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Sexual Differentiation and Development of Forebrain Reproductive Circuits

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Summary

Males and females exhibit numerous anatomical and physiological differences in the brain which often underlie important sex differences in physiology or behavior, including aspects relating to reproduction. Neural sex differences are both region- and trait-specific and may consist of divergences in synapse morphology, neuron size and number, and specific gene expression levels. In most cases, sex differences are induced by the sex steroid hormonal milieu during early perinatal development. In rodents, the hypothalamic anteroventral periventricular nucleus (AVPV) is sexually differentiated as a result of postnatal sex steroids, and specific neuronal populations in this nucleus are also sexually dimorphic, with females possessing more kisspeptin, dopaminergic, and GABA/ glutamate neurons than males. The ability of female rodents, but not males, to display an estrogen-induced luteinizing hormone (LH) surge is consistent with the higher levels of these neuropeptides in the AVPV of females. Of these AVPV populations, the recently-identified kisspeptin system has been most strongly implicated as a critical component of the sexually-dimorphic LH surge mechanism, though GABA and glutamate have also received some attention. New findings have suggested that the sexual differentiation and development of kisspeptin neurons in the AVPV is mediated by developmental estradiol signaling. Although apoptosis is the most common process implicated in neuronal sexual differentiation, it is currently unknown how developmental estradiol acts to differentiate specific neuronal populations in the AVPV, such as kisspeptin or dopaminergic neurons.

Keywords

sex difference; sexually dimorphic; sexual differentiation; hypothalamus; kisspeptin; *KissI*; tyrosine hydroxylase; development; reproduction; AVPV

Introduction

In mammals and many other vertebrates, both the brain and reproductive system are anatomically and physiologically differentiated between males and females. These sex differences range from disparities between men and women in the prevalence of certain

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reproductive health disorders to gender differences in normal reproductive physiology and reproductive circuits in the brain. In addition to girls typically entering puberty earlier than boys, girls are also more likely to present with early onset pubertal disease, whereas boys are more likely to present with diseases of delayed puberty or the inability to reach full sexual maturation. Often, these conditions are linked to defects in the secretion of reproductive hormones, which is controlled by forebrain and hypothalamic circuits. Although the key hypothalamic populations and neuronal mechanisms that underlie sex differences in reproductive disorders and physiology remain poorly understood, a specific region of the hypothalamus, the anteroventral periventricular nucleus (AVPV), is sexually differentiated in rodents, being larger in volume and containing more cells in females than males. Moreover, certain neuronal populations within the rodent AVPV are also sexually dimorphic, such as dopaminergic, GABA/glutamate, and *Kiss1*-expressing neurons, with females also having more of each of these specific cell types than males [1–3]. It has been postulated that these sexually dimorphic neuronal populations in the AVPV influence reproductive function, such as the ability of adult females, but not males, to produce the preovulatory gonadotropin surge, as well as possibly influencing sex differences in puberty onset. Although the *Kiss1* sexual dimorphism in the AVPV is particularly compelling because kisspeptin signaling has been implicated in fertility and puberty in many species, including humans [4–6], it is currently unknown if the other sexually dimorphic AVPV populations, such as dopaminergic or GABA/glutamate neurons, also play similar or complementary roles. This brief review discusses the functional relevance of the sexual differentiation of these various AVPV neural circuits, with an emphasis on the newly identified *Kiss1* system, and highlights some of the most recent findings in the field from the past few years. Additionally, based on known mechanisms of the sexual differentiation the brain, we also discuss some of the potential developmental mechanisms that could be underlying the sex-steroid mediated sexual differentiation of neuronal populations in the AVPV.

Sexual Differentiation of Neural Circuits and Reproductive Physiology

Mammals exhibit numerous sex differences in physiology and behavior, including several indices of reproductive biology [3,7,8]. Many of the physiological and anatomical differences between females and males reflect sex differences within the brain. In fact, there are many well-documented sex differences in the brains of many species, including insects, fish, birds, and mammals (reviewed in [9]). These neural sexual dimorphisms are present in many different areas of the brain, including the hypothalamus, hippocampus, and medial pre-optic nucleus [3,8,10,11]. Sex differences in the brain may include morphological differences in synapses, differential gene expression, and disparities in cell size or regional volume, including the number of neurons present (Table 1) [7,10,12]. For example, the medial preoptic nucleus (mPOA) and the principal bed nucleus of the stria terminalis (BNST) are both larger and possess more neurons in male rodents than in females [7,12,13]. In addition, the rodent BNST exhibits sexually dimorphic vasopressin (VP) gene expression, with adult males having more VP neurons in the BNST than females [14]. Interestingly, neuronal projections from the BNST to the AVPV are also sexually differentiated, with males having an abundance of projections compared to females [15]. Conversely, the AVPV of the rodent hypothalamus is greater in size and cell number in females than in males [16]. Particular neuronal populations in the AVPV are also sexually differentiated, with females having greater numbers of dopaminergic, GABA/glutamate, and *Kiss1*-expressing neurons [1,2,17]. In many cases, the functional significance of sexually dimorphic brain regions is currently unclear, and it remains unknown why neuron size or gene expression levels are higher in one sex than another. However, in a few cases, accumulating data has allowed for conjecture of potential functions of specific neural sex differences. For example, it has been postulated that the sexually dimorphic BNST is involved in modulating well-documented sex differences in anxiety and stress responses [18]. The AVPV, on the other hand, is known to be involved in reproduction and sex differences in this

region may mediate numerous sex differences related to reproductive function, a possibility discussed in more detail below.

In some cases, gender-biased genetic contributions by sex chromosomes lead to sexual dimorphisms in the brain [19]; however, the majority of known sex differences are induced by the sex steroid hormonal milieu during early postnatal development [7]. During the postnatal “critical period”, which is the first 7–10 days of life in rodents, males secrete gonadal testosterone (T) whereas females secrete negligible levels of sex steroids. Experiments manipulating the postnatal steroid milieu support the model that the presence or absence of postnatal T secretion determines whether sexually dimorphic traits develop to be male-like or female-like in adulthood (Figure 1). Thus, in newborn males, postnatal T [or its aromatized product, estradiol (E₂)] organizes the developing brain to exhibit a male-like phenotype in adulthood [8]. In contrast, newborn females are not exposed to sufficient levels of sex steroids and therefore, their brains develop to be female-like in adulthood [3,7]. Supporting this model, castration of newborn males (which removes postnatal T secretion) results in the development of feminized neural populations, whereas sex steroid treatment to newborn females (to simulate T secretion in normal males) results in the development of masculinized neuronal circuitry (reviewed in [4]).

Like the brain, the neuroendocrine reproductive axis also contains multiple discernible sexual dimorphisms. In rodents, these include sexually differentiated neural mechanisms controlling sex-specific reproductive behaviors [20], earlier pubertal maturation in females [21,22], and the presence of neural circuitry that generates preovulatory hormone surges in females but not males [1,23]. Besides sex differences in normal reproductive physiology and behavior, there are a number of reproductive health disorders and diseases in humans which present with disparate frequency between the sexes, such as idiopathic hypogonadotropic hypogonadism (more common in men), precocious puberty (more common in girls), and constitutional delayed puberty (more common in boys) [24,25]. Presumably, many of the sex differences in reproductive physiology, behavior, and health disorders reflect underlying sex differences in brain circuitries (Figure 1). For example, as mentioned earlier, if male rodents are castrated during the postnatal critical period, thereby preventing their ability to secrete postnatal T, they display female-like brain circuits and reproductive physiology in adulthood, including the ability to display an E₂-induced luteinizing hormone (LH) surge, similar to normal adult females [7,8,26]. Conversely, if a female rodent is given a single injection of T or E₂ during the postnatal critical period to mimic the male postnatal T secretion, she displays male-like brain phenotypes and reproductive physiology in adulthood, including her inability to produce a preovulatory LH surge or to display normal female sexual behaviors such as lordosis [7,8,26].

Sexually Differentiated Neurons of the AVPV and Their Role in Reproduction

Neurons that secrete gonadotropin releasing hormone (GnRH) are the final common pathway by which the brain controls reproduction. Interestingly, GnRH neurons are not themselves sexually dimorphic, suggesting that other neuronal populations influence sex differences in reproductive status. In rodents, the hypothalamic AVPV, which is responsible for conveying hormonal and environmental signals to GnRH neurons, does display sex differences in various parameters [7,27]. Importantly, the rodent AVPV is considered to be one of the main anatomical sites that drive the sexually differentiated preovulatory LH surge [28]. In accordance with this conjecture, the AVPV exhibits several robust sex differences [7,27,29]: both the overall size of the AVPV nucleus and the total number of cells in this region are greater in adult females than males [30]. Moreover, the AVPV contains a number of sexually dimorphic subpopulations which have been implicated to varying degrees in modulating or regulating GnRH cells, including dopaminergic neurons, which express the tyrosine

hydroxylase (TH) enzyme [3,31], cells expressing both GABA and glutamate [2], and *Kiss1* neurons [1,32,33]. In all three cases, the cell number of these specific AVPV neuronal populations is greater in females than males.

Tyrosine Hydroxylase and GABA/Glutamate Neurons

Dopaminergic neurons were the first sexually dimorphic neuronal population identified in the AVPV [17]. These sexually differentiated neurons, more prevalent in females than males, were originally detected by Simerly and colleagues in the rat AVPV with the use of immunohistochemical staining of the TH enzyme that synthesizes dopamine; the TH sex difference has since been confirmed in the mouse AVPV [17,30,31,34]. Although the higher amount of these dopaminergic AVPV neurons in females correlates with their ability to generate an LH surge, the evidence supporting the role of dopamine in the surge event is inconclusive [7]. There is evidence, however, that the second identified sexually dimorphic population in the AVPV, GABA/glutamate neurons, may contribute to signaling between the AVPV and GnRH neurons. Earlier work showed that antagonists to GABA and glutamate receptors blocked GnRH release and the proestrus LH surge in rats [2,35], and GABA treatment also affects the electrical activity of GnRH neurons [36,37]. More recently, it was discovered that the synaptic terminals of dual phenotype GABA/glutamate neurons in the AVPV directly contact GnRH neurons in rats, and that E₂ inhibits GABA release and increases glutamate release during the LH surge [2]. Although these findings suggest that this particular neuronal population may be involved in the sexually dimorphic LH surge, at present, the role of GABA/glutamate AVPV neurons in eliciting the LH surge still remains poorly defined.

Kiss1 Neurons

More recently, the neuropeptide kisspeptin has been implicated in the direct regulation of GnRH secretion and sexually dimorphic reproductive function (reviewed in [4]). Encoded by the *Kiss1* gene, kisspeptin is a ligand for the G-protein-coupled receptor, Kiss1R, formerly known as GPR54 [38–40]. In mammals, *Kiss1* mRNA expression and kisspeptin immunoreactivity have been detected in two discrete regions of the hypothalamus, the arcuate nucleus (ARC) and the AVPV [29]. Recent studies have identified the importance of *Kiss1* for reproductive function in humans and other species, including mice. Deletions or mutations in the *Kiss1* or kisspeptin receptor genes result in infertility and impaired pubertal maturation [41–43]. Exogenous treatment of rodents, sheep, monkeys, and humans with kisspeptin increases circulating LH and FSH levels [44,45]. The stimulation of gonadotropin secretion by kisspeptin is likely via activation of GnRH neurons since kisspeptin induces c-Fos expression in GnRH neurons, increases electrical activity of GnRH neurons, and causes the secretion of GnRH in hypothalamic explants [34,46–48]. Moreover, kisspeptin-containing fibers directly contact GnRH neurons, which express the kisspeptin receptor [32,46], suggesting that kisspeptin acts directly on GnRH neurons.

In 2007, *Kiss1*-expressing neurons in the AVPV were shown to be sexually differentiated, with adult females possessing more *Kiss1* mRNA than males [1]. Similar observations have been reported for kisspeptin protein levels in the AVPV [32,33]. Thus, like TH and GABA/glutamate, the presence of numerous *Kiss1* neurons in the AVPV correlates with the ability of an animal to generate an LH surge: adult females have high *Kiss1* levels and can generate an LH surge, while adult males have little AVPV *Kiss1* expression and cannot surge (even with E₂ treatment). However, in contrast to TH and GABA/glutamate neurons, mounting evidence supports a critical involvement of kisspeptin in the sexually differentiated LH surge. For example, E₂ dramatically stimulates *Kiss1* expression in the AVPV [49] and *Kiss1* neuronal activity in this region is upregulated in a circadian pattern in complete synchrony with the circadian timing of the LH surge [50]. Furthermore, *Kiss1*-null or *Kiss1R*-null mice are unable to produce an LH surge, even in the presence of E₂ [5]. Most recently, it was shown that central

infusion of a kisspeptin receptor antagonist in cycling female rats blocks the proestrus LH surge, emphasizing the requirement for kisspeptin signaling in this sexually dimorphic reproductive event [6]. In contrast to the AVPV population, *Kiss1* cells in the ARC are not sexually dimorphic in adult rodents, regardless of adulthood sex steroid milieu [1,51]. However, it is noteworthy that sex differences in the regulation of *Kiss1* expression in the ARC are present in mice during the prepubertal period, which may relate to sex differences in sexual maturation [51].

Regulation of Sexual Differentiation of the AVPV by Sex Steroids

Like many other sexually dimorphic brain traits, at least two of the AVPV populations, *Kiss1* and TH, have been shown to be sexually differentiated by sex steroids during early postnatal development [1,31,33,52]. Postnatal T treatment to newborn female rats masculinizes the development of the AVPV TH population; the effects of T on the sexual differentiation of these TH neurons has been shown to be mediated by estrogen signaling (via aromatization of T to E₂), likely via estrogen receptor α [31,52]. Although circulating sex steroids in adulthood can dramatically stimulate *Kiss1* expression in the AVPV [49,53], the adult sex steroid milieu does not account for sex differences in AVPV *Kiss1* expression [1]. Male and female rats that are gonadectomized as adults and treated with identical E₂ levels still display sexually dimorphic *Kiss1* expression in the AVPV [1,54]. Rather, the *Kiss1* sex difference, like that of TH, appears to be permanently organized early in development by sex steroid signaling during the postnatal critical period (Figure 1). Indeed, castrating male rats at birth causes a permanent feminization of the developing AVPV *Kiss1* system [33]. Conversely, postnatal female rats treated once with T or E₂ exhibit a robust reduction of *Kiss1*- or kisspeptin-expressing cells in the AVPV in adulthood, similar to normal males [1,33,55].

The fact that postnatal E₂ treatment can, like T, alter the development of the *Kiss1* system suggests that masculinization of the AVPV *Kiss1* system is likely mediated via aromatization of T to E₂ during the critical period (as is also the case for the TH system). Importantly, the masculinization (i.e., reduction) of AVPV *Kiss1* expression in female rats treated at birth with sex steroids is consistent with their inability to generate an LH surge as adults [33], highlighting the link between the sexually dimorphic AVPV *Kiss1* system and LH surge event. However, while the development of the sexually dimorphic *Kiss1* system is dependent on the postnatal sex steroid milieu, it is unclear exactly how postnatal sex steroids, primarily E₂, direct the sexual differentiation of this system.

Mechanisms of Sex Steroid-Mediated Sexual Differentiation of the Brain

How does T or E₂ direct the sexual differentiation of the AVPV, and more specifically, subpopulations within the AVPV? Several hormone-dependent mechanisms, such as differential neurogenesis, migration, epigenetics, and apoptosis, have been implicated in the sexual differentiation and development of various neuronal populations (Figure 1) [7,11,56, 57]. E₂, 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 [7]. Likewise, in the developing rat hippocampus, sex steroids (which are higher in postnatal males than females) increase the number of new cells, leading to more neurons present in males [58]. Because the AVPV as a whole (i.e., total size of the nucleus) does not undergo differential neurogenesis in response to postnatal sex steroids [7], neurogenesis may not be a major contributor to the sexual differentiation of specific subpopulations, such as *Kiss1* neurons, within the AVPV. However, this assertion has not yet been directly tested.

Many sexually dimorphic populations in the brain arise via apoptotic mechanisms (i.e., programmed cell death) [30,59,60]. In fact, sex differences in the overall size and total cell

number of the AVPV, as well as other brain regions such as the BNST, are induced by apoptosis. Most of these apoptotic-induced sex differences are dependent on the pro-apoptotic gene, *Bax* [30,59,61]. BAX is a pro-apoptotic 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. Cytochrome c release activates caspases, which degrade target substrates in the cell, resulting in cell death [62]. Interestingly, in the developing rat AVPV, postnatal males have higher *Bax* expression than females (and hence, potentially more cell death) [63], which correlates with the presence of fewer AVPV cells present in adult males than females. The differential postnatal expression of *Bax* also correlates with higher sex steroid levels in postnatal males than females, suggesting that sex steroids might affect *Bax* expression [63]. Indeed, neonatal E₂ treatment of female rats increases the number of apoptotic AVPV neurons, supporting this conjecture [64]. Finally, a recent study determined that the sex difference in the total number of AVPV and BNST neurons is eliminated in *Bax* knockout mice [30]. Thus, at least one AVPV trait, total cell number, is sexually differentiated via *Bax*-dependent apoptotic mechanisms. Despite these findings, the sexual differentiation of TH neurons in the AVPV appears to occur via a *Bax*-independent mechanism, because *Bax* knockout mice do not have altered sexual differentiation of AVPV TH neurons [30]. Thus, this TH population is sexually differentiated by either other apoptotic pathways or non-apoptosis mechanisms. Likewise, although the sex difference in total cell number and volume of the BNST is due to *Bax*-dependent apoptosis, the mechanism of sexual differentiation of the BNST neuronal population expressing VP is not due to sexually differentiated cell death [65]. Intriguingly, however, recent data indicate that sexual differentiation of AVPV GABA-ergic neurons may involve sex-specific apoptosis that is mediated via tumor necrosis factor α (TNF α)-dependent and -independent mechanisms [60], raising the possibility that TH and VP cells are also differentiated this way. The possibility that the AVPV *Kiss1* system is also regulated via BAX- or TNF α -dependent apoptotic pathways has not yet been tested.

Epigenetic changes precipitated by sex hormones early in life are emerging as critical contributors to alterations in neuronal cell number and gene expression [56]. Modifications to histones (proteins that organize DNA into chromatin) or DNA methylation are thought to influence the transcriptional activity of specific genes, and histone acetylation in particular is associated with transcriptional activation [66]. Recently, histone acetylation has been implicated in the postnatal sex steroid-induced sexual differentiation of the total number of neurons and overall density of the BNST [57]. In rodents, the BNST contains more cells and is larger in volume in males than females. Pharmacological disruption of histone deacetylation during the postnatal critical period, thereby increasing the level of acetylated histones, resulted in the feminization of the BNST region [57]. In this case, it is likely that epigenetic changes directly or indirectly affected the process of apoptosis in the BNST, as sex differences in this region are known to be governed specifically by BAX-mediated apoptosis [59]. The concept that postnatal sex steroid milieu influences the sexual differentiation and phenotypic identity of numerous neuronal populations via epigenetic mechanisms is particularly attractive, especially in light of new studies implicating promoter DNA methylation (an epigenetic modification affecting transcriptional activity) in the sexual differentiation of estrogen receptor α expression in the developing rat pre-optic area [67]. Males had higher levels of estrogen receptor α methylation than females, which correlated with lower expression levels of the gene in the male pre-optic area [67]. Overall, these findings implicate the involvement of chromatin remodeling in the sexual differentiation of the brain, and suggests that other sexually dimorphic traits elsewhere in the brain, such as the AVPV, may arise in part due to epigenetic changes.

Conclusions

The hypothalamic AVPV region and particular neuronal populations within the AVPV are sexually differentiated under the influence of postnatal sex steroid milieu, with adult females

possessing more AVPV neurons overall as well as more *Kiss1*, TH, and GABA/glutamate neurons than males. The sexual differentiation of AVPV neurons expressing GABA/glutamate, TH, and *Kiss1*, may underlie the ability of females, but not males, to display a GnRH/LH surge. However, of these sexually dimorphic AVPV populations, *Kiss1* has been most strongly linked to both reproductive function and the regulation of sexually-dimorphic LH surge, though additional evidence supports a possible role of GABA/Glutamate as well. Although many advances have been made in the understanding of these sexually differentiated neuronal populations, there are important issues that have yet to be resolved. Such issues include defining the specific mechanisms for the sex steroid-induced sexual differentiation of these AVPV neuronal populations, and identifying if other sexually differentiated AVPV neurons, besides just *Kiss1* cells, play a critical role in sexually dimorphic reproductive function. Importantly, understanding the mechanisms underlying the development of sexually dimorphic AVPV populations and neuropeptide expression levels may have clinical relevance due to the abundance of sexually differentiated disorders, including some relating to reproductive function.

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Figure 1.

Table 1

A partial list of some neural sexual dimorphisms in the rodent brain. In some cases, the possible function of each sex difference is either unknown or only hypothesized based on available evidence. See text for more details.

Brain Region	Phenotype	Sex Difference	Possible Function	References
AVPV	Cell number/size	females>males	unknown (perhaps reproduction)	[7,30]
AVPV	<i>Kiss1</i> expression	females>males	puberty and/or reproduction	[1,32]
AVPV	<i>TH</i> expression	females>males	unknown	[3,31]
AVPV	GABA/glutamate	females>males	puberty and/or reproduction	[2]
BNST	Cell number/size	males>females	stress/anxiety/reproduction	[13,18]
BNST	VP expression	males>females	stress/anxiety/reproduction	[65]
BNST	Projections to AVPV	males>females	unknown	[15]
mPOA	Cell number/size	males>females	sexual behavior	[7]
Hippocampus	Cell number/size	males>females	memory formation	[7,58]