical” ER α signaling, as *Kiss1* expression in the AVPV/PeN cannot be increased by E2 in mice carrying a mutation to the DNA binding domain of ER α [50]. Parenthetically, T can also increase *Kiss1* expression in the AVPV/PeN of mice, but non-aromatizable androgens, such as

dihydrotestosterone (DHT) have no effect, suggesting that the stimulatory effects of T are due to aromatization to E2 [135].

Besides the fact that E2 stimulates *Kiss1* neurons in the AVPV/PeN, there is additional evidence linking this kisspeptin population to the mechanism underlying the E2-invoked LH surge. First, the expression of *Kiss1* increases at the time of the LH surge [120; 137], and there is also increased c-fos co-expression (a marker of neuronal activation) in AVPV/PeN kisspeptin neurons during the LH surge [18; 120; 136; 155]. Second, like the LH surge, the *Kiss1* gene and activation of AVPV/PeN *Kiss1* neurons are circadian regulated in synchrony with the LH surge, and kisspeptin neurons in the AVPV/PeN receive direct neural inputs from the suprachiasmatic nucleus, the master circadian clock [73; 120; 155]. Third, in adult rodents, the preovulatory LH surge is sexually dimorphic, with only females being able to produce an LH surge in response to sex steroid treatments. Fittingly, *Kiss1* mRNA and kisspeptin protein are sexually differentiated in the AVPV/PeN (greater in females than males), matching the sexual dimorphism in the LH surge [17; 58; 66; 156]. Lastly, functional studies have shown that the LH surge is dependent on kisspeptin signaling, as it cannot occur in *Kiss1r* KO or *Kiss1* KO mice or after pharmacological blockade of Kiss1r [18; 122].

3.3 Sex steroid regulation of *Kiss1* in the adult rodent ARC

In contrast to the AVPV/PeN population, kisspeptin neurons in the ARC are negatively regulated by E2 in both male and female rodents [134; 135]. As in the AVPV/PeN, T can also regulate *Kiss1* expression in the ARC (in an inhibitory fashion, like E2) [74; 135]. However, unlike in the AVPV/PeN, some of the effects in the ARC may be mediated by androgen receptors (AR), since DHT is also able to repress *Kiss1* levels in the ARC [74; 135]. Supporting these data, ER α is co-expressed in most ARC *Kiss1* neurons in mice of both sexes, and AR is found in over 60% of *Kiss1* cells in the ARC of male mice [134; 135]. AR and *Kiss1* co-expression have not yet been investigated in female rodents. In mice, E2's negative regulation of *Kiss1* in the ARC can occur through "non-classical" signaling pathways (i.e. not by ER α binding estrogen response elements in the *Kiss1* promoter), since E2 can still repress *Kiss1* expression in the ARC of mice lacking a functional ER α DNA binding domain [50].

Based on the ability of E2, T, and DHT to inhibit *Kiss1* expression in the ARC, the ARC kisspeptin population is believed to mediate sex steroid-induced negative feedback of the reproductive axis and to comprise part of the proposed pulse generator that regulates GnRH pulsatility (as opposed to the AVPV/PeN, which is responsible for GnRH surge generation but not GnRH pulsatility) [88]. This pulsatile role of the ARC is supported by data demonstrating that discrete pharmacological blockage of kisspeptin signaling in the ARC, but not the AVPV/PeN, decreases LH pulse frequency data in rats [86]. Additional data comes from electrophysiological recordings of ARC activity in rodents and larger animals, demonstrating that LH pulses correlate with electrical pulses in neurons in this region (reviewed in [88]), and further supported by the proposed autocrine/paracrine actions of NKB and dynorphin on ARC kisspeptin neurons [46; 99; 152].

In rodents, quantification of kisspeptin protein immunoreactivity in the ARC is difficult, although not impossible, due to a very dense plexus of kisspeptin immunoreactive fibers found in this region. This challenge is especially true in mice. While measuring *Kiss1* mRNA by *in-situ* hybridization allows for quantification of discrete *Kiss1*-expressing cell bodies, similar quantification of kisspeptin immunoreactive cell bodies cannot easily be made in the rodent ARC due to these numerous dense overlapping kisspeptin fibers. Nevertheless, ARC kisspeptin immunoreactive fibers and cell bodies have been quantified in the rat during development [7; 31; 32; 144]. The source of these kisspeptin fibers is still not

clear, as they could originate from kisspeptin neurons in the ARC, AVPV/PeN, or even the MeA. These immunoreactive kisspeptin fibers in the ARC appear to be regulated by sex steroids (not surprising since kisspeptin synthesis is sex steroid-regulated), with reduced levels in adult gonadectomized mice, as well as in mouse models with impaired sex steroid production [19; 41] or mutated sex steroid receptors [89]. In these cases, ARC kisspeptin cell bodies are more easily detected than in the gonadally-intact or hormone-treated condition where the fibers obstruct the soma. Because the source of the fibers is unknown, and also because the reduced fiber staining could represent either increased kisspeptin secretion/release or increased storage/decreased transport, it is difficult to interpret what the changes in fiber levels reflect in terms of kisspeptin function.

3.4 Sex steroid regulation of kisspeptin expression in the adult rodent amygdale

As mentioned above, in rodents, kisspeptin and *Kiss1* mRNA can be found at lower levels in regions outside the hypothalamus. For example, in addition to the AVPV/PeN and ARC, *Kiss1* is also expressed in the rodent medial amygdala (MeA), but only with elevated levels of circulating sex steroids [74]. *Kiss1* mRNA in MeA is higher in males than females in gonadally-intact mice and rats, but gonadectomy removes this sex difference and E2 or T replacement can induce MeA *Kiss1* expression to a similar degree in both sexes [74]. As in the AVPV/PeN, DHT does not have an effect on *Kiss1* expression in the MeA, suggesting that *Kiss1* in this region is controlled exclusively through estrogen receptor pathways. The role of kisspeptin in the MeA is unknown, but we have speculated that kisspeptin in this region may be involved in pheromone processing, which is channeled through the MeA and integrated into sexual behavior and reproduction [6]. Exposure to opposite sex pheromones can induce c-fos co-expression in AVPV/PeN kisspeptin neurons, demonstrating that these kisspeptin cells are responsive to social odor cues [3], but whether this also occurs in the amygdala kisspeptin population remains to be determined.

3.5 Distribution and regulation of Kiss1 mRNA in non-rodent species

Outside of rodents, neuronal kisspeptin in mammals has been studied primarily in two other animal models: sheep and rhesus macaques. The ability of kisspeptin to stimulate the reproductive axis is conserved in all three models, and many aspects of kisspeptin neurons (such as co-expression of ER α) are conserved across species. However, there are several differences in the distribution, and potential function, of kisspeptin neurons between each of these animal models. In sheep, the distribution of *Kiss1* mRNA and kisspeptin protein is rather similar to that of rodents [141], with *Kiss1* mRNA (and kisspeptin immunoreactivity) present in the rostral preoptic area (POA) as well as the ARC [35; 37; 138]. Kisspeptin neuronal fibers in sheep make close appositions to GnRH neurons [139]. Additionally, the number of these kisspeptin appositions increases after the onset of the breeding season [139], similar to data obtained from mice regarding changes in kisspeptin appositions on GnRH neurons at puberty [17]. A difference between sheep and rodents is that kisspeptin fibers have been identified in the external zone of the median eminence of sheep [37] but not in mice [157]. E2 has a similar inhibitory effect on *Kiss1* expression in the ARC of sheep as it does rodents, and gonadectomy increases *Kiss1* expression the ARC of both animal models. Also like rodents, kisspeptin neurons in the ARC of sheep co-express NKB and dynorphin [46], as well as ER α [37]. In rodents, positive and negative feedback effects of sex steroids are split between the AVPV/PeN and ARC, respectively, but in sheep these two physiological processes appear to both occur in the ARC region [8; 13]. Although ARC *Kiss1* expression in sheep is primarily inhibited by E2 [138; 139], one study suggests that *Kiss1* mRNA in the caudal portion of the ovine ARC is upregulated just before the preovulatory LH surge [35], suggesting that there may be subpopulations of kisspeptin neurons in the ovine ARC continuum, with some kisspeptin neurons being dedicated to positive and/or negative feedback. If kisspeptin neurons in the ARC are in fact responsible

for both positive feedback and negative feedback, it is unclear what role POA kisspeptin neurons might have. These POA neurons also express ER α and are upregulated by E2 [37; 138; 139], similar to the AVPV/PeN population in rodents, but their specific function remains unclear.

In rhesus macaques, *Kiss1* mRNA and kisspeptin protein cell bodies are found in the infundibular nucleus of the hypothalamus, which is homologous to the ARC of rodents and sheep [116; 121], and kisspeptin immunoreactive fibers are found in apposition to GnRH fibers in the median eminence [116]. Unlike in rodents or sheep, there are no reports in monkeys of *Kiss1* or kisspeptin cells in the anterior hypothalamus/POA. However, the vast majority of kisspeptin monkey studies have been performed in males, whose AVPV/PeN population in rodents is very small relative to that of females. Whether *Kiss1* or kisspeptin neurons in the POA region of monkeys are more readily detectable in females remains to be addressed. As in rodents and sheep, sex steroids can regulate the neuronal expression of kisspeptin in monkeys, with E2 and T suppressing *Kiss1* levels in the infundibular nucleus [34; 121; 128]. An additional similarity between monkeys and other mammals is that the infundibular population of kisspeptin neurons co-expresses NKB and dynorphin [117]. To date, kisspeptin or *Kiss1* expression has yet to be identified in the MeA of sheep or monkeys.

4. Development of the rodent AVPV/PeN kisspeptin system

4.1 Sex differences in kisspeptin and *Kiss1* in the rodent AVPV/PeN

The expression of *Kiss1* and kisspeptin in the AVPV/PeN mirrors the sexually dimorphic nature of this brain region: in female rodents, the AVPV/PeN is considerably larger than that of males due to an increased number of cells and greater cell volume [10; 123; 129; 142]. Following this pattern, females have significantly more *Kiss1* mRNA and kisspeptin immunoreactivity in the AVPV/PeN than males (Figure 1). This sex difference was first characterized in rats by *in-situ* hybridization [66], but has been also observed in mice by both *in-situ* hybridization and immunocytochemistry [3; 17; 45; 58; 149; 156] as well as qPCR [74]. In terms of mRNA, the sex difference typically presents as both more *Kiss1* mRNA-expressing neurons in the region as well as greater *Kiss1* mRNA levels per neuron in females than males. This kisspeptin sex difference goes hand-in-hand with previously-identified sex differences in several other neurotransmitters expressed in this region [106; 123; 129]. Although the precise biological function of the kisspeptin sex difference is not yet definitively determined, it has been proposed that the presence of higher levels of kisspeptin in females versus males relates to the ability of females, but not males, to display E2-induced positive feedback (i.e., generate an LH surge in response to E2) [57; 69; 73].

4.2 Ontogeny of AVPV/PeN *Kiss1* expression in rodents

When does the AVPV/PeN kisspeptin system first develop, and when does the sex difference in this population first become evident? Initial reports using immunocytochemistry demonstrated that kisspeptin protein could be detected in the AVPV/PeN in peripubertal rodents (~3 weeks old), but not before postnatal day 15 (PND 15, day of birth = PND 1) [17]. It therefore remained unclear at what age kisspeptin is first expressed in this region. Subsequent studies looking at mRNA levels over postnatal development (using *in situ* hybridization or qPCR) found that *Kiss1* mRNA is not detectable during the first postnatal week of life, but is detectable sometime in the second week of life. In mice, *Kiss1* expression was observed at very low levels in both sexes at PND 10 [12; 124; 145]. The sex difference in *Kiss1* mRNA emerged slightly later, around PND 12 [124] (Figure 1), although it is unclear if the sex difference has any physiological relevance at this early age. In rats, *Kiss1* expressing neurons was detected in the AVPV/PeN of males and females as early as

PND 8 [145]. Similar results were obtained by other investigators, where they observed *Kiss1* expression in the rat AVPV/PeN on PND 12 by *in-situ* hybridization [12]. The first appearance of *Kiss1* neurons in the second postnatal week of life is inconsistent with the pattern of neurogenesis of the AVPV, which occurs embryonically in rodents [101]. It is therefore likely that the AVPV/PeN neurons that express *Kiss1* or kisspeptin in the prepubertal period and adulthood are actually present in early neonatal and postnatal life, but do not begin to express the *Kiss1* gene until around PND 10 and beyond. The mechanism(s) that regulate this possible developmental change in *Kiss1* gene expression are currently unknown. To date, it has been difficult to examine kisspeptin protein over these early postnatal ages, due to complications in detecting low levels of this protein using traditional immunocytochemistry methods without colchicine pretreatment to prevent vesicle release [59].

4.3 Evidence for an organizational effect of perinatal sex steroids on *Kiss1* development

Long before the functional link between kisspeptin and the LH surge was established, it was shown that female rodents exposed to T or E2 during late prenatal and early neonatal life would be unable to produce a hormonal-primed LH surge in adulthood [4; 42; 43; 132]. Likewise, removal of sex steroids in neonatal males (via castration within a few hours of birth) allowed these males to produce an LH surge in response to E2 treatment in adulthood [24; 42; 56; 132]. These data, in addition to similar results for other sexual dimorphisms (mating behavior, brain morphology, etc.), have supported the “organizational hypothesis” of sexual differentiation. This hypothesis, first proposed in the seminal 1959 paper by Phoenix et al. [110], has proven to be an essential principle of sexual differentiation, though evidence now indicates that a few sex differences are governed by differences in genes on the sex chromosomes [9]. From late fetal life through early postnatal life, male rodents have significantly higher circulating T of gonadal origin than their female littermates [25; 93; 154] and this T travels to and acts in the brain [119]. It is believed that this circulating perinatal T is critical for directing most sex differences in the brain, though in most cases, it is actually E2, produced locally in the brain by aromatization from the circulating T, that is ultimately responsible for organizing neural sex differences. In support of this model, injection of E2 into neonatal female rodents masculinizes the animal equally or more potently than T [24; 130; 131; 132] and E2-mimicking compounds, such as bisphenol-A, can similarly masculinize the brains of perinatal female rodents. In contrast, DHT, which can only bind the AR, typically has no major effect on sexual differentiation of the brain, highlighting the importance of E2 signaling in this developmental process.

Like many other sex differences, the sex difference in AVPV/PeN kisspeptin neurons is regulated (i.e., “organized”) by sex steroids during early postnatal development (Figure 2). Female rats given T on the day of birth or shortly after have low, male-like numbers of *Kiss1* neurons in the AVPV/PeN in adulthood [66], regardless of the levels of circulating sex steroids in adulthood. This matches well with the abolishment of the hormonal-primed LH surge and changes in the size of the AVPV in similarly-treated females [24; 65; 123; 129]. Neonatal injections of E2 are also able to masculinize the *Kiss1* in the AVPV/PeN, suggesting that the effects of neonatal T are mediated through an estrogen receptor mechanism, after aromatization of the T into E2 [58; 108]. Furthermore, ER α specific agonist propylpyrazoletriol, and ER β specific agonist diarylpropionitrile, can permanently masculinize the AVPV/PeN kisspeptin system when given during the neonatal critical window [108; 109]. Additional data supporting this model has come from KO female mice lacking α -fetoprotein, which acts to protect developing fetuses from high levels of E2 produced by the mother during gestation. Adult female α -fetoprotein KO mice have reduced, male-like levels of kisspeptin immunoreactivity in the AVPV/PeN and fail to produce an LH surge with the proper hormonal priming [45]. α -fetoprotein KO females are

also infertile, which may be due to their abnormally low kisspeptin expression in the AVPV/PeN [28; 39; 45].

In conjunction with the ability of exogenous sex steroid treatments during the perinatal period to masculinize the AVPV/PeN kisspeptin system of females, removing T from neonatal males has the opposite effect. Castration of newborn rats on the day of birth (to remove elevated T) results in higher *Kiss1* expression in the AVPV/PeN in adulthood compared to gonadally-intact males. Although this “rescue” did not completely increase *Kiss1* levels to that of females [58], this may be because the males’ brains were briefly exposed to some sex steroid just prior to castration, since the testes secrete elevated T almost immediately at birth. Likewise, males castrated at birth are able to produce LH surges in adulthood, though again, these surges are less robust than in females [24; 42; 56; 132]. Therefore, the presence of T (and E2) in perinatal life actively changes the developmental trajectory of AVPV/PeN *Kiss1* cells from being female-like to being male-like (Figure 2). Thus, high *Kiss1* expression in the AVPV/PeN can be considered a trait that is essentially *lost* in normal male rodents in response to sex steroids during development, along with the ability to generate an LH surge. This is in contrast to the development of a male-biased sex difference, such as aggressive behaviors, which are often *gained* due to neonatal T (or E2) exposure [40].

Organizational sex differences can only be programmed during specific perinatal ages in rodent development. Outside of these narrow developmental ranges, or “critical periods”, sexual differentiation re-programming is unsuccessful. For example, newborn female rats given T on the day of birth will exhibit a male-like volume in the sexually dimorphic nucleus of the POA, while T given later in postnatal life does not masculinize this or other brain regions [61; 118]. The critical periods of sexual differentiation for sexually dimorphic traits are often species- and trait-specific, and the exact critical periods of the AVPV/PeN kisspeptin sex difference are still not fully characterized in most species.

4.4 The developmental mechanisms underlying sexual differentiation of *Kiss1* expression in rodents

How do sex steroids, and particularly E2, act during the perinatal “critical period” to induce sexual differentiation of AVPV/PeN *Kiss1* neurons? In the past several years, our lab has attempted to better understand this important question. We have considered a number of possibilities for explaining the sexual differentiation mechanism, including (but not limited to) differential apoptosis, neurogenesis, and gene silencing, and each of these will be discussed in turn.

Apoptosis (i.e., programmed cell death) may contribute to sex differences when the cells of a brain nucleus undergo a defined process of “cell suicide” in one sex, but not the other. This mechanism appears to be involved in some cases of sexual differentiation, as apoptotic markers can be observed in the brain of one sex but not the other during the period of sexual differentiation [47]. Additionally, targeted deletion of essential neural pro-apoptotic genes, such as *Bax*, abolish the normal development of some sexually dimorphic phenotypes in the brain [36; 78]. Intriguingly, previous data from our colleagues indicated that BAX-mediated apoptosis is required for sexual differentiation of the overall size of the AVPV region in mice [36]. This led us to hypothesize that BAX-mediated apoptosis was also required for sexual differentiation of *Kiss1* expression in the AVPV/PeN. This model would predict that males and females would begin with the same number of *Kiss1* neurons, and then, in response to perinatal sex steroid exposure, developing males would lose a larger portion of these *Kiss1* cells due to E2-induced apoptosis than females, producing a *Kiss1* sex difference. If so, *Bax* KO mice, lacking the ability to induce BAX-mediated apoptosis, would not be expected to have a *Kiss1* sex difference. However, despite the logic of such a

model, we surprisingly found that the sex difference in *Kiss1* persisted in *Bax* KO mice [124]. It was previously shown that TH immunoreactivity, which is also sexually dimorphic in the AVPV, is not regulated by BAX-mediated apoptosis, which we confirmed with *in-situ* hybridization analysis of *TH* mRNA [36; 124]. Therefore, it seems clear that the *Kiss1/TH* population within the AVPV/PeN, despite being sexually dimorphic, does not develop via a BAX-mediated apoptotic mechanism.

Differential neurogenesis between the sexes is, in theory, another possible mechanism by which the AVPV/PeN *Kiss1* sex difference could develop. Postnatal neurogenesis events are a less common mode of sexual differentiation, as neurogenesis is a somewhat rare event in normal postnatal neurobiology. However, there are examples of sex difference in neurogenesis in the olfactory bulb and hippocampus [55; 62; 158]. Thus, one might speculate that low sex steroid levels in perinatal females would promote mitosis in AVPV/PeN *Kiss1* neurons, generating more *Kiss1* neurons, whereas high T secretion in perinatal males would inhibit mitotic processes in *Kiss1* cells, thereby keeping *Kiss1* neuron number lower in males. However, there is little evidence to support this model. First, the birth of AVPV neurons occurs during fetal life, suggesting that the AVPV neurons that express *Kiss1* in adulthood are already fully present during the perinatal critical period, though this is difficult to experimentally confirm [101]. Thus, this model would require a second wave of mitotic division in the AVPV during the critical period under the influence of perinatal sex steroids that specifically influence *Kiss1* cells. Although unlikely, there is one report of sex-specific neurogenesis in the AVPV during postnatal life [2], though in this case, the new neurons were born after the known time of the initial appearance of *Kiss1* neurons, suggesting that they are not *Kiss1* cells.

One mechanism which may be involved in directing the sexual differentiation of *Kiss1* expression is epigenetic modifications, which encompasses silencing or activating the expression of a gene via changes in chromatin structure [44; 91]. There is a growing interest in epigenetic regulation of sexual differentiation [91], and some epigenetic mechanisms, such as histone acetylation and DNA methylation, have been recently reported to relate to some neuronal sex differences, including those in the BNST [95] and the preoptic area [79]. We recently performed a series of experiments to ascertain whether sex differences in either DNA methylation and/or histone modifications may be responsible, either fully or in part, for the AVPV/PeN *Kiss1* sex difference [125]. First, we assessed the possibility that the sex difference in AVPV/PeN *Kiss1* expression is influenced by histone acetylation [16], which typically promotes increased gene transcription by relaxing the chromatin around a given gene locus. Histone deacetylases are enzymes that act to remove the acetyl group from histones, thereby promoting closing of chromatin and repression of gene transcription. We tested whether *Kiss1* sexual differentiation reflects an inequality in histone deacetylase activity between the sexes, such that developing males experience greater histone deacetylation surrounding the *Kiss1* locus than females. To test this, histone deacetylase inhibitors were given to newborn males and females during the critical period of *Kiss1* sexual differentiation, which is shortly after birth. Although this inhibition of postnatal histone deacetylase activity abolished the sex difference in the size of the BNST, this treatment did not affect the sex difference in AVPV/PeN *Kiss1* neurons [125]. We therefore concluded that male and female differences in postnatal histone acetylation are not responsible for the *Kiss1* sex difference, though this does not rule out the possible involvement of other histone modifications, such as sex specific histone methylation or phosphorylation.

A second epigenetic mechanism that could explain the *Kiss1* sex difference is DNA methylation. Methylation of DNA occurs specifically at cytosine-phosphate-guanine (CpG) sites 5' to or within a gene's sequence producing 5-methylcytosine at these sites.

Methylation of CpG sites can occur anywhere in the genome, but tends to occur more frequently at CpG sites clustered together (so-called CpG islands). CpG methylation acts to prevent transcription factor and RNA polymerase binding, and is therefore generally considered a repressive modification since a more methylated locus will have less transcriptional activity. We hypothesized that there may be more CpG methylation in the *Kiss1* gene of males than females, serving to reduce or silence transcription of the *Kiss1* gene in males, thereby producing a sex difference in *Kiss1* expression. Using DNA obtained from tissue punches of the AVPV/PeN from adult males and females, we analyzed ~75 CpG sites in the *Kiss1* promoter and coding region, including a CpG island in the third exon. We found a significant difference in the degree of methylation between males and females at four *Kiss1* CpG sites, three in the promoter and one in the first intron [125]. Intriguingly, in all four cases, males had *less* methylation than females, a result that was opposite of what was expected [125]. However, *in-silico* analysis of the specific DNA sequences encompassing the differentially methylated CpG sites revealed a binding sites for transcriptional repressors [125], suggesting that differential methylation between the sexes may allow for higher repressor binding to the *Kiss1* gene in males than females, producing the sex difference in *Kiss1* expression. Despite this possibility, it is currently unknown if the predicted repressors act on the *Kiss1* gene *in-vivo*. For comparison, methylation of the *Kiss1* gene was similarly examined in DNA obtained from the non-sexually dimorphic ARC and revealed no sex difference in methylation of the same *Kiss1* CpG sites, demonstrating a nucleus-specific epigenetic action. While this is a first step, further experimentation is required to determine the significance of these differentially methylated CpG sites in the transcription of the *Kiss1* gene, as well as the effects of sex steroids during the critical period on this methylation pattern.

4.5 Sex steroid regulation of *Kiss1* in the AVPV/PeN during prepubertal/pubertal life

Since kisspeptin signaling is required for normal pubertal progression, a number of studies have examined changes in *Kiss1* mRNA and kisspeptin protein levels before and after puberty. Within the AVPV/PeN, there is an obvious increase in the number of *Kiss1*-expressing cells, total *Kiss1* mRNA levels, and kisspeptin immunoreactivity in both mice and rats after puberty compared to juvenile animals [17; 54; 144]. The developmental increases in both kisspeptin immunoreactivity and *Kiss1* mRNA in the AVPV/PeN actually begin before puberty, in the second week of life, and continue throughout much of the pubertal transition, reaching adult levels sometime in mid-to-late puberty [17; 19; 71; 124]. The mechanism underlying the increase in the expression of kisspeptin at these ages is not entirely clear. It is possible that the increasing concentrations of ovarian-secreted E2, which rise as puberty proceeds, induces higher levels of *Kiss1* expression. If so, the developmental increases in AVPV/PeN *Kiss1* and kisspeptin levels may simply be secondary to, or a side effect of, other pubertal driving factors. On the other hand, the developmental increase in AVPV/PeN *Kiss1* may be independent of sex steroids and reflective of central changes that occur to promote pubertal progression. One study in rats concluded that the pubertal increase in *Kiss1* in the AVPV/PeN is dependant on the current hormonal milieu, as gonadectomized prepubertal female rats did not show a significant increase in AVPV/PeN *Kiss1* expression at later ages [144]. It is likely that combination of factors, both hormonal and cell intrinsic, may be involved. In addition to a pubertal increase in *Kiss1* and kisspeptin cell number in the AVPV/PeN, there are also an increasing number of kisspeptin-GnRH appositions observed as puberty progresses, with the most appositions detected after the completion of puberty [17]. However, it is unknown if the increased number of kisspeptin-GnRH appositions is a morphological change in axonal fibers, or simply reflective of enhanced kisspeptin synthesis with age, resulting in immunoreactive kisspeptin fibers simply being more easily detectable.

A currently unresolved issue is whether or not puberty represents a “critical period” for kisspeptin neuron development in the AVPV/PeN. Clarkson et al. [19] observed that gonadectomy in female mice before puberty (~PND 15) reduced the number of kisspeptin neurons detected in the AVPV/PeN later in early adulthood, and that E2 replacement during puberty could rescue the adulthood deficit in kisspeptin levels. It has therefore been proposed that ovarian sex steroids produced in prepubertal life have feed-forward activity on AVPV/PeN kisspeptin and assist in advancing pubertal development. Additionally, it has been proposed that peripubertal E2 organizes the trajectory of kisspeptin development in the female AVPV/PeN by further feminizing kisspeptin in another “critical period” occurring around the time of puberty. However, the initial data that supported this hypothesis are difficult to interpret because there were uncontrolled group differences in circulating E2 at the time of sacrifice [19]. That is, mice gonadectomized before puberty did not have circulating E2 at sacrifice in adulthood, whereas the gonadally-intact and E2-replaced groups both had E2 at sacrifice. It is therefore difficult to determine if the observed group differences in adult AVPV/PeN kisspeptin levels are due to long-lasting developmental effects of E2 acting during a critical pubertal period, or merely differences in activational effects of E2 on kisspeptin at the time of sacrifice. We recently directly addressed this issue using prepubertal (PND 14) female mice that were gonadectomized (to remove gonadal E2) or left intact and then given E2 later in early adulthood. In this case, with all animals exposed to similar E2 levels in adulthood—regardless of their pubertal E2 exposure or lack thereof—no differences in either the number of *Kiss1* cells in the AVPV/PeN or the level of *Kiss1* mRNA per cell were observed between prepubertally-gonadectomized and prepubertally-intact females [70]. Thus, by controlling for activational effects of E2 in the adulthood, this finding demonstrates that the maturation of *Kiss1* in the AVPV/PeN is *not* dependent on E2 signaling exclusively during the peripubertal period. The pubertal period is therefore not a “critical period” for E2’s actions on *Kiss1* neuron development.

Despite the preceding conclusion, data from female aromatase KO mice and hypogonadal (hpg) mutant mice (lacking GnRH and hence, gonadal sex steroid synthesis) suggest that developmental E2 exposure may still be required for proper *Kiss1* neuronal maturation. This is evidenced by the fact that both aromatase KO and hpg females display very low kisspeptin staining in the AVPV/PeN in adulthood, even after E2 replacement in adulthood [3; 41]. Thus, it appears that E2 signaling is required at *some point* in development for proper female-like *Kiss1* development. Exactly when and for how long E2 is needed to ensure proper *Kiss1* neuron development in females is unknown, though our data would argue that any requisite E2 is likely needed sometime *before* PND 14.

Because the vast majority of kisspeptin neurons in the AVPV/PeN and ARC express ER α , and E2’s activational effects on *Kiss1* expression in both regions requires intact ER α signaling [134], it was recently hypothesized that ER α expression specifically in kisspeptin neurons is required for normal pubertal development and reproductive function. To test this hypothesis, Mayer et al. used conditional gene targeting to generate kisspeptin neuron specific ER α KO mice (KERKO) [89]. Developmental characterization of these mice revealed abnormally advanced pubertal progression, as determined by day of vaginal opening, as well as irregular cyclicity, and minimal ovarian luteinization. The phenotype of kisspeptin neurons in KERKO mice was also abnormal, as the number of immunoreactive kisspeptin cells in the AVPV/PeN was markedly reduced in adult KERKO animals. However, the developmental regulation of AVPV/PeN kisspeptin cells by ER α is difficult to interpret in these mice, because the activational and organizational effects of E2 cannot be teased apart owing to a permanent absence of ER α in kisspeptin neurons throughout all life stages. With this model, it is impossible to know if the scarcity of detectable AVPV/PeN kisspeptin neurons in adulthood is due to organizational (i.e., permanent) effects due to an absence of ER α early development or merely a deficit in the activational effects of E2 in

adulthood. Additional experiments on the age-specific roles of ER α throughout life are needed to fully understand the role of E2 in the development of *Kiss1* neurons.

5. Development of the ARC kisspeptin system

5.1 *Kiss1* and kisspeptin in the fetal and neonatal ARC of rodents

Unlike in the AVPV/PeN, *Kiss1* mRNA can be detected in the rodent ARC prenatally [31; 147] and at birth in both sexes [12; 30; 113] (Figures 1, 3). A recent study in rats determined that *Kiss1* neurons in the ARC are born between embryonic days 12.5 and 17.5 [31], and are present at all ages after that. Moreover, unlike in adulthood, there is a significant sex difference in *Kiss1* expression in the ARC of perinatal rodents, with females expressing more ARC *Kiss1* cells and higher levels of *Kiss1* mRNA/cell than males (Figure 2, 3). This ARC *Kiss1* sex difference is apparent both before birth and during early postnatal life in both mice and rats [12; 31; 113; 145]. It is interesting to note that this perinatal sex difference in ARC *Kiss1* parallels the sex difference in serum gonadotropins at this time, with perinatal females having higher FSH and LH than perinatal males [33; 112; 113; 133]. This suggests a possible functional relationship between kisspeptin and the pituitary (presumably through GnRH) at this early developmental stage, mirroring kisspeptin's reproductive role later in puberty and adulthood. Indeed, recent evidence demonstrates that kisspeptin and GnRH signaling are each essential for neonatal gonadotropin secretion in rodents, since newborn *Kiss1* KO and hpg mice both have undetectable serum FSH and LH levels, unlike their newborn WT littermates [113]. However, the physiological significance of kisspeptin regulating GnRH and gonadotropins in perinatal life is unclear, especially since testicular function in rodents appears to be completely kisspeptin- and GnRH-independent at this early age (see below).

The mechanism(s) producing the early postnatal sex difference in ARC *Kiss1* expression in rodents not yet elucidated. One likely possibility is that the elevated circulating T present in perinatal males but not females acts to negatively feedback and repress ARC *Kiss1* expression, paralleling the inhibitory action of T on ARC *Kiss1* expression in adults [135]. Under this model, perinatal females lack elevated gonadal sex steroid secretion and therefore their ARC *Kiss1* neurons receive less negative feedback than their male siblings, resulting in higher *Kiss1* expression in females (Figure 2). Since elevated T secretion in newborn male rodents is independent of kisspeptin and GnRH signaling [113], the T-mediated repression of *Kiss1* in males would remain constant during the perinatal period. Despite this logic underlying this possibility, this model has not yet been tested; thus it still remains unknown if the perinatal sex difference in ARC *Kiss1* levels is truly caused, fully or in part, by activational effects of circulating sex steroids.

What is the function of ARC-derived kisspeptin during prenatal and neonatal life? One possibility is that kisspeptin regulates the perinatal endocrine events that govern sexual differentiation [67], such as the elevated gonadal T secretion that occurs in rodents in prenatal and neonatal males but not females [5; 23; 24; 25; 93; 107; 113]. Since kisspeptin signaling in adulthood is essential for gonadal T secretion (via kisspeptin's upstream stimulation of GnRH), ARC-derived kisspeptin may also be critical for regulating neonatal T secretion. However, despite this, we recently determined that kisspeptin signaling is *not* required for sexually-dimorphic neonatal T secretion, as newborn *Kiss1* KO males have elevated serum T levels that are similar to newborn WT males and higher than those of newborn females [113]. A similar outcome was observed in hpg mice, in which newborn hpg males exhibit elevated circulating T levels just like their WT counterparts [113]. Thus, unlike later in life, neonatal T secretion is independent of kisspeptin and GnRH control, at least in rodents. Moreover, because kisspeptin and GnRH are each required for gonadotropin secretion in neonatal rodents, there appears to be a disconnect between the brain/pituitary

and gonads in early perinatal development, such that the neonatal testes secrete T independently of kisspeptin, GnRH, and gonadotropin influence. Based on these findings, the function, if any, of ARC-derived kisspeptin early in perinatal life still remains unknown.

As discussed in earlier sections, it is well-established that neonatal E2 permanently organizes the development and sexual differentiation of the AVPV/PeN kisspeptin population. Interestingly, neonatal E2 treatment also appears to reduce adult levels of kisspeptin fibers in the ARC [108]. In agreement with this result, neonatal E2 significantly reduces total hypothalamic *Kiss1* mRNA levels, measured by RT-PCR, in adult male rats [96; 100]. While these results suggest that perinatal E2 may exert permanent organizational effects on the ARC kisspeptin population, as it does in the AVPV/PeN, there are some caveats in making this interpretation. First, while exogenous E2 given in development clearly has long-term effects on ARC kisspeptin levels, it remains to be shown that endogenous neonatal E2 has similar developmental effects. Second, some of the kisspeptin fibers in the ARC may in fact originate from the AVPV/PeN population [157]. Thus, the effects of neonatal E2 treatment on kisspeptin fibers in the ARC could simply reflect changes in AVPV/PeN-derived kisspeptin levels, which are highly influenced by E2 signaling both neonatally (organizational) and in adulthood (activational). Third, with respect to the RT-PCR data [96; 100], RNA from whole hypothalamus was used, and therefore includes a mixed population of AVPV/PeN and ARC kisspeptin neurons, making it difficult to interpret the relative contribution of each population to the data. Moreover, circulating sex steroid levels, which strongly affect kisspeptin levels, were not controlled for in adulthood in either experiment. Lastly, in all cases, the diminished ARC kisspeptin levels in adult animals that were given E2 neonatally could be caused fully or in part by elevated E2-induced apoptosis of kisspeptin neurons. Indeed, a significant number of kisspeptin neurons in the ARC seem to normally die during development via BAX-dependent apoptotic mechanisms, as evidenced by significantly more ARC *Kiss1* cells present in adult *Bax* KO than WT mice [124]. Interestingly, these “additional” ARC *Kiss1* cells in adult *Bax* KO mice were often situated dorsally to the traditional *Kiss1* neuron population in this region [124]. This suggests that the ARC *Kiss1* cell population begins as a much bigger population that is then trimmed during development via programmed cell death. The exact developmental age(s) when BAX-mediated apoptosis acts to reduce the number of *Kiss1* cells in the ARC has not been determined. Regardless, since sex steroids have been shown to modulate apoptosis in the brain [123; 153], it is therefore possible that exogenous E2 treatment early in neonatal development accentuates normal apoptotic mechanisms that serve to reduce kisspeptin cell numbers.

5.2 Sex differences in the regulation of ARC Kiss1 neurons during prepubertal life

Unlike in prenatal and early postnatal life, *Kiss1* expression in the ARC of peripubertal rodents is not as sexually dimorphic, with gonadally-intact peripubertal males and females exhibiting fairly similar numbers of ARC *Kiss1* neurons and *Kiss1* mRNA levels [12; 68; 145] (Figure 3). However, gonadectomy of prepubertal mice, performed in the third postnatal week, results in dramatic increases in both ARC *Kiss1* expression and serum LH when measured several days later, but only in females [68]. Age-matched gonadectomized male mice show no appreciable change in ARC *Kiss1* expression or serum LH levels during this prepubertal stage. The gonadectomy-induced increase in *Kiss1* mRNA in prepubertal females suggests that gonadal hormones are acting at this time to negatively feedback and inhibit ARC *Kiss1* expression, as they normally do in adulthood. However, the lack of a similar increase in *Kiss1* expression in gonadectomized prepubertal males suggests that gonadal-independent mechanisms are acting at this time to repress ARC *Kiss1* levels in males. This sex difference in ARC *Kiss1* responsiveness to gonadectomy is only observed in prepubertal mice, as gonadectomy in early adulthood robustly increases ARC *Kiss1* mRNA

equally in both sexes [68]. These data suggest that the regulation of *Kiss1* in the ARC during prepubertal development is sexually dimorphic, with males at this age experiencing some inhibition of their ARC *Kiss1* system that is independent of gonadal hormones.

The mechanism(s) and factors underlying the sex difference in the regulation of ARC *Kiss1* neurons is still unclear, as is the sex difference's functional significance, though it could relate to known differences in puberty onset between males and females (females initiate and complete puberty earlier than males). If so, the heightened ARC *Kiss1* response to gonadectomy in prepubertal female mice may reflect a more advanced and mature kisspeptin system which is much closer to triggering puberty than in similarly-aged males who are farther from pubertal onset at this time. Since this sex difference in ARC *Kiss1* regulation is no longer present in adulthood, we are currently investigating the possibility that as males develop closer to puberty, their *Kiss1* system will begin to respond to gonadectomy in a similar manner as younger prepubertal female.

5.3 Changes in *Kiss1* and kisspeptin in the rodent ARC during puberty

The developmental changes that occur in rodents in the ARC *Kiss1* population at puberty are less clear than those of the AVPV/PeN population. There are several conflicting reports on the pubertal pattern of ARC *Kiss1*/kisspeptin which may partly be attributed to species differences between mice and rats, differences between sexes, and differences in methodologies (measuring mRNA or protein, or measuring at different ages). Even so, there slowly appears to be some consensus forming between a number of rodent studies that demonstrate no robust changes in *Kiss1* in the ARC during puberty (Figure 3). qPCR analysis of *Kiss1* in the ARC in male and female mice showed no significant changes in *Kiss1* mRNA over several days of pubertal development [41]. This data matches that of a previous report in male mice that observed no significant differences in the number of ARC *Kiss1* cells between juvenile and adult male mice, as measured by *in-situ* hybridization [54]. Furthermore, *in-situ* hybridization analysis of *Kiss1* levels in male and female rats during peripubertal development (assayed at 3, 4 and 5 weeks of age) also found no significant differences in ARC *Kiss1* between these ages in either sex [145]. Lastly, our lab recently reported similar preliminary data demonstrating no major changes in *Kiss1* expression from day to day over pubertal development in female mice [71]. Despite the agreement of these studies, there are a few reports that conflict with the idea of no robust change in ARC *Kiss1* mRNA during puberty. First, an increase in *Kiss1* mRNA in the ARC, as measured by *in-situ* hybridization, was reported in male rats between juvenile and pubertal ages, with a subsequent decline again after puberty [7]. However, the quantification method of this *in-situ* hybridization data differs than that of other reports, as only the posterior ARC was quantified instead of the entire ARC, which may account for this different result. Another study in rats used qPCR to measure *Kiss1* levels from microdissected tissue of the ARC region and found higher *Kiss1* mRNA during and after puberty relative to the juvenile stage [144]. Unfortunately, in this study and most of the others, only one or two pubertal ages were studied, giving an incomplete picture of how *Kiss1* changes across the entire pubertal transition, which in rodents may be as long as 10–14 days. This limitation makes it difficult to compare findings between studies, especially when the same particular age is not analyzed in each case.

While the majority of studies thus far suggest that ARC *Kiss1* mRNA does not change considerably during pubertal development in rodents, kisspeptin immunoreactivity in the ARC does appear to increase during puberty. Assays examining kisspeptin immunoreactive area (cell bodies plus fibers) in mice [41] and rats [59; 144] showed an increase in the total kisspeptin immunoreactive level in the ARC after the completion of puberty. In both species, the pubertal increase in kisspeptin immunoreactivity appears to be gradual, with maximal immunoreactive levels observed once puberty is actually completed [41; 59].

However, in one rat study, there was a sharp increase in kisspeptin immunoreactivity in the ARC observed during early puberty (between PND 26 and 31) [144]. These pubertal increases in kisspeptin protein levels in the ARC contradict the relatively minimal (or absent) pubertal changes observed in *Kiss1* mRNA in this region. However, it is possible that a significant number of kisspeptin fibers detected in the ARC actually originate from the AVPV/PeN [157]. Thus, pubertal increases in kisspeptin-immunoreactive fibers in the ARC may reflect—fully or in part—increases in kisspeptin synthesis in the AVPV/PeN (a possibility supported by multiple findings of increasing *Kiss1* expression in the AVPV/PeN during puberty). Certainly, the inability to discern the source of individual kisspeptin fibers in the ARC based on single-label assays makes interpretation of that data challenging. Future studies may divert this problem by employing double-label immunohistochemical assays with kisspeptin and co-factors known to be solely expressed in either the AVPV/PeN (e.g., TH) or ARC (e.g., NKB) populations.

Several sex steroid-related manipulations have been performed in rodents to determine how *Kiss1* in the ARC is regulated during puberty. Using qPCR in normal and hpg mice, ARC *Kiss1* mRNA was found to be higher in hpg mice as early as the second postnatal week [41]. This genotype difference in *Kiss1* mRNA further increased by the fourth postnatal week (early puberty), with hpg mice having several fold higher ARC *Kiss1* levels than WT mice. Since hpg mice are hypogonadal, their higher ARC *Kiss1* levels during puberty could be due to a lack of gonadal sex steroid feedback. If so, it suggests that sex steroids at early pubertal ages normally act to inhibit *Kiss1* expression in WT mice. This has not yet been tested, as hpg mice have not had sex steroid replacement during puberty to determine if this treatment corrects the high *Kiss1* expression. However, it seems likely that *Kiss1* in the ARC during the pubertal period would in fact be repressed by sex steroids, based on evidence in younger animals [19; 68] and the effect of sex steroids on pubertal kisspeptin fiber staining (see below) [41]. Regardless, given the developmental increase in ARC *Kiss1* levels between postnatal weeks 2 and 4 in the hpg animals, it suggests that there may be a gonad-independent mechanism responsible for increasing *Kiss1* expression around the time of puberty. If so, the high levels of *Kiss1* observed in the ARC of pubertal hpg mice may be reflective of both a lack of steroid negative feedback and a gonad-independent enhancement of *Kiss1* expression that occurs around puberty. Thus, kisspeptin neurons in the ARC may integrate both non-gonadal factors that promote pubertal progression and sex steroid negative feedback signals that modulate this process.

In contrast to an abnormal pubertal increase in ARC *Kiss1* mRNA, hpg mice also show decreased kisspeptin fiber immunoreactivity in the ARC over pubertal development [41]. Similar reductions in kisspeptin immunoreactive levels in the ARC are observed over development in KERKO mice [89] as well as in adult aromatase KO mice [19], suggesting that the presence of detectable kisspeptin fibers in the ARC is partly E2-dependent. This effect is likely due to an activational defect rather than a permanent organizational impairment, because hpg females given E2 in adulthood display normal kisspeptin fibers levels, similar to WT mice [41]. It is not known if the same result would occur in aromatase KO mice. Moreover, as in previous cases discussed earlier, it is not clear if the diminished kisspeptin fiber levels in the ARC of hpg, KERKOs, and aromatase KOs reflect changes in kisspeptin levels in the AVPV/PeN, which sends some projections to the ARC region.

5.4 Changes in *Kiss1* in the primate ARC during puberty

While the majority of studies have examined kisspeptin during puberty using rodent models, there is some evidence for changes in *Kiss1* with puberty in rhesus macaques. qPCR analysis from the medial basal hypothalamus (homologous to the rodent ARC) revealed that *Kiss1* was significantly higher in pubertal than juvenile gonadal male monkeys [127]. This finding mirrors that in pubertal hpg mice discussed above. Furthermore, *Kiss1* levels in the

medial basal hypothalamus were significantly higher in midpubertal than juvenile intact female monkeys, but there was no significant difference between juvenile and early pubertal females [127]. This midpubertal increase in *KissI* in female monkeys appears to match a similar increase in kisspeptin release in the median eminence, as measured by push-pull perfusates [72]. In addition to changes in *KissI*, *KissIr* expression is also increased in the medial basal hypothalamus in intact female monkeys in early and midpuberty, relative to the juvenile stage [127]. These changes in *KissIr* appear to have functional implications, as release of GnRH in response to infusions of kisspeptin is greater in pubertal than prepubertal female monkeys [52]. These data provide a small glimpse into the developmental regulation of kisspeptin in primates, but further studies are needed to determine if the changes and regulation of *KissI* observed in rodents can be applied to primates, including humans.

6. Summary and Conclusions

For the better part of a decade, neuroendocrinologists have vigorously studied the role of kisspeptin in puberty and reproduction and the development of *KissI*-expressing nuclei. To this end, there have been several advancements in our understanding of the actions of gonadal sex steroids on the kisspeptin system during different phases of development. Indeed, it is now clear that the activity of sex steroids on kisspeptin neurons is both age- and region-specific. First, we have learned that perinatal sex steroids program the sexual differentiation of AVPV/PeN kisspeptin neurons and it that epigenetic mechanisms, rather than apoptotic pathways, may be responsible for organizing this important sex difference. However, further studies are needed to determine exactly what epigenetic modifications regulate this *KissI* sex difference, at what specific perinatal and postnatal ages they are induced, and how they are developmentally controlled. At later developmental stages, AVPV/PeN kisspeptin neurons may also be further regulated by pubertal E2 to become fully feminized. However, this conclusion is equivocal and currently unresolved, as published reports have not always accounted for activational effects of E2.

In rodents, *KissI* is expressed in the ARC both before birth and in early neonatal life, but the role of ARC-derived kisspeptin at these early developmental stages is unknown. Although kisspeptin was initially hypothesized to be responsible for regulating perinatal T secretion, especially in males, recent results from *KissIr* KO mice indicate this is likely not the case. Rather, elevated T secretion in perinatal males appears to be kisspeptin signaling-independent, at least in mice. Since, apart from this short-lived perinatal T surge in males, the rodent reproductive axis of both sexes is virtually quiescent during the majority of early development, it is not entirely clear what role kisspeptin might serve during this stage of life, though it is possible it is unrelated to reproduction. This important issue is worthy of future experimentation. In regards to the AVPV/PeN population, *KissI* expression is not detectable in rodents until the second week of life, but it is likely that *KissI* neurons in this region are born well before this time. When these AVPV/PeN *KissI* neurons are actually born and why they do not express *KissI* until later ages remains to be elucidated. Additionally, what role, if any, these few AVPV/PeN *KissI* cells have in pubertal development needs to be resolved.

With respect to the ARC kisspeptin population, which is not markedly sexually dimorphic in adulthood, there are two stages in rodent development, the neonatal and juvenile periods, when *KissI* cells in the ARC display sexually dimorphic characteristics. Although not yet tested, it is likely that the sex difference in neonatal ARC *KissI* levels is due to activational effects caused by temporary variances in circulating sex steroids, separating it from the organizational sex difference in the AVPV/PeN *KissI* population that is permanently induced by the same neonatal sex steroids. In contrast, the mechanisms, hormonal or otherwise, that underlie the sex difference in the regulation of juvenile ARC *KissI* neurons are less understood and require further experimentation. In addition, studies in transgenic

mice suggest that there are gonadal factors, likely sex steroids, that normally inhibit ARC *Kiss1* expression during the pubertal period, but the identify of these regulatory factors, their specific mechanism of action, and their functional relationship to pubertal progression remain to be determined.

Lastly, to date, the majority of developmental kisspeptin studies have been in rodents, with only a handful of studies examining the development of kisspeptin in other species, including sheep and monkeys. In order to better understand the role of kisspeptin in human reproductive development, additional studies are needed in non-rodent models, such as non-human primates. While there are likely many parallels in the development of *Kiss1* neurons between species, there are certainly known species differences in the regulation and distribution of kisspeptin neurons, highlighting the need for developmental data from other animal models.

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Highlights

- Kisspeptin, encoded by *Kiss1*, is a neuropeptide critical for puberty and fertility
- Different *Kiss1* populations have different phenotypes and developmental patterns
- Sex steroids regulate kisspeptin neurons in both development and adulthood
- Sex steroids effects on kisspeptin neurons can be organizational or activational
- Sex steroids at birth govern sexual differentiation of select *Kiss1* populations

Figure 1.

Representative photomicrographs of *in-situ* hybridization for *Kiss1* mRNA in the ARC and AVPV/PeN of mice. A) *Kiss1* staining in the ARC of postnatal day 1 (PND 1, day of birth) female (F) and male (M) mice, as well as adult gonadally-intact F (diestrous) and M mice. . B) *Kiss1* staining in the AVPV/PeN of PND 14 F and M mice, as well as adult gonadally-intact F (diestrous) and M mice.

Figure 2.

Schematic representation of the effects of the neonatal testosterone surge on *Kiss1* expression in the AVPV/PeN and ARC. Neonatal male (M) rodents have considerably higher testosterone (T) on the day of birth than female (F) rodents. This increased T has known organizational effects on the *Kiss1* system in the AVPV/PeN, after aromatization to E₂, possibly through epigenetic modifications that induce a permanent sex difference later in development (with F rodents having more *Kiss1* neurons than M). These high levels T (or E₂) may have short-term negative feedback effects (activational) in the ARC during the neonatal period. The higher T (or E₂) represses *Kiss1* in neonatal M, but neonatal F have less circulating T (or E₂) and therefore less repression of *Kiss1* expression. This presumed

difference in negative feedback produces a sex difference in *KissI* expression in the neonatal period, which is not present later in life.

* One report of increased *Kiss1* at puberty in male rats

** One report of increased *Kiss1* in female rats after puberty

Figure 3.

Schematic representation of general changes in *Kiss1* in the AVPV/PeN and ARC over postnatal development and puberty. The difference in *Kiss1* over development is in females (pink line) and males (blue line) are traced through the neonatal/prepubertal periods, the pubertal period and adulthood. Expression of *Kiss1* in the AVPV/PeN is not detectable until the second postnatal week of life, which is a prepubertal age in rodents. The sex difference in *Kiss1* expression in this region is present shortly after *Kiss1* is first detected and continues throughout postnatal life. In the ARC, *Kiss1* is present at birth and is different between sexes, but this sex difference disappears before puberty begins in intact animals. Most published data suggests that *Kiss1* expression does not change much during puberty in

gonadally-intact animals. However, there is one report on increased *KissI* expression in male rats during puberty which then drops later in adulthood [7] and another report of an increase in *KissI* from before puberty into adulthood in females [144].