Chapter 11
The Development of Kisspeptin Circuits in the Mammalian Brain

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Abstract The neuropeptide kisspeptin, encoded by the *Kiss1* gene, is required for mammalian puberty and fertility. Examining the development of the kisspeptin system contributes to our understanding of pubertal progression and adult reproduction and sheds light on possible mechanisms underlying the development of reproductive disorders, such as precocious puberty or hypogonadotropic hypogonadism. Recent work, primarily in rodent models, has begun to study the development of kisspeptin neurons and their regulation by sex steroids and other factors at early life stages. In the brain, kisspeptin is predominantly expressed in two areas of the hypothalamus, the anteroventral periventricular nucleus and neighboring periventricular nucleus (pre-optic area in some species) and the arcuate nucleus. Kisspeptin neurons in these two hypothalamic regions are differentially regulated by testosterone and estradiol, both in development and in adulthood, and also display differences in their degree of sexual dimorphism. In this chapter, we discuss what is currently known and not known about the ontogeny, maturation, and sexual differentiation of kisspeptin neurons, as well as their regulation by sex steroids and other factors during development.

Introduction

The status of the neuroendocrine reproductive axis is in flux during various stages of perinatal and pubertal development, ranging from being entirely quiescent to fully active. Additionally, the neuroendocrine reproductive system is anatomically and physiologically differentiated between males and females, and these sex differences

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originate during key stages of development. Sex differences are also seen in several reproductive health disorders, such as idiopathic hypogonadotropic hypogonadism, constitutional delayed puberty, and precocious puberty [1–5]. Many of these reproductive health disorders have been attributed to known, or in many cases, unknown developmental defects in the brain. Specific neuronal circuits located in the forebrain and hypothalamus have been implicated as control centers responsible for proper development of reproductive physiology, converging on neurons that release gonadotropin releasing hormone (GnRH). GnRH stimulates the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary, thereby driving the maturation and activation of the gonads. How and when the developmental changes in the neuroendocrine axis are induced is not completely known, but gaining a clearer understanding may help pinpoint the cause and timing of defects in reproductive maturation.

One of the key upstream hypothalamic circuits involved in the control of GnRH secretion consists of neurons expressing the *Kiss1* gene and its protein product, kisspeptin. Kisspeptin signaling has been implicated as an essential regulator of fertility and puberty in numerous mammalian species, including humans [6–8]. Alterations in the expression of *Kiss1* or kisspeptin over development, along with differences in expression between the sexes, especially during key developmental periods, may be a critical driving force in the maturation of the neuroendocrine reproductive system. Indeed, changes in the *Kiss1* system likely contribute to the timing of puberty onset, sex differences in LH secretion, and other facets of reproductive physiology. It is therefore essential to understand how kisspeptin neurons develop, when and how *Kiss1* gene and protein expression are modified during development, and what possible regulatory mechanisms govern the development of the kisspeptin system. This chapter discusses the current knowledge for these topics in mammals and also pinpoints several important unanswered questions involving the development of kisspeptin neuronal circuits.

Kiss1 and Kisspeptin Expression in the Adult Brain

In order to study the development of the kisspeptin system, it is essential to first understand the localization and phenotype of kisspeptin neurons in the adult state. Until the recent generation of transgenic mice, which label kisspeptin cells with markers such as GFP [9–11], three techniques were used to examine the localization of kisspeptin neurons in the brain: reverse transcriptase PCR (RT-PCR)/quantitative PCR (qPCR), in situ hybridization (ISH), and immunohistochemistry (IHC). RT-PCR was used in early studies to identify high *Kiss1* mRNA expression in large regions of the brain, such as the hypothalamus [12]. However, this technique was weakened by an inability to specifically visualize and separately analyze discrete *Kiss1* populations within these large brain areas. This issue was resolved through the subsequent use of ISH and IHC, which allow for precise neuroanatomical mapping of *Kiss1*- and kisspeptin-synthesizing neurons, respectively. However, in earlier

IHC studies, some kisspeptin antibodies were not very specific, as they were shown to cross react with other RFamide family members [13]. The recent use of more specific kisspeptin antibodies has allowed for more precise detection of kisspeptin immunoreactivity in the brain [14–19]. Utilizing these various methods, many studies have confirmed that in adult rodents, *Kiss1* (or kisspeptin) is expressed in just a few discrete brain regions, including a small population in the medial amygdala (MeA) [20] and two larger hypothalamic populations in the anteroventral periventricular nucleus and neighboring periventricular nucleus (AVPV/PeN) and the arcuate nucleus (ARC) [21–23]. In non-rodent species, such as sheep and non-human primates, *Kiss1* gene expression and kisspeptin immunoreactivity have, for the most part, a similar distribution as in rodents, with expression localized to the pre-optic area (POA) and the ARC/infundibular nucleus (INF) [19, 24–27]. Expression of *Kiss1* or kisspeptin in the MeA of non-rodent species has not yet been examined.

In contrast to *Kiss1*/kisspeptin cell bodies, which are found in just a few discrete brain regions, kisspeptin-immunoreactive (ir) fibers are scattered throughout the brain (discussed in detail in Chap. 3). In adult rodents and sheep, terminals of kisspeptin fibers are found within the POA (and regions containing GnRH neurons), the ARC and medial basal hypothalamus, the paraventricular nucleus, and the median eminence (perhaps targeting GnRH axons/terminals) [14, 15, 28]. In mice, additional regions have been identified containing kisspeptin fibers, including the lateral septum, dorsal-medial nucleus of the hypothalamus, bed nucleus of the stria terminalis (BNST), and the MeA [14]. It also appears that ARC and AVPV/PeN *Kiss1* neurons send a number of projections to one another, perhaps allowing these two populations to directly communicate [29], although currently there is no evidence that *Kiss1* neurons themselves express *Kiss1r* [30].

In adult rodents, the AVPV/PeN region displays sex differences in various morphological parameters [31, 32] and is considered the main anatomical site that drives the sexually dimorphic preovulatory luteinizing hormone (LH) surge that occurs in adult females [33]. Mounting evidence supports a critical involvement of AVPV/PeN kisspeptin neurons in the sexually differentiated LH surge. For example, Estradiol (E$_2$) dramatically stimulates *Kiss1* expression in the AVPV/PeN, and *Kiss1* neurons in this region co-express sex steroid receptors, including ERα [34]. Moreover, *Kiss1* neuronal activity (as measured by *cfos* induction) in the AVPV/PeN is upregulated in a circadian pattern in complete synchrony with the circadian timing of the LH surge [35]. Additionally, as will be discussed in more detail later, the AVPV/PeN *Kiss1* population itself is sexually differentiated, just like the preovulatory LH surge, with females expressing greater *Kiss1* and kisspeptin expression in this region than males [36]. It is also worth noting that the AVPV/PeN region contains several other sexually dimorphic subpopulations that have been implicated in regulating reproduction, such as dopaminergic neurons, which express the tyrosine hydroxylase (TH) enzyme [37, 38], and cells expressing both GABA and glutamate [39]. Interestingly, most *Kiss1* neurons in the AVPV/PeN co-express TH [40, 41], though the functional significance of such co-expression has yet to be determined.

Kisspeptin neurons in the ARC comprise the largest kisspeptin population in the brain [15, 24, 25, 34, 36, 42, 43]. In contrast to the AVPV/PeN population, *Kiss1*

cells in the ARC are not highly sexually dimorphic in adult rodents, especially when the adulthood sex steroid milieu is similar between the sexes [36, 44]. However, there are sex differences in the regulation of *Kiss1* expression in the ARC that are present in mice during the prepubertal period, an intriguing finding that will be discussed more below [44]. Moreover, some species, such as sheep, exhibit sex differences in ARC *Kiss1* neurons in adulthood, which may reflect slightly different roles of these ARC kisspeptin neurons between species. While *Kiss1* expression is upregulated in the AVPV/PeN by E_2 and testosterone (T), the opposite is true in the ARC: sex steroids potently inhibit ARC *Kiss1* expression [22, 34, 36, 45], and *Kiss1* neurons in this region have a high degree of colocalization with ERα, androgen receptor, progesterone receptor, and to a lesser degree, ERβ [15, 22, 24, 46–49]. It is suggested that the inhibition of *Kiss1* expression in the ARC by sex steroids reflects the involvement of ARC kisspeptin neurons in the negative feedback effects of sex steroids on pulsatile GnRH secretion in both sexes [6, 50, 51]. Additionally, ARC *Kiss1* cells co-express several other regulatory neuropeptides, neurokinin B (NKB) and dynorphin (DYN), which appear to also play a role in regulating GnRH/LH secretion [52–57]. Moreover, ARC kisspeptin neurons have been shown to have abundant reciprocal connections with each other [58–60] and to highly express the NKB receptor (NK3R) [16, 55, 58, 60], which may allow these neurons to communicate and synchronize with each other [55, 60] (discussed more in Chap. 15).

The Development of the AVPV/PeN *Kiss1* Population

Ontogeny of Kiss1 Expression in the AVPV/PeN

In adulthood, *Kiss1* is highly expressed in the hypothalamic AVPV/PeN and ARC regions, but this is not always the case earlier in development. In rodents, *Kiss1* expression is present in the embryonic brain but is limited to the ARC region [61], with no detectable AVPV/PeN *Kiss1* expression at this age. Rather, *Kiss1* mRNA and kisspeptin protein expression in the AVPV/PeN of rodents first occurs later in postnatal life. The developmental timing of AVPV/PeN *Kiss1* expression has been studied in both mice and rats. However, different studies describing the development of *Kiss1* (or kisspeptin) expression sometimes use different nomenclature to describe age of birth: some studies define the day of birth as postnatal day 1 (PND 1), others as PND 0 or P0. For consistency, and to permit for direct comparison between studies, in this chapter the day of birth will be denoted as PND 1; if the day of birth used in the original paper was noted as P0 or PND 0, it will be changed to PND 1 and subsequent ages modified accordingly.

Neither *Kiss1* mRNA nor kisspeptin protein expression has been detected in the AVPV/PeN of mice or rats on the day of birth. The earliest documented AVPV/PeN *Kiss1* expression in mice was recently shown, using ISH, to be ~PND 10 [40], though this expression was of very low magnitude compared to the next several days examined, PND 12, and PND 14, when moderately higher levels were observed (Fig. 11.1).
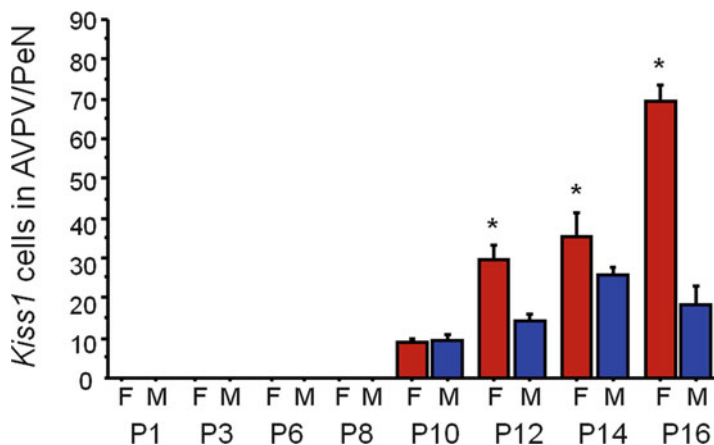
Fig. 11.1 Development of the *Kiss1* sex difference in the anteroventral periventricular nucleus and neighboring periventricular nucleus (AVPV/PeN) of mice. Mean number of *Kiss1* neurons in the AVPV/PeN of female and male mice over the course of early postnatal development. *Kiss1* cells were first detected in each sex on PND 10. The number of *Kiss1* neurons was significantly higher in females than males on PND 12 and later. *Significantly different from males of same age. Modified from Semaan SJ, Murray EK, Poling MC, Dhamija S, Forger NG, Kauffman AS 2010 BAX-dependent and BAX-independent regulation of Kiss1 neuron development in mice. Endocrinology 151:5807–5817

Another developmental study of kisspeptin protein expression in female mice found no kisspeptin-immunoreactive (ir) cells in the AVPV/PeN at PND 10 and only a small number of kisspeptin cells at PND 15, which was the next chronological age examined [62]. Whether the absence of detectable kisspeptin-ir cells at PND 10 reflects differences in the sensitivities of the techniques used (IHC vs. ISH) or a developmental difference at the level of either post-transcriptional processing or translation is currently unknown.

Looking beyond the first 2 weeks of life, kisspeptin-ir cell number was found to steadily increase in the mouse AVPV/PeN from PND 15 to adulthood (assessed every 5 days of age) [62]. Extending this protein data, our lab recently performed a detailed, day-by-day analysis of *Kiss1* mRNA expression in prepubertal and pubertal female mice and found that *Kiss1* cell number in the AVPV/PeN steadily and continually increases from PND 15 through PND 28, at which point it resembles adulthood levels [63]. In rats, like mice, AVPV/PeN kisspeptin expression is not detectable on PND 4 or PND 8, but is noticeably present by the next age examined, PND 22 [64]. Developmental changes in *Kiss1* mRNA expression have also been documented in the POA of ewes [65] at 25, 30, and 35 weeks of age, and an increase in POA *Kiss1* cell number was observed at 30 weeks, corresponding to the time of puberty, with no further increase detectable at 35 weeks [65]. In non-human primates, *KISS1* expression also increases in the hypothalamus during puberty, but this was attributed to kisspeptin neurons in the medial basal hypothalamic region (i.e., the ARC) rather than the POA population [25].
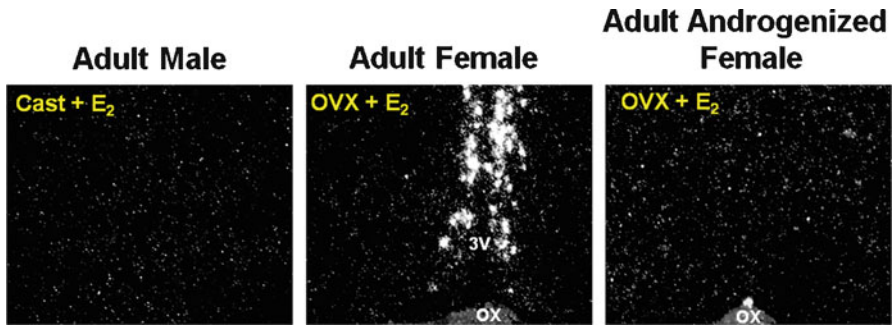
Fig. 11.2 Sex differences in AVPV *Kiss1* cells are organized early in development by perinatal hormones and are unaffected by the activational effects of adult hormones. Representative photomicrographs showing *Kiss1* mRNA-expressing cells in the AVPV of adult male, female, and neonatally androgenized female rats. All animals were treated in adulthood with E$_2$. *3V* third ventricle; *Cast* castrated; *OVX* ovariectomized. Modified from Kauffman AS, Gottsch ML, Roa J, Byquist AC, Crown A, Clifton DK, Hoffman GE, Steiner RA, Tena-Sempere M 2007 Sexual differentiation of Kiss1 gene expression in the brain of the rat. Endocrinology 148:1774–1783. With permission from The Endocrine Society

In addition to developmental changes in AVPV/PeN kisspeptin cell number, the development of kisspeptin fibers apposing GnRH neurons has been reported to change over development [28]. Detectable kisspeptin fiber appositions to GnRH neurons were absent in female mice younger than PND 25, but such appositions were present on PND 25 and further increased in numbers by PND 31 [28]. The authors postulated that many of these GnRH-apposing kisspeptin fibers were derived from the AVPV/PeN region, though this was not experimentally determined. However, if this assumption is true, then the appearance and subsequent pubertal increase in kisspeptin-GnRH appositions may be a function of the known increase in kisspeptin synthesis in the AVPV/PeN during this age, increasing the visibility and detection of the fibers. Alternatively, it is also possible that a physical increase in the degree of kisspeptin fiber innervation of GnRH neurons occurs peripubertally, perhaps affecting GnRH activation around puberty onset.

Sexual Differentiation of AVPV/PeN Kiss1 Expression During Development

A few years ago, it was discovered that *Kiss1*-expressing neurons in the adult rat AVPV/PeN are sexually differentiated, with adult females possessing more *Kiss1* mRNA (and detectable *Kiss1* cells) than males (Fig. 11.2) [36]. Similar observations have now been reported for *Kiss1* mRNA levels in mice [40] and kisspeptin protein levels in mice and rats [28, 66]. No documented *Kiss1* sex differences have yet been reported in the POA of the ewe or monkey, although a sex difference is observed in the ARC *Kiss1* population of sheep [67] and is discussed later in further detail.

Although activational effects of sex steroids play a role in transiently increasing AVPV/PeN *Kiss1* expression levels in adulthood [22], the adult sex steroid milieu does not account for the observed sex differences in AVPV/PeN *Kiss1* expression [36]. This is evidenced by the fact that male and female rats that are gonadectomized as adults and treated with identical E_2 levels still display sexually dimorphic *Kiss1* expression in the AVPV/PeN [36, 42]. In fact, the AVPV/PeN *Kiss1* sex difference appears to be permanently organized by sex steroid signaling early in postnatal development. In the postnatal "critical period," which is typically the first week of postnatal life in rodents, males normally secrete elevated gonadal T, whereas females secrete little sex steroids at this time. Experiments manipulating the postnatal sex steroid milieu of rodents support the model that the presence of elevated levels of postnatal sex steroids determines whether many sexually dimorphic traits develop to be male-like in adulthood. Thus, in newborn males, elevated sex steroids act to organize neural circuits to permanently develop a male-like phenotype [68]. In contrast, newborn females are not exposed to sufficient levels of sex steroids, and therefore their brains permanently develop to be female-like [32, 37]. Supporting this "organizational" model of sexual differentiation, castration of newborn males, to remove high postnatal T, results in the permanent development of feminized neural populations. Conversely, sex steroid treatment to newborn females, mimicking elevated T secretion in postnatal males, results in the permanent development of masculinized brain circuitry (reviewed in ref. [6]).

A number of studies have determined that the AVPV/PeN *Kiss1* system is organized postnatally by sex steroids. For example, castrating male rats at birth causes a permanent feminization of the developing AVPV/PeN *Kiss1* system (Fig. 11.3) [66]. Conversely, neonatal female rats treated once with T or E_2 exhibit a permanent reduction of *Kiss1*- or kisspeptin-expressing cells in the AVPV/PeN in adulthood, similar to what is exhibited in normal males (Figs. 11.2 and 11.3) [36, 66, 69]. The fact that postnatal E_2 treatment can, like T, permanently alter the development of the AVPV/PeN *Kiss1* system suggests that postnatal masculinization of this system is likely mediated via aromatization of T to E_2. In rats, the effects of postnatal E_2 on *Kiss1* sexual differentiation are likely mediated by ERα and not ERβ, because neonatal treatment with the ERα agonist, PPT, caused a reduction in female AVPV/PeN kisspeptin levels [70], while neonatal administration of the ERβ agonist, DPN, had no significant effect on adulthood *Kiss1* levels [71]. In these experiments, however, males and females were not compared, and further studies are needed to determine if PPT can completely masculinize the female *Kiss1* AVPV/PeN population to male levels. Additionally, the reduction of AVPV/PeN *Kiss1* expression in female rats that were treated neonatally with sex steroids correlates with the inability of these females to generate an E_2-mediated LH surge as adults [66], linking the sexually dimorphic AVPV/PeN *Kiss1* system and the sexually dimorphic LH surge event.

As discussed earlier, *Kiss1* mRNA is expressed in the AVPV/PeN as early as PND 10 in mice of both sexes. However, there are no sex differences in AVPV/PeN *Kiss1* neuron number or *Kiss1* mRNA levels/neuron at this age, even though both of these parameters are well-established sex differences in adulthood [40]. The sex difference in *Kiss1* cell number, however, is evident by PND 12 and becomes even
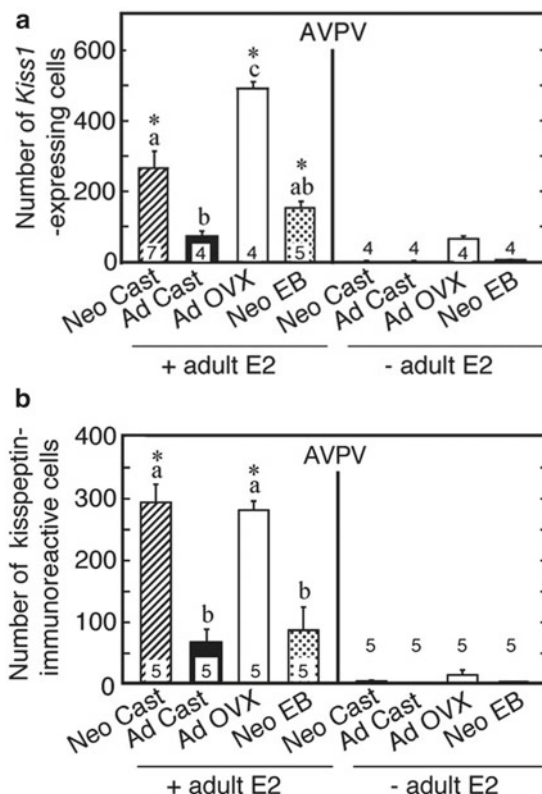
Fig. 11.3 *Kiss1* mRNA and kisspeptin expression in the AVPV of adult rats. Mean level of *Kiss1* mRNA expression (**a**) and kisspeptin immunoreactivity (**b**) in the AVPV of male and female rats that were either neonatally castrated (Neo Cast), neonatally treated with estradiol benzoate (Neo EB), or untreated. All animals were gonadectomized and treated with E_2 or without E_2 as adults to equalize hormone levels. Values with same letters are not significantly different within the group with the same adult E_2 treatment. Values with asterisks are significantly different from corresponding animals without adult E_2 treatment. The number in or on each column indicates the number of animals used. Values are means ± SEM. Modified from Homma T, Sakakibara M, Yamada S, Kinoshita M, Iwata K, Tomikawa J, Kanazawa T, Matsui H, Takatsu Y, Ohtaki T, Matsumoto H, Uenoyama Y, Maeda K-i, Tsukamura H 2009 Significance of Neonatal Testicular Sex Steroids to Defeminize Anteroventral Periventricular Kisspeptin Neurons and the GnRH/LH Surge System in Male Rats. Biology of Reproduction 81:1216–1225. With permission from The Society for the Study of Reproduction

more robust on PND 14 and 16 (Fig. 11.1) [40]. Semi-quantitative analysis of *Kiss1* mRNA levels/cell revealed a significant sex difference only beginning around PND 16 [40]. Thus, AVPV/PeN *Kiss1* mRNA expression first arises at the same time in both sexes (~PND 10), but the sex difference in both cell number and mRNA expression/cell takes several more days to develop, at least as assessed via ISH in mice. As mentioned above, in other rodent studies utilizing IHC, neither sex displays
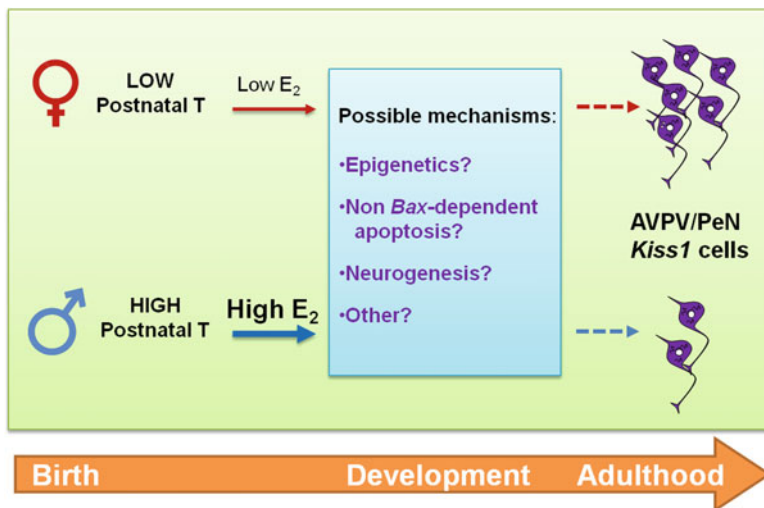
Fig. 11.4 Schematic of the development of sexually dimorphic AVPV/PeN *Kiss1* neurons. Males secrete elevated testosterone (T) at birth, while newborn females secrete negligible levels of sex steroids. Postnatal T converted to estradiol influences the development of neural circuits, leading to their masculinization in adulthood. AVPV/PeN *Kiss1* neurons are more abundant in adult females than males. It is currently unknown how the perinatal sex steroid milieu organizes the sexual differentiation of *Kiss1* neurons, although the AVPV as a whole is differentiated through *Bax*-dependent apoptotic mechanisms. However, the sexual differentiation of *Kiss1* neurons is likely not due to *Bax*-dependent apoptosis [40], though other apoptotic mechanisms have not been ruled out. Current data indicate that the *Kiss1* gene may be more transcriptionally active in females, pointing to epigenetic alterations as a putative mechanism

detectable kisspeptin-ir cells in the AVPV/PeN on or before PND 10 [28, 64]. Currently, kisspeptin protein levels have not been directly compared between sexes from PND 11 through PND 21, but the sex difference in kisspeptin-ir cell number was readily apparent in mice on PND 25 [28] and in rats on PND 21 [64].

Possible Mechanisms of Steroid-Mediated Sexual Differentiation of AVPV/PeN Kiss1 Neurons

Sexual differentiation of the AVPV/Pen *Kiss1* system is dependent on the postnatal sex steroid milieu, but it is unclear exactly how E_2 (aromatized from T) directs this developmental process. Several sex steroid-dependent mechanisms, such as differential neurogenesis, migration, epigenetics, and apoptosis, have been implicated in the sexual differentiation and development of other neuronal populations (Fig. 11.4) [32, 72–74]. E_2, for example, can promote neurogenesis in the olfactory bulb and dentate gyrus of the adult rat hippocampus, leading to more newly formed neurons in females [32].

Likewise, in the developing rat hippocampus, higher levels of postnatal sex steroids in males increase the number of new cells, leading to more neurons present in males than in females [75]. However, because the AVPV/PeN as a whole does not undergo differential neurogenesis during the postnatal critical period [32], differential neurogenesis between males and females may not be a major contributor to the sexual differentiation of specific subpopulations within the AVPV/PeN, such as the kisspeptin neurons. However, this assertion has not yet been directly tested.

One of the primary mechanisms implicated in the sexual differentiation of a number of sexually dimorphic brain populations is programmed cell death (apoptosis) [76–78]. In fact, in rodents, sex differences in the overall size and total cell number of the AVPV region, as well as other brain regions such as the BNST, are induced by apoptosis. Most of these apoptosis-induced sex differences are dependent on the pro-apoptotic gene, *Bax* [77–79]. BAX is a protein located primarily in the cytosol in a healthy cell. In response to cell death signals, BAX translocates to the mitochondria where it precipitates the release of cytochrome c, thereby triggering caspase pathways that culminate in cell death [80]. Interestingly, in the developing rat AVPV, postnatal males have higher *Bax* expression than postnatal females, which possibly initiates more cell death in the former sex [81]. Higher *Bax* expression in postnatal males than females therefore correlates with the presence of fewer AVPV cells in adult males. The sexually dimorphic postnatal *Bax* levels also coincide with higher sex steroid levels in postnatal males than females, suggesting that sex steroids might affect postnatal *Bax* expression [81]. Supporting this prediction, E_2 treatment of neonatal female rats increases the number of apoptotic AVPV neurons [82]. Importantly, a recent study determined that the sex difference in total number of AVPV neurons is eliminated in *Bax* knockout mice [76]. Thus, total cell number in the AVPV is sexually differentiated via *Bax*-dependent apoptotic mechanisms. Despite these findings, the sexual differentiation of *Kiss1* neurons in the AVPV/PeN is surprisingly unaltered in *Bax* knockout mice [40]. When *Kiss1* cell numbers are compared between adult male and female *Bax* KO mice, the *Kiss1* sex difference was still incredibly robust [40]. Thus, the *Kiss1* population is sexually differentiated either by other apoptotic pathways, such as tumor necrosis α-dependent or -independent mechanisms (as may be the case for AVPV GABA-ergic neurons [78]) or, by non-apoptosis-related mechanisms (Fig. 11.4).

It is likely that the sexual differentiation of AVPV/PeN *Kiss1* cells is not induced by mechanisms that affect the physical existence of cells (like apoptosis). Rather, sex differences in *Kiss1* cell number may be induced by developmental mechanisms affecting transcriptional activity of the *Kiss1* gene (Fig. 11.4) [5, 83]. In fact, epigenetic changes, such as histone modifications and DNA methylation, precipitated by postnatal sex steroids are emerging as critical contributors to alterations in neuronal cell number and gene expression between the sexes [73, 84–88]. Histones are proteins that allow for the packaging of DNA into chromatin. When histones are modified, such as by acetylation, transcriptional activity is altered [89]. Histone acetylation, which is generally associated with increased transcriptional activity, has recently been implicated in the sex steroid-induced sexual differentiation of the BNST [73]. For example, inhibiting histone deacetylase (HDAC) during the early

postnatal period in mice blocks the sexual differentiation of the size of the BNST and also alters sexually dimorphic vasopressin fiber projections [73, 88]. Postnatal HDAC inhibition also alters the sexual differentiation of male sexual behavior in rats [87]. It is likely that these postnatal alterations in HDAC activity directly or indirectly affected apoptosis in the BNST, as sex differences in this region are known to be governed specifically by *Bax*-dependent apoptosis [77]. Other sexual differentiation studies have addressed the role of DNA methylation, which occurs at CpG sites or on CpG islands in a gene or its promoter, and which is generally associated with the repression of gene expression. Studies investigating the involvement of DNA methylation in neural sex differences have found that DNA methylation levels in the hypothalamus correlate with sexually differentiated expression of sex steroid receptor genes in this region [85, 86]. In addition, the expression of DNA methyl transferase 3a (DNMT3a) in newborn rats was found to be sexually dimorphic in the amygdala, which is a known sexually dimorphic brain region [90].

With all the recent evidence implicating the involvement of epigenetics in the sexual differentiation of various brain parameters, we investigated whether either histone deacetylation and/or DNA methylation contributes to the AVPV/PeN *Kiss1* sex difference [83]. We pharmacologically blocked histone deacetylation during the postnatal period by administering an HDAC inhibitor, valproic acid (VPA), or vehicle to mice on PND 1 and PND 2 and then analyzed AVPV/PeN *Kiss1* expression in adulthood. This postnatal HDAC inhibitor treatment significantly increased the number of detectable *Kiss1* cells in the adult AVPV in each sex. However, the sex difference in *Kiss1* expression was not eliminated, indicating that histone acetylation is not a key process for inducing the *Kiss1* sex difference [83]. Although the sex difference was still robust, the fact that overall *Kiss1* levels were higher in mice treated with HDAC inhibitor suggests that the level of histone H3 acetylation during the critical period may be involved in modulating the development of *Kiss1* neurons in the AVPV.

Interestingly, we found significant sex differences in the CpG methylation status of the AVPV/PeN *Kiss1* gene, predominantly in the putative promoter region (Fig. 11.5). In all cases, these sexually dimorphic *Kiss1* CpG sites were more methylated in females than males. Methylation of CpG sites can have multiple modes of affecting gene activity. We tested if methyl-CpG binding protein-2 (MeCP2) was involved in the sex difference by assessing AVPV/PeN *Kiss1* levels in male and female *Mecp2* mutant mice. The AVPV/PeN sex difference was not eliminated in *Mecp2* mutant mice, suggesting that if DNA methylation influences the AVPV/PeN *Kiss1* sex difference, it likely does so via non-Mecp2 mechanisms, possibly by blocking the binding of transcriptional repressors [83]. Although these experiments increase our knowledge about the involvement (or lack of involvement) of several epigenetic processes in the development of the AVPV/PeN *Kiss1* sex difference, more work is needed to elucidate the exact extent that certain processes, like DNA methylation, are involved. In fact, the AVPV/PeN *Kiss1* sex difference may be induced by several epigenetic processes affected by the postnatal sex steroid milieu, causing a silencing of the *Kiss1* gene in males while simultaneously allowing increased transcriptional activity in females.
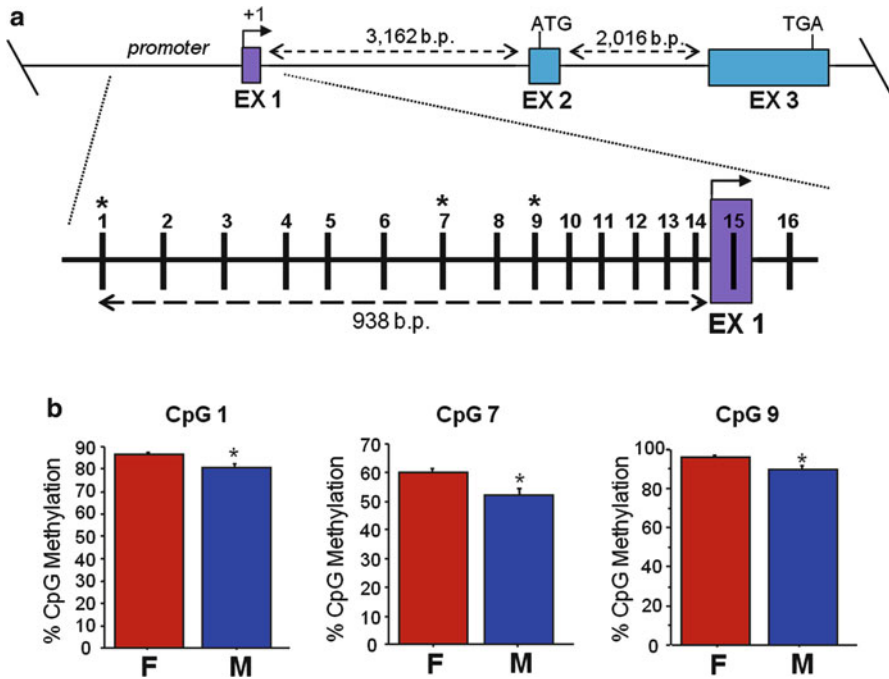
Fig. 11.5 CpG methylation analysis of the putative murine *Kiss1* promoter region in the AVPV/PeN. (**a**) Map of pyrosequenced CpG sites in the putative *Kiss1* promoter region using bisulfite-treated DNA derived from AVPV/PeN micropunches of adult males and females that were E_2-treated for 1 week before sacrifice. (**b**) Mean percentage of methylation of sexually dimorphic CpGs (CpG 1, 7, 9) in the *Kiss1* promoter. *Significantly different than females ($P < 0.05$). Modified from Semaan SJ, Dhamija S, Kim J, Ku EC, Kauffman AS 2012 Assessment of epigenetic contributions to sexually dimorphic kiss1 expression in the anteroventral periventricular nucleus of mice. Endocrinology 153:1875–1886. With permission from The Endocrine Society

Regulation of AVPV/PeN **Kiss1** *Neurons by Gonadal and Non-gonadal Factors During Peripubertal Development*

The steady developmental increase in kisspeptin cell number in the female AVPV/PeN is likely dependent on the presence of ovarian steroids. As mentioned previously, *Kiss1* gene expression in the AVPV/PeN increases markedly over the pubertal transition [12, 28, 62, 63, 91]. In adult animals, *Kiss1* expression levels in the AVPV/PeN are transiently increased by elevations in the sex steroid milieu and, conversely, decreased by removal of sex steroids [22, 36]. Therefore, it is likely that the observed developmental increase in *Kiss1* gene expression during puberty is caused by increased ovarian sex steroid secretion at this time.

Several studies have addressed the effects of sex steroids or their receptors on the developmental increase of kisspeptin cell number in the AVPV/PeN. In one study,

female mice were either ovariectomized (OVX) or sham treated on PND 15 and killed on either PND 30 or PND 60. Mice that were OVX on PND 15 had dramatically reduced levels of kisspeptin in the AVPV/PeN later in adulthood, suggesting that the primary cause of the developmental kisspeptin increase is due to ovarian sex steroid secretion [62]. This was supported by the observation that estrogen replacement in OVX animals from either PND 15–30 or PND 22–30 rescues kisspeptin expression when examined at PND 30 [62]. Thus, kisspeptin neurons are sensitive to ovarian steroids peri-pubertally, which is not surprising given their robust regulation by sex steroids in adulthood. It is currently unknown if kisspeptin neurons, however, are responsive to E_2 at the first time of visible protein expression (PND 15) or mRNA expression (PND 10), or if OVX at earlier time periods has a more permanent effect on the development of kisspeptin expression.

Several studies have used aromatase knockout (ArKO) mice to examine the effects of E_2 signaling on kisspeptin neuron development. One study reported a complete elimination of kisspeptin expression in the AVPV/PeN of adult female ArKO mice [62]. However, E_2 was not replaced in these mice prior to sacrifice, and it was therefore unclear if the absence of AVPV/PeN kisspeptin cells in ArKO females mirrored a chronically OVX condition (since removal of E_2 via OVX in adulthood reduces AVPV/PeN kisspeptin synthesis). More recently, another study looked at kisspeptin cells in ArKO mice that were given E_2 in adulthood. This study found that AVPV/PeN kisspeptin cells were in fact present in adult ArKO mice after E_2-treatment. However, surprisingly, the sex difference in kisspeptin cell number was eliminated in these E_2-treated ArKO mice [92]. But, instead of ArKO males exhibiting high kisspeptin levels similar to that of wild-type (WT) females, as would be predicted due to the lack of E_2-signaling in these males during the postnatal critical period, AVPV/PeN kisspeptin cell number was instead intermediate in level in ArKOs of both sexes, being significantly lower than in normal WT females and significantly higher than in normal WT males [92]. This finding suggests that E_2 during development may normally actively contribute to complete feminization of the AVPV/PeN kisspeptin system in females, although when and how this would occur is unknown.

A similar story has emerged concerning the development of the AVPV/PeN kisspeptin system in hypogonadal (*hpg*) mice. Hypogonadal mice possess a deletion in the *Gnrh* gene and therefore do not secrete GnRH or gonadal sex steroids [93]. In female *hpg* mice, AVPV/PeN kisspeptin-ir cell number during development never reaches the level of WT females and is similar to that of *hpg* males (i.e., the normal kisspeptin sex difference is absent in *hpg* mice) [94]. Similarly, *Kiss1* mRNA expression in pubertal *hpg* females is decreased compared to that of WT females, and sexually dimorphic *Kiss1* expression is eliminated in *hpg* mice [94]. Hormone replacement was not compared in pubertal *hpg* females and males in this particular study and may be critical to fully interpret the results. Interestingly though, 1 week of E_2 replacement in adult *hpg* females did *not* increase AVPV/PeN kisspeptin protein levels to WT female levels, suggesting that gonadal sex steroids may be required at some time during development in order for AVPV/PeN kisspeptin expression to fully mature [94].

Finally, in a different study, ERα was specifically ablated in kisspeptin neurons using cre-lox technology (generating "KERKO" mice) in order to test the role of ERα signaling in kisspeptin neurons [10]. In this model, AVPV/PeN kisspeptin-ir was significantly diminished in adult KERKO females compared to WT females, further implicating the involvement of E_2 and ERα in the development of the AVPV/PeN kisspeptin population in females [10]. Likewise, a number of recent studies have implicated endocrine disruptors (which often mimic the effects of E_2) in affecting the development of AVPV/PeN *Kiss1* gene expression [69, 95, 96], a topic discussed in detail in Chap. 21.

The Development of the ARC *Kiss1* Population

Ontogeny of Kiss1 Expression in the ARC

The second major population of kisspeptin neurons in the hypothalamus is located in the ARC, which is equivalent to the INF in humans [7, 14, 21, 62]. The ARC is the most consistently detected kisspeptin population in mammalian species, although its precise distribution and role may vary from species to species [15, 16, 19, 21, 22]. In general, the developmental pattern of *Kiss1* expression in the ARC, and the specific factors regulating the development of this neuronal population, are not completely understood and are less well-characterized than the AVPV/PeN.

Unlike in the AVPV/PeN, *Kiss1* is expressed in the rodent ARC during embryonic development. Analysis of both kisspeptin-ir and *Kiss1* mRNA (via qPCR) in embryonic rats determined that expression in both sexes begins around E14.5 and increases by E18.5, with a sharp drop in levels just prior to birth [61]. Additionally, double-labeling of kisspeptin-ir/BrdU-ir demonstrated that kisspeptin cell neurogenesis in the ARC begins around E12.5 and peaks several days later [97]. In other recent studies, when crossed to reporter mice that permanently mark all cells that ever expressed *Kiss1* at any point in development, two *Kiss1*-Cre transgenic mouse lines were reported to have "extra" cells in the greater ARC region [9, 11]. One of these reports quantified these "extra" *Kiss1* cells to comprise ~25 % of the total cells that have ever expressed *Kiss1* in the ARC region at some point during development or adulthood [11]. This suggests that the pattern of ARC kisspeptin expression may differ significantly between early developmental and older ages, but it is not clear at what developmental age these "extra" kisspeptin cells become undetectable. Additionally, both male and female adult mice with impaired BAX-mediated apoptosis display "extra" *Kiss1* cells in the ARC compared to adult WT mice, particularly in the male, suggesting that the number of ARC *Kiss1* neurons is also regulated in early development by apoptosis [40]. The extra *Kiss1* cells in the ARC of both the *Bax* KO and the *Kiss1*-Cre mice show a similar expression pattern that is located dorsally in relation to the normal ARC *Kiss1* population (Fig. 11.6). The ontogeny, developmental pattern, and role, if any, of these extra kisspeptin neurons remain unknown.
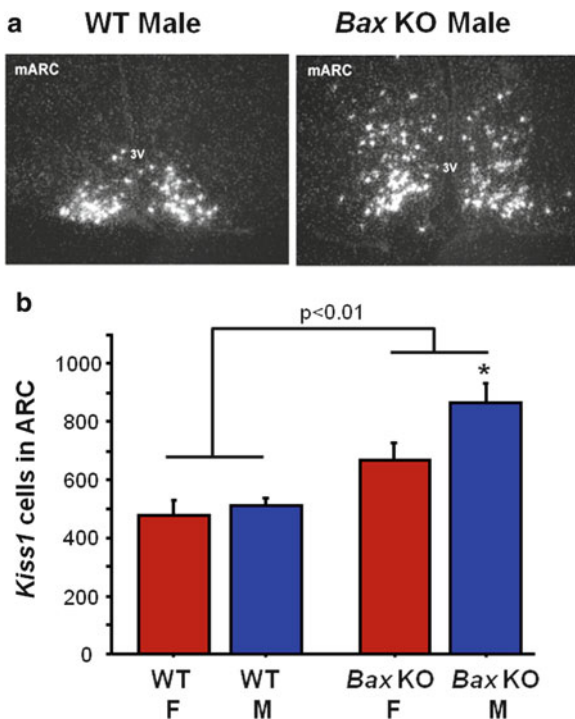
Fig. 11.6 Regulation of *Kiss1* expression in the ARC by apoptosis. (**a**) *Kiss1* expression in the ARC of GDX adult WT and *Bax* KO male mice show extra *Kiss1* cells in the ARC, suggesting that ARC *Kiss1* neurons are regulated in early development by apoptosis. The "extra" *Kiss1* cells in the ARC show an expression pattern that is located dorsally in relation to the normal ARC *Kiss1* population typically found in WT mice. *3V* third ventricle; *mARC* medial ARC. (**b**) Mean number of *Kiss1* cells in the ARC of adult female (F) and male (M) *Bax* KO and WT mice that were GDX for 9 day before sacrifice. *Kiss1* gene expression in the ARC was significantly higher in *Bax* KO mice than WT mice (*P*<0.01), especially in males. *Significantly different from female mice of same genotype. (**a**, **b**) Modified from Semaan SJ, Murray EK, Poling MC, Dhamija S, Forger NG, Kauffman AS 2010 BAX-dependent and BAX-independent regulation of Kiss1 neuron development in mice. Endocrinology 151:5807–5817. With permission from The Endocrine Society

In rats, initial studies detected *Kiss1* expression in the whole hypothalamus on PND 1 using RT-PCR, but it was not determined which specific nuclei (ARC, AVPV/PeN, etc.) were responsible for this neonatal *Kiss1* expression [12]. Based on the AVPV/PeN developmental time-course discussed earlier, it is unlikely that the source of this PND 1 *Kiss1* expression is the AVPV/PeN. Rather, most, if not all, of the hypothalamic *Kiss1* detected in PND 1 rats was probably from the ARC population. Several recent studies have now provided supporting evidence for this likelihood. Using ISH, *Kiss1* expression in the ARC was readily detected at PND 1 in mice [98] and rats [47, 99], as well as in rats in another study at the first collected
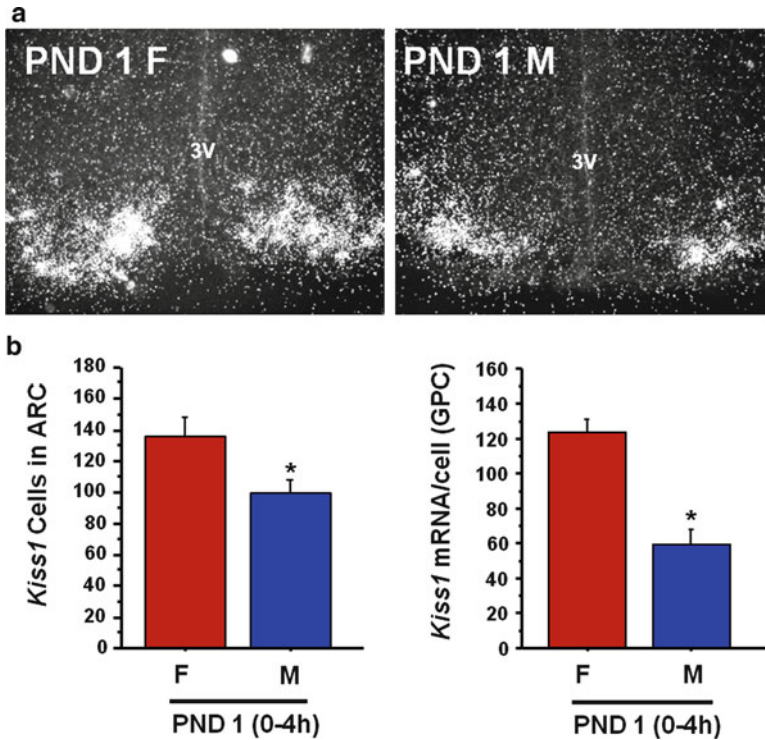
Fig. 11.7 *Kiss1* gene expression in the murine ARC on PND 1. (**a**) Representative photomicrographs for *Kiss1* expression in newborn females (F) and males (M) that were 0–4 h old. (**b**) The mean number of *Kiss1* neurons in the ARC, as well as the relative level of *Kiss1* mRNA/cell, for PND 1 male and female mice that were 0–4 h old. Newborn males had significantly fewer ARC *Kiss1* cells and lower mRNA per cell than newborn females ($P < 0.05$). *3V* third ventricle; *GPC* grains per cell. Modified from Poling MC, Kauffman AS 2012 Sexually dimorphic testosterone secretion in prenatal and neonatal mice is independent of kisspeptin-kiss1r and GnRH signaling. Endocrinology 153:782–793. With permission from The Endocrine Society

age of PND 4 [64]. Poling and Kauffman provided greater temporal resolution of expression in PND 1 mice: they found notable *Kiss1* expression in both sexes at both 0–4 h and 16–20 h after birth (Fig. 11.7), without a significant change in *Kiss1* levels between these time-points [98]. Parenthetically, NKB, which is co-expressed in ARC *Kiss1* neurons in adulthood, was also readily expressed in the ARC of both sexes on PND 1 [98]. One rat study noted an increase in *Kiss1* expression in the ARC between PND 1 and 3 in both sexes [47], with an additional increase at PND 8 in females only. Another study noted similar but non-significant trends in both males and females [64].

The role of *Kiss1* in the ARC during early neonatal life is unknown. Because kisspeptin signaling regulates the pubertal and adult reproductive axis, it is possible that kisspeptin plays a similar role earlier in neonatal development. However, since the reproductive axis is typically quiescent at this time, it is not clear what functional

significance neonatal kisspeptin signaling would have. The kisspeptin receptor (Kiss1r), also known as Gpr54, is already present in some GnRH neurons at birth and begins appearing in other areas of the brain by PND 6 [100], suggesting the possibility of a functional kisspeptin neuronal network at early postnatal ages. It is possible that neonatal kisspeptin regulates gonadal T secretion, causing the elevation in T that is seen in newborn males but not females [98, 101–105]. Recent evidence has shown that, unlike their WT littermates, newborn *Kiss1r* KO and *hpg* mice both have undetectable FSH and LH, demonstrating that kisspeptin and GnRH signaling are each essential for neonatal gonadotropin secretion [98]. However, the same study found that newborn male *Kiss1r* KO and *hpg* mice both had elevated serum T levels at birth that were similar to their WT male littermates, indicating that neither GnRH nor kisspeptin signaling is required for neonatal T secretion. These results suggest that, while kisspeptin regulates gonadotropin secretion in neonatal rodents, the neonatal T surge in males is independent of regulation by gonadotropins and kisspeptin.

Prepubertal and Pubertal Changes in Kiss1 Expression in the ARC

Changes in peripubertal *Kiss1* expression have been examined in the ARC of rodents, but not as thoroughly as in the AVPV/PeN, and the results are not entirely consistent between studies. Early studies in the whole hypothalamus of the rat showed that *Kiss1* levels increased between PND 1–20 and PND 30 in females, and between PND 1–30 and PND 45 in males, followed by decreased expression in adulthood. These studies demonstrated that increases in *Kiss1* levels correlate with pubertal timing in males and females [12]. However, since the whole hypothalamus was analyzed, these pubertal changes in *Kiss1* expression could be due to changes in AVPV/PeN and/or ARC *Kiss1* levels. To get better spatial resolution of the developmental changes in kisspeptin/*Kiss1*, more recent studies in rodents have used ISH or IHC. Studies using IHC detected kisspeptin-ir in the ARC of juvenile, prepubertal, and peripubertal mice of both sexes, although this kisspeptin-ir was not quantified [28, 94]. An early study using ISH found similar numbers of ARC *Kiss1* cells in PND 18 vs. adult male mice [106], suggesting no difference in ARC *Kiss1* levels between juvenile and adult stages; however, peripubertal ages in between PND 18 and adulthood were not analyzed. This finding was mirrored by another recent ISH study in female rats that reported no difference in ARC *Kiss1* levels between juvenile and late-pubertal animals [107]. Likewise, in male and female mice, *Kiss1* expression in the ARC, determined with qPCR, remained virtually unchanged during several stages of postnatal and pubertal development (PND 11–61), and no significant sex differences were noted at any of the ages studied [94].

In contrast to the above studies showing no major peripubertal changes in ARC *Kiss1* levels, additional experiments demonstrated increases in ARC *Kiss1* expression around the time of early puberty in both female [91] and male rats [108], suggesting

that increased ARC *Kiss1* expression may be involved in puberty onset. More recently, initial elevations in ARC *Kiss1* expression in neonatal/juvenile female rats were reportedly followed by a decrease in *Kiss1* levels by 3 weeks of age [47, 64], perhaps concurrent with increased sex steroid production (and negative feedback) at this time. One study also detected another increase in ARC *Kiss1* of females between weeks 5 and 8 [64]. Several studies reported that ARC *Kiss1* levels in male rats remained essentially unchanged during the first several weeks of life (up to around 4 weeks of age) [47, 64], followed in one case by a small temporary increase in ARC *Kiss1* levels around week 5 [64]. Overall, the current data on rodent ARC *Kiss1* expression during development, especially at prepubertal and pubertal stages, is incomplete and fairly inconsistent. Thus, full characterization of *Kiss1* expression in the ARC during development and puberty is still needed in rodents, as well as other species (see below).

Very few studies on developmental changes in ARC kisspeptin neurons have been conducted in non-rodent species. One study using agonadal male and intact female monkeys described an increase in *Kiss1* expression in the ARC during puberty [25]. In peripubertal female lambs with controlled sex steroid levels, a non-significant increase in ARC *Kiss1* cell number was seen over time when comparing weeks 25, 30, and 35. When analyzed specifically in lambs that showed increased LH pulse frequency, the number of *Kiss1* cells in the middle ARC was found to have a significant linear increase between weeks 25, 30, and 35, possibly implicating the middle ARC in puberty onset [65]. However, another recent report in sheep found that kisspeptin-ir cell number increased in the caudal ARC of females between the prepubertal and postpubertal periods [109]. Thus, the specific location(s) within the ovine ARC where *Kiss1* might increase during puberty occurs still needs to be resolved. Collectively, these initial studies in monkeys and sheep suggest that increased levels of *Kiss1* in the ARC of pubertal animals correlate with increased activation of the reproductive axis, but these studies surveyed a low resolution of ages over development, necessitating additional data on this subject.

Sex Differences in ARC Kisspeptin Neurons During Development

Most data agree that, unlike the sexually dimorphic AVPV/PeN [31, 47, 70], the number of ARC *Kiss1* or kisspeptin cells and the quantity of *Kiss1* mRNA per cell are not majorly different between sexes in adult rodents, especially when circulating sex steroid levels are equalized between the sexes [28, 36, 40, 42, 44, 66, 94]. In the few cases where an ARC *Kiss1* sex difference was observed, it was typically in gonadally intact adult animals that have unequal sex steroid levels between the sexes. For example, Adachi et al. [42] reported that intact adult male and female rats had equivalent ARC *Kiss1* expression at all stages of the female estrous cycle except for at diestrous 2. Moreover, adult males and females exhibit similar increases in *Kiss1* levels in the ARC after gonadectomy (GDX) and similar inhibition of ARC *Kiss1* expression when treated equivalently with sex steroids [36, 40, 42, 44]. It should be noted that sex differences exist in the ARC in several other parameters,

such as the morphology of astroglia, the number of spine and somatic synapses [110–112], the expression of growth hormone-releasing hormone, and the axonal projections of NKB/Dyn neurons [113–115]. Additionally, the kisspeptin fibers visualized in the ARC by IHC are often denser in adult females, but it is likely that some of these fibers are derived from the sexually dimorphic AVPV/PeN kisspeptin population [28, 29, 62, 116]. The ARC population of *Kiss1* neurons in rodents is hypothesized to provide tonic stimulatory input to GnRH neurons and to relay negative feedback effects of sex steroids to the GnRH axis [36, 47, 117–119]. These processes occur in both sexes, which is consistent with the lack of a major sexual dimorphism in these neurons in adult rodents.

In contrast to adulthood, recent analysis of *Kiss1* expression in the ARC of rodents during development has revealed a different story regarding sexual dimorphism. Work in the *Bax* KO mouse suggests that male mice may initially have more ARC *Kiss1* neurons (or neurons that have the potential to express *Kiss1* later in development) than females prior to birth, which is later offset by a higher rate of perinatal apoptosis [40]. However, a recent report in the rat did not find sex differences in the number of kisspeptin-ir cells during prenatal development, but did not look at sex differences in *Kiss1* expression levels [97]. A recent study in mice found that newborn PND 1 pups exhibit sexual dimorphism in ARC *Kiss1* expression, with newborn females having significantly more *Kiss1* and *NKB* cells in the ARC, as well as higher cellular expression of these mRNAs, than newborn males [98]. Similarly, male rats also have lower ARC *Kiss1* levels than females at PND 3 [47] and PND 4 [64]. Studies using both ISH and IHC found that the number of *Kiss1* neurons in the ARC of rats is still sexually dimorphic between PND 5 and PND 11 [47, 64, 99, 120], with juvenile females having approximately 2–4 times as many ARC *Kiss1* (or kisspeptin) neurons than juvenile males of the same age. This ARC *Kiss1* sex difference begins to diminish with the approach of puberty; while males maintain a slight non-significant trend in increasing ARC *Kiss1* cell numbers from juvenile life through puberty, *Kiss1* expression in the ARC of females decreases around 3 weeks of age to reach levels similar to males [44, 47, 63].

The neonatal and juvenile sex differences in *Kiss1* expression in the rodent ARC may be a temporary sex difference due to sexual dimorphism in the circulating sex steroid milieu: higher levels of circulating sex steroids in young males may provide more negative feedback inhibition than in young females, resulting in lower ARC *Kiss1* expression in the males. This could certainly be the case in newborn animals, in which males secrete elevated T (to drive sexual differentiation) whereas females do not [98]. However, it is not entirely clear what normal sex steroid levels are in male and female rodents, especially mice, during each phase of neonatal and juvenile development, and most studies that have examined *Kiss1* expression early in development have not measured serum sex steroid levels. Moreover, it is also possible that non-steroidal mechanisms also influence the ARC *Kiss1* sexual dimorphism, as PND 1 male mice that are 16–20 h old (when circulating T levels are no longer different between the sexes) still display sex differences in ARC *Kiss1* levels [98]. Other findings support the role of sex steroid-independent factors in regulating *Kiss1*/*NKB* expression in the ARC of juvenile mice in a sexually dimorphic manner

[44] (discussed more later). Additionally, the functional significance, if any, of the sexual dimorphism in the ARC *Kiss1* neuronal population during neonatal and juvenile development is completely unknown, especially since the reproductive axis is essentially quiescent at this time.

Some non-rodent species, such as the sheep, display clear adulthood sex differences in kisspeptin cells in the ARC. Ewes display a greater number of *Kiss1* cells in the ARC than adult males [67]; this may be due to differences in the functional roles of *Kiss1* cells in the ARC/INF of rodents vs. sheep. However, as of yet, no direct comparison has been made between adult ewes and rams with equivalent sex steroid levels. Additionally, while prenatal androgen treatment reduced NKB and DYN expression in the ARC in adult sheep, this prenatal treatment did not alter ARC *Kiss1* expression, which remained sexually dimorphic [67]. Thus, there may be different critical periods for the sexual differentiation of these ARC genes, but it is also possible that the ovine ARC *Kiss1* system is not affected by prenatal sex steroids. Dissimilarities between species may be due to the difference in the role of the ARC; while ARC kisspeptin cells in rodents may mediate negative feedback regulation of GnRH secretion, which is not sexually dimorphic [6, 22, 46, 51, 118], the same kisspeptin population in sheep appears to be key for *both* negative feedback *and* positive feedback (i.e., the preovulatory GnRH surge) [51, 121–124]. Similar sex differences in kisspeptin expression have been recently reported in humans in the INF, as well as the POA, with females showing greater number of cells than males in each region [125]. However, note that in these human studies, regulatory factors that are known to alter kisspeptin levels, such as circulating sex steroids, metabolic status, stress hormones, and circadian status, were not controlled for and could differ dramatically between subjects, thereby confounding the results. Whether non-human primates display adult sex differences in ARC/INF kisspeptin levels is currently unexamined [126].

Regulation of ARC Kiss1 Neurons by Gonadal and Non-gonadal Factors During Development

As noted earlier, under normal developmental conditions, the rodent ARC displays a dense network of kisspeptin-ir fibers that likely originate from both the ARC and the AVPV/PeN. Supporting the hypothesis that some of these fibers are projections from the AVPV, data has shown that when gonadal hormones are removed during development, whether by GDX or genetically, as in the case of the *hpg* and KERKO mice, the number of kisspeptin fibers detected in the ARC are reduced in adulthood [10, 62, 94]. These data also suggest that gonadal steroids during development are necessary for either the physical development of kisspeptin fibers (organizational effects) or for the transient expression of kisspeptin protein within the cells and fibers (activational effects). Considering that the number of kisspeptin fibers in the ARC was restored in adult *hpg* mice treated with E_2, it seems likely that these kisspeptin fibers develop normally but only contain detectable levels of kisspeptin under the

correct adult sex steroidal milieu [94]. Thus, the reduction of these fibers in non-E$_2$ treated *hpg* (and KERKO) mice is likely due to an activational defect (i.e., absence of circulating sex steroids). However, caution must be used in interpreting fiber data since the origin of the fibers cannot always be determined.

The *hpg* and KERKO mouse models have recently been utilized to shed more light on the role of gonadal sex steroids in the development of ARC *Kiss1* neurons. First, kisspeptin cell bodies are more visible in the ARC of adult *hpg* mice than WT mice [94]. This difference in detectable kisspeptin cells is likely due to the lack of obstruction by dense kisspeptin fibers in *hpg* mice, as well as a possible increase in ARC kisspeptin synthesis in *hpg* mice lacking steroid negative feedback (Fig. 11.8). Since differences in ARC kisspeptin-ir expression and cell number were not easily measurable by IHC due to the obstructive overlaying fiber network, *Kiss1* mRNA levels were examined instead using ISH. Both the number of *Kiss1* neurons and the density of silver grains were increased in the ARC of adult *hpg* mice compared to WT littermates, suggesting an increased level of kisspeptin synthesis in *hpg* mice, perhaps due to less steroid negative feedback. Interestingly, at PND 11, only a small difference in *Kiss1* levels was present between WT and *hpg* littermates (as measured by RT-PCR), but by PND 31 this difference was much larger, with dramatically higher ARC *Kiss1* expression in *hpg* mice. This indicates that, at PND 11, there may be non-gonadal hormone factors inhibiting *Kiss1* in the ARC, but by PND 31 this gonadal-independent mechanism is reduced (or absent). These *hpg* data therefore suggest that changes in gonad-independent factors may induce ARC *Kiss1* expression around puberty.

The robust ARC *Kiss1* expression seen in cell bodies of adult *hpg* and ArKO mice may be similar to the hypertrophied *Kiss1* neurons seen in postmenopausal women [43, 127]; in all cases, the elevated *Kiss1* levels may reflect a lack of negative feedback of sex steroids. Finally, female KERKO mice show decreased kisspeptin-ir fibers in the ARC at PND 16, 26, and 35, similar to *hpg* mice [10]. Surprisingly, unlike *hpg* mice, adult KERKO mice did not display the high-expressing kisspeptin-ir cell bodies in the ARC, even though the same kisspeptin antibody was used in both studies. However, when mRNA was analyzed by qPCR in the mediobasal hypothalamus (which includes the ARC), *Kiss1* levels in KERKO mice were in fact increased relative to WT levels, similar to data from *hpg* mice. This unexpected difference between protein and mRNA levels in the KERKO mice has not yet been explained.

Recent work has shown that during the prepubertal period of mice, the *Kiss1* response to GDX is sexually dimorphic. While prepubertal females display elevated *Kiss1* expression in the ARC, along with increased LH levels, several days after GDX, prepubertal male mice do not (Fig. 11.9). Instead, if prepubertal male mice are GDX at PND 14, no increase is seen in ARC *Kiss1* cell number or serum LH levels when measured 2–4 days later (PND 16–18) [44]. Because this prepubertal sex difference is only observable after the removal of gonadal hormones (there is no sex differences in ARC *Kiss1* cell number at this age in gonadally intact mice), these results suggest a sexually dimorphic difference in the *regulation* of ARC *Kiss1* cells at this point in development: *Kiss1*/LH levels in PND 16–18 females appear to
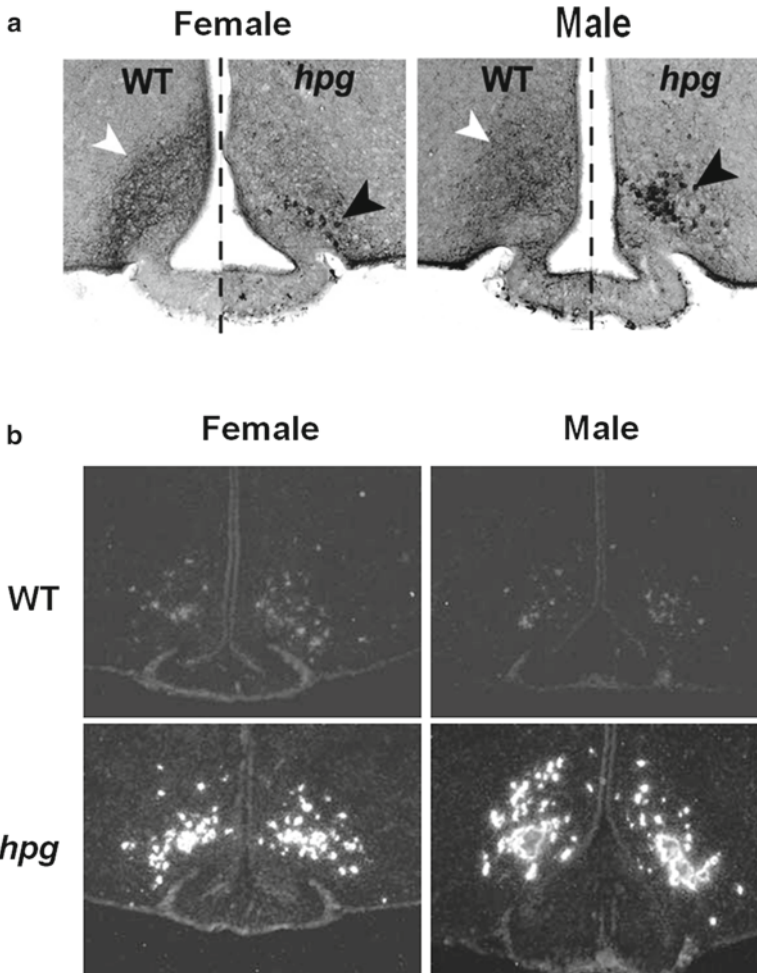
Fig. 11.8 Kisspeptin and *Kiss1* expression are increased in the ARC of female and male *hpg* mice. (**a**) Kisspeptin staining pattern in the ARC of both female and male WT mice (*left* of *dashed line*) is dominated by densely stained fibers that obscure kisspeptin-positive cell bodies (*white arrowheads*). In *hpg* mice (*right* of *dashed line*), kisspeptin immunoreactivity shows reduced fiber staining and increased clusters of large, darkly stained cell bodies (*black arrowheads*). (**b**) Representative dark-field images of silver grain *Kiss1* mRNA signal as measured by ISH in the ARC of pubertal-aged WT and *hpg* females (30 days) and males (45 days). Scale bar = 200 mm. Figure modified from Gill JC, Wang O, Kakar S, Martinelli E, Carroll RS, Kaiser UB 2010 Reproductive hormone-dependent and -independent contributions to developmental changes in kisspeptin in GnRH-deficient hypogonadal mice. PLoS One 5:e11911

be controlled primarily by gonadal sex steroid feedback, whereas PND 16–18 males have an additional mechanism of regulation of their ARC *Kiss1* neurons by unknown non-gonadal factors. Whether this divergence between the sexes is due to a unique, male-specific mechanism not present in similarly aged females or to sex differences
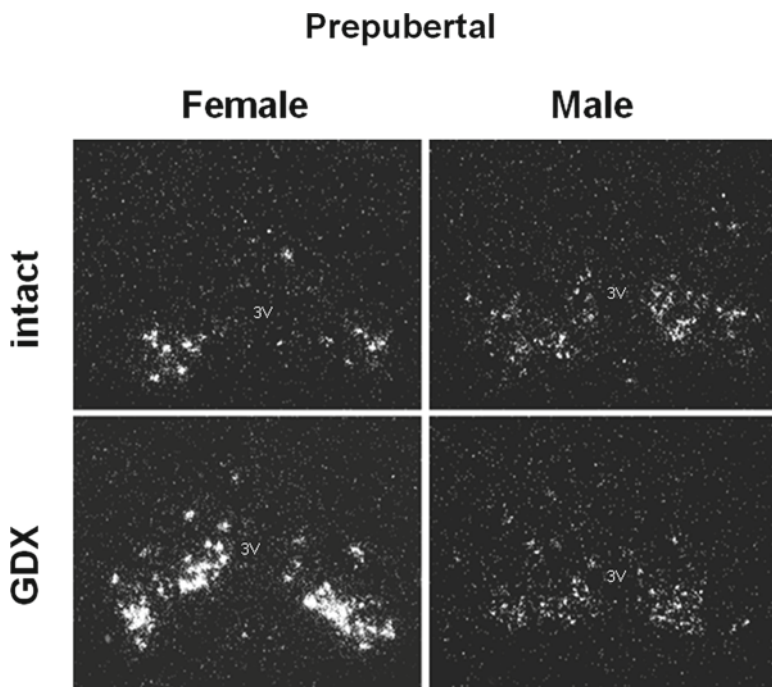
Prepubertal

Fig. 11.9 *Kiss1* expression in the ARC of prepubertal mice under different sex steroid conditions. Gonadectomized prepubertal female mice 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* third ventricle. Figure modified from Kauffman AS 2010 Gonadal and non-gonadal regulation of sex differences in hypothalamic Kiss1 neurons. J Neuroendocrinol 22:682–691 with permission from John Wiley and Sons

in the rate of pubertal maturation (females initiate puberty earlier than males) remains to be seen. This gonadal hormone-independent regulation of *Kiss1* neurons in developing males appears to disappear sometime between PND 18 and 45, as male mice GDX at PND 14 exhibit the expected elevation of ARC *Kiss1* and serum LH levels when analyzed at PND 45. The exact function of this sex difference still needs to be elucidated, though it is possible it relates to the sexually dimorphic timing of pubertal progression.

Conclusions and Perspectives

Kiss1 neurons are critical for puberty and reproduction, and this chapter is focused on the development of these neurons in the hypothalamic AVPV/PeN and ARC (Fig. 11.10). In terms of the AVPV/PeN *Kiss1* population, which emerges in the
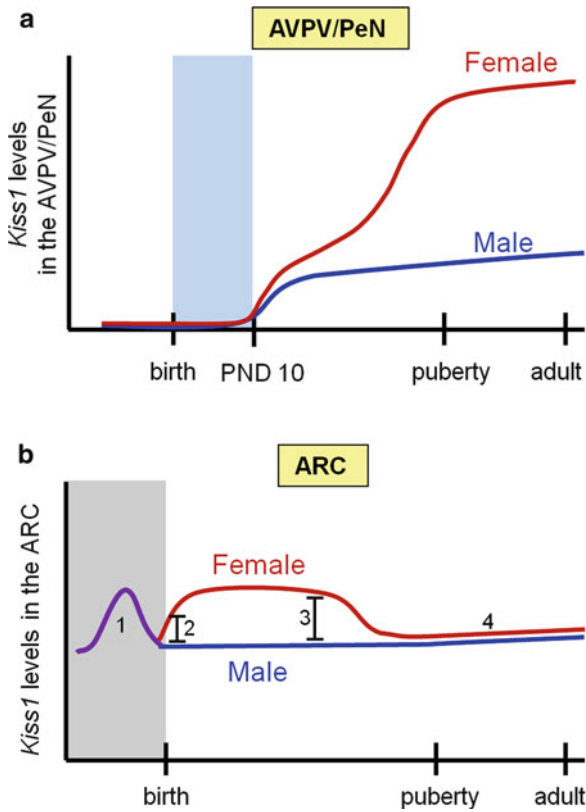
Fig. 11.10 Schematic diagram developmental changes in *Kiss1* expression in the AVPV/PeN (**a**) and ARC (**b**) of rodents. (**a**) *Kiss1* expression in the AVPV/PeN has not been detected on the day of birth or during other days of the "critical period" of perinatal development (*light blue shading*). *Kiss1* mRNA expression is first detected in the AVPV/PeN on postnatal day 10 (PND 10) in both males and females. The sex difference emerges by PND 12. Male *Kiss1* gene expression in the AVPV/PeN only slightly increases after that time to adulthood, whereas females have a steady increase in *Kiss1* expression that reaches adulthood levels around the time of puberty. (**b**) *1 Kiss1* is expressed in the ARC before birth. Analysis of combined male and female embryonic rat brains demonstrates increasing levels of *Kiss1* in the ARC throughout prenatal development (*gray shading*), with a slight drop before birth. Comparative analysis of embryonic *Kiss1* mRNA levels between sexes has not yet been determined. *2* During the neonatal period, *Kiss1* expression in the rodent ARC is sexually dimorphic, with females expressing more *Kiss1* than males. This may correlate with differences in the circulating sex steroid milieu, but requires further investigation. *3* Peripubertal *Kiss1* levels in the ARC decrease significantly in females to levels similar to males. *4* There may be a slight increase in ARC *Kiss1* expression during puberty leading to adulthood levels, though such an increase is controversial at present

second postnatal week of life and is sexually dimorphic, it remains to be answered how this population becomes sexually differentiated. Recent data suggest that epigenetic processes, precipitated by postnatal sex steroid signaling, may induce the AVPV/PeN *Kiss1* sex difference [5], especially because a role for the major neuronal

pathway of apoptosis was ruled out [40]. This model predicts that the differences in sex steroid exposure between male and female rodent brains early in neonatal life eventually result in permanent changes in the transcriptional activity of the *Kiss1* gene in the AVPV/PeN, resulting in greater *Kiss1* silencing in males and greater transcriptional activation in females. Supporting this, DNA methylation of the putative promoter of the *Kiss1* gene in the AVPV/PeN is sexually differentiated [83], though we still do not know when or how in development this *Kiss1* methylation difference first occurs.

Although E$_2$ signaling in males masculinizes the AVPV/PeN *Kiss1* system during the neonatal "critical period," it now appears that exposure to E$_2$ in developing females also shapes and promotes the complete feminization of AVPV/PeN *Kiss1* expression [10, 62, 94]. Indeed, several studies in mice have suggested that E$_2$ is required at *some time* during development in order for AVPV/PeN kisspeptin expression to fully develop a female phenotype [10, 62, 94]. However, the mechanism by which this occurs currently remains a mystery and may also involve epigenetics, neurogenesis, or a combination of these and other processes.

The role of the ARC/INF population of kisspeptin neurons in mediating sex steroid negative feedback in rodents appears to be different than some other mammals, such as sheep, in which ARC kisspeptin cells also play a role in positive feedback. Recent studies suggest that *Kiss1* is expressed in the ARC of developing rodents long before the AVPV/PeN, including prenatally (Fig. 11.10). The role of ARC *Kiss1* expression during prenatal/neonatal development is currently unknown and requires further investigation. In many species, the regulation of ARC kisspeptin fibers and their projections in development remains under-studied, in part because, until recently, there has not been a good way to distinguish the anatomical origin of kisspeptin-ir fibers. The recent findings that the ARC *Kiss1* population co-expresses *NKB* and *DYN* (in all species examined to date), whereas AVPV/PeN *Kiss1* neurons do not, should facilitate future studies characterizing the development of kisspeptin neuron projections, as should the use of several newly created transgenic *Kiss1* mouse models. Additionally, some studies suggest that there may be an increase in *Kiss1* expression in the ARC around puberty (Fig. 11.10), but the data is incomplete and inconsistent between studies, and more experiments looking at pubertal *Kiss1*/kisspeptin expression with higher temporal resolution are needed, including in non-rodent species.

While sex differences in ARC *Kiss1* cell number have not been observed by most studies examining adult rodents, such sex differences do exist in the ARC of neonatal and juvenile rodents (Fig. 11.10), as do sex differences in the gonad hormone-independent regulation of ARC *Kiss1* neurons in prepubertal life, at least in mice. The functional significance of these early ARC *Kiss1* sex differences is unknown, as are the specific factors controlling *Kiss1* expression in the ARC at early developmental ages. For example, sex steroids may transiently inhibit ARC *Kiss1* neurons (activational effects) during prenatal and postnatal development, as occurs in adulthood, but this has not been directly tested. Indeed, there is a need for examination of normal sex steroid levels in developing rodents, especially mice, during neonatal, juvenile, and prepubertal stages. Interestingly, it appears that in the complete

absence of gonadal sex steroids during development, ARC *Kiss1* neurons still develop normally and have intact hormone responsiveness later in adulthood, suggesting that this *Kiss1* population may only be affected by activational effects of sex steroids, rather than by permanent organizational effects that occur in the AVPV/PeN.

Overall, the development of kisspeptin circuits appears to be complex, region-specific, sex-specific, and influenced by multiple factors. The upstream mechanism(s) driving developmental and pubertal changes in kisspeptin neurons in each hypothalamic region, and how gonadal and non-gonadal factors work together to control development of the *Kiss1* system, remain important questions whose answers will provide a better understanding of the regulation of the reproductive neuroendocrine axis at multiple life stages.

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