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Location, location, location: Genetic regulation of neural sex differences

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Abstract

Sex differences in many behaviors such as cognition, mood, and motor skills are well-documented in animals and humans and are regulated by many neural circuits. Sexual dimorphisms within cell populations in these circuits play critical roles in the production of these behavioral dichotomies. Here we focus on three proteins that have well described sexual dimorphisms; calbindin-D28k, a calcium binding protein, tyrosine hydroxylase, the rate limiting enzyme involved in dopamine synthesis and vasopressin, a neuropeptide with central and peripheral sites of action. We describe the sex differences in subpopulations of these proteins, with particular emphasis on laboratory mice. Our thrust is to examine genetic bases of sex differences and how the use of genetically modified models has advanced our understanding of this topic. Regional sex differences in the expression of these three proteins are driven by sex chromosome complement, steroid receptors or in some instances both. While studies of sex differences attributable to sex chromosome genes are still few in number it is exciting to note that this variable factors into expression differences for all three of these proteins. Different genetic mechanisms, which elaborate sex differences, may be employed stochastically in different cell populations. Alternately, general patterns involving the timing of differentiation of the sex differences, relative to the “critical period” in hormonal differences between males and female neonates may emerge. In conclusion, future directions in this area should include examination of the importance of location, timing, steroidal receptor/sex chromosome gene synergy and epigenetics in molding neural sex differences.

Keywords

Sex chromosomes; Estrogen receptors; Calbindin-D28k; Tyrosine hydroxylase; Dopamine; Vasopressin; Sexually dimorphic; Cerebellum; Frontal cortex; Epigenetic

1 Introduction

Raisman and Field in the early 1970's [1, 2] discovered a sex difference (females>males) in the incidence of synapses on dendritic spines in adult rats. The region of interest was in the medial preoptic area (MPOA) below the anterior commissure at the point where the bed nucleus of the stria terminalis and MPOA converge in the coronal plane. Castration of neonatal males within the first 12 h after birth resulted in female-like numbers of synapses. Their observation followed studies by Barraclough, Harris and their colleagues [3–5] who demonstrated that males produced in this manner were capable of ovulation when implanted with surgically grafted ovaries. Moreover, the opposite was true when females were

neonatally exposed to androgens [4, 6]. Thus, the suggested function of this sexual dimorphism was ovulatory capacity of females. Several years later, another sex difference was reported in the medial preoptic area. Gorski's laboratory had studied many ramifications of the neonatal hormone environment on behavior, body weight and neuroendocrinology in adult rats. Finally, in 1978 [7] they reported structural differences, at the light microscopic level, between male and female MPOA (males>females) and these differences in cell size, number and volume of the nucleus were modified by early hormone exposure. Specifically perinatal females exposed to androgens had larger sexually dimorphic nuclei than controls. These data combined with behavioral studies that were ongoing in many laboratories including the classic work from William Young's laboratory [8]. These researchers noted that when guinea pigs were exposed to androgens during gestation, and tested as adults, females were more likely to display male-typical sexual behaviors.

In the past 30 years many research laboratories have independently examined these sex differences in the MPOA and have mapped other sexual dichotomies in the brain, primarily in the hypothalamus [9, 10]. The parallels between the actions of early steroids on ovulation, behavior and these morphological sex differences are truly elegant, but at the same time, this paradigm has concentrated work on the hypothalamus and on the actions of steroid receptors. Thus, alternative mechanisms and brain regions have been less well studied.

In addition to the role of steroid hormones and their receptors for the elaboration of sex differences in brain and behavior, just over a decade ago Art Arnold offered a complementary alternative hypothesis. He argued that sex differences in brain may not be completely explained by steroid receptors, particularly in song birds [11]. In birds and mammals, sex chromosomes are a source of genetic variation between males and females. Arnold and colleagues proceeded to ask if sex chromosome genes might be instrumental in the development of sexual dichotomies in brain and behavior [12]. This work was conducted in a mouse model, now referred to as the Four Core Genotypes (FCG) [13]. A spontaneous mutation on the Y chromosome (referred to as Y-) effectively deletes the testes determining gene (*Sry*) and this leads to the possibility of XY females [14]. In addition to this mutation the FCG mice have a *Sry* transgene, incorporated into an autosome; the transgene is able to rescue fertility in XY mutant mice [15]. Thus sex chromosome complement and gonadal sex are uncoupled and when normal XX females are mated with XY-*Sry* males the four core genotypes are produced; XX females (ovary-bearing), XY females (ovary-bearing), XX males (testes-bearing) and XY males (testes-bearing) [12].

In this review, we will focus on several well-studied sex differences in the mouse brain. We will discuss historical aspects of these dimorphisms as illustrated by work done with other species, in particular rats, but the bulk of the review is on laboratory mice because the FCG, and other engineered models, allows us to assess the contributions of these potentially non-steroidal mechanisms. We concentrate on three markers, calbindin-D28k, tyrosine hydroxylase (dopamine) and vasopressin. Again, we are limiting ourselves to these markers because there are enough data, from mice and in particular the FCG, that we can at least begin to speculate about the actions of sex chromosome versus steroid receptors and which mechanism(s) underlie these sex differences. As a preview of our conclusion, it is clear that regional differences, which certainly map to differences yet to be discovered in cellular phenotypes, are critical as to whether the dimorphisms are regulated by sex chromosome complement and/or steroid receptors. Moreover, in some cases both factors are in play. We are in the early days in our discovery of how these genes sculpt neural development in general and sex differences in particular, but progress can be rapidly made using state of the art techniques. We will highlight some paths that should be explored in our conclusions.

2 Calbindin-D28k

Calbindin-D28k (calbindin) is a calcium binding protein which is heterogeneously expressed in a subset of neurons and highly abundant in rat brain [16]. Calbindin functions as a high affinity calcium buffer and sensor in neurons that demonstrate a high level of calcium activity during neuronal signaling and communication [17–19]. Studies using calbindin knockout or transgenic mice show subtle, but distinct phenotypic changes in motor coordination, hippocampal long-term potentiation, spatial learning, Purkinje cell Ca^{+} signaling and resetting of the circadian clock [19–25].

2.1 Calbindin expression in rat SDN-MPOA

Calbindin has a special significance for the study of sex differences in the brain as it serves as a “biomarker” for the sexually dimorphic nucleus of the medial preoptic area (SDN-MPOA) of the rat [26–28]. In this area, males express more and larger neurons than females and the neurons are visualized by their calbindin immunoreactivity [29]. In males, aromatization of testosterone to estradiol increases cell number and hence calbindin expression by regulating apoptosis [30–32]. In fact, the estrogen receptor α (ER α) is likely the ER responsible for these effects since calbindin co-localizes with ER α positive neurons in the SDN-MPOA of the rat [33]. An androgen-dependent mechanism may also influence calbindin expression in rats because blocking androgen with the antagonist flutamide, decreased calbindin levels prenatally in the MPOA of male and female rats [34]. However it is not clear if the suprachiasmatic nucleus, a region that expresses calbindin and androgen receptors, was included in the tissue used for these protein blot studies [35].

2.2 Calbindin in mouse SDN-MPOA

Unlike rats, many strains of mice do not exhibit a sexual dimorphism in the SDN-MPOA when Nissl preparations alone are examined [36–38]. However, similar to rats, calbindin is a sexually dimorphic marker of the SDN-MPOA in C57BL/6J mice with males showing more calbindin expression than females [39, 40]. The critical period of testicular production of steroids in the neonatal male mouse is similar to the rat; starting at E17/18 and ending shortly after birth [41–43]. Thus, the sex difference in mice may very well depend on gonadal secretions. In fact, this has been proven in *SF-1* knockout (SF-1 KO) mice who fail to differentiate gonads and adrenal glands and therefore are not exposed to steroids during the fetal/neonatal period [40]. The SF-1 KO mice do not show a distinct calbindin expressing cell nucleus on the SDN-MPOA in either sex whereas the wild type (WT) males and females show the expected difference.

In C57BL/6J male mice, castration on the day of birth reduces calbindin immunoreactive cell numbers to levels found in the female. Similarly, in female mice either estradiol (five daily injections) or a single injection of testosterone administered on the day of birth increased adult calbindin cell numbers [44]. This finding demonstrates that, like rats, the ERs are modulating this sex difference during development. However, studies using mice with a spontaneous AR mutation (Tfm) show that androgen receptors (AR) may also regulate calbindin. Cell numbers in male Tfm mice were intermediate to WT males and female littermates and statistically no different from either control group [39]. Two studies have treated females with a single injection of dihydrotestosterone (DHT) on the day of birth, and examined adult calbindin defined SDN-POA. In one study no effect of DHT was found; control females had the same numbers of cells as DHT injected females whereas estradiol treatment increased cell numbers in females and males were not examined [44, 45]. In the other report control males and females were significantly different in cell number and the DHT treated females were numerically intermediate [45]. Since the effects of the Tfm mutation extend beyond the day of birth, and a single exposure to DHT, it is likely that both

the AR and the ERs are involved synergistically in the sexual dimorphism of the mouse SDN-MPOA.

2.3 Extra-hypothalamic sex differences in calbindin

The population of calbindin-containing cells in the MPOA is a tiny group compared with the overall distribution of this protein in the brain. There are several other distinct populations of neurons that show robust calbindin expression in the frontal cortex, thalamus, hippocampus, amygdala and cerebellum, where calbindin serves as a marker for Purkinje cells. Interestingly, we have recently discovered striking sex differences in calbindin gene and protein expression in the frontal cortex and cerebellum of juvenile (PN21-25) mice [46]. However, unlike the SDN-MPOA, in which calbindin levels are higher in males, females have about 2-fold more expression than males in the cortex and cerebellum. We examined the mechanisms that underlie sex differences in these extra-hypothalamic regions with two mouse lines: the ER α KO mouse and the four core genotypes (FCG). As explained in the introduction the four sex chromosome genotypes produced in the FCG are; XX females (ovary-bearing), XY females (ovary-bearing), XX males (testes-bearing) and XY males (testes-bearing) [12].

In the frontal cortex, calbindin levels in females are primarily affected in both mouse models (Fig. 1a,b); ER α KO and XY females had reduced calbindin as compared with WT females, in fact their calbindin levels were equivalent to that of males. This suggests an interaction between ER α and sex chromosome genes. In the cerebellum (Fig. 1c,d), both males and females were affected by the genetic manipulations afforded by the two mice models. XX males showed calbindin levels that approximated XX females and both were greater than XY animals regardless of gonadal sex (Fig. 1c). In contrast to the pattern observed in the frontal cortex, in the cerebellum, both male and female ER α KO mice showed a decrease in calbindin expression relative to their WT littermates (Fig. 1d). Importantly, the loss of the ER α did not eliminate the sex difference. Thus, in the cerebellum, sex chromosome complement completely dictated calbindin expression levels with XX mice of either sex having more calbindin than XY mice [46]. Therefore, although calbindin levels are increased in females relative to males in the frontal cortex and cerebellum, the mechanisms underlying this sexual dimorphism differs in these areas. The frontal cortex sexual dimorphism is due to both ER α and sex chromosomal complement, whereas in the cerebellum sex chromosomal complement is the only factor we have identified to date.

3 Tyrosine hydroxylase (dopamine)

Tyrosine hydroxylase (TH), the rate-limiting enzyme involved in dopamine synthesis, is commonly used as a marker of dopaminergic neurons. The vast majority of dopamine producing cells reside either in the substantia nigra and project to the dorsal striatum, the mesocortical and limbic regions of the forebrain, or in the ventral tegmental area with projections to the frontal cortex and limbic areas including the amygdala and the nucleus accumbens [47, 48]. Obviously, with this broad distribution of target areas the midbrain cells are influential in a large number and variety of behaviors. On the other hand the small population of dopaminergic cells in the anteroventral periventricular nucleus (AVPV) of the MPOA has fewer projections [49]. The AVPV has ascending projections to the lateral septum, periventricular nucleus, and the arcuate nucleus. But the projections to the vascular organ or the lamina terminalis which is a region containing many gonadotropin releasing (GnRH) neurons have received the most attention. The GnRH neurons project to the median eminence and affect GnRH regulation of gonadotropin secretion and ovulation. [5].

3.1 Sex difference in TH expression in AVPV

The AVPV is in the most medial part of the MPOA, just a few cell layers thick, surrounding the third ventricle. In many species, this area is larger in volume and cell number in females as compared with males [32, 50, 51]. This sex difference results from androgen dependent apoptosis in males; cell numbers in females are reduced to that of males when they are treated with testosterone propionate before or shortly after birth [52–54]. Bax, a pro-death member of the Bcl-2 protein family [55], has also been shown to regulate this sex difference as demonstrated by the absence of the sex difference in the AVPV of Bax knockout mice, thereby suggesting an androgen-Bax dependent mechanism of cell death in this area [56, 57]. Further, the anti-apoptotic gene, Bcl-2, is reduced in male rat AVPV compared to females via an increased expression of tumor necrosis factor receptor associated factor 2-inhibiting protein (TRIP) [58]. The AVPV contains a subset of dopaminergic neurons, indicated by TH immunoreactivity, used as a marker for this area. The number of these TH-positive cells is about three times greater in female rats and mice compared to males and treatment of female rats with testosterone eliminates this difference [51, 59–61].

Interestingly, this sex difference was not absent in Bax knockout mice, in contrast to total neurons in this area [56] suggesting a mechanism of regulation other than the androgen-Bax link. Instead, feminization of the dopamine neurons in the AVPV, at least in mice, is ER dependent since ER α KO, ER β KO and double KO males have fewer TH neurons as compared to their WT male littermates [45, 60]. Further, unlike the MPOA, feminization of the AVPV is independent of a functional AR receptor; TH immunoreactivity in the AVPV of Tfm male mice was no different from WT males [60]. Estradiol appears to act on both ERs during development since treatment of perinatal female pups with either an ER α or β specific agonist, tends to masculinize the number of TH neurons in the AVPV [45]. When the AVPV TH cell number was examined in the FCG mice, no role for sex chromosome complement was found [12].

3.2 Estrogenic actions and sexual differentiation in SDN versus AVPV

Masculinization of TH cells in the AVPV is driven by estrogens and depends on a caspase-dependent mechanism of apoptotic cell death. *In vitro*, TH neurons respond to estradiol with increased cell death, which can be blocked by estradiol antagonists or the pan-caspase inhibitor ZVAD (N-benzyloxycabonyl-Val-Ala-Asp-fluoromethylketone) [62]. This is in marked contrast to the neuroprotective role estrogens appear to play in the masculinization of the SDN-MPOA. In this area, the increased volume of the SDN in males, early in development, is ER-dependent [29]. In this capacity, estrogens may be preventing cell death in the SDN through caspase dependent pathways indirectly via the up-regulation of calbindin, which can inhibit caspase 3 [63]. Alternatively, estrogens may act directly by up-regulating the anti-apoptotic protein, *bcl-2*, and down-regulating caspase-3 [64]. In contrast, overexpression of *bcl-2* in mice could not rescue TH neurons in the AVPV from cell death when estrogens mediated cell death [65]. Since the number of cells immunoreactive for caspase-3 in the SDN are greater in rat postnatal day (PND) 8 females, compared to males, either scenario of estrogen regulation may be responsible for the apoptosis that occurs during development [63]. Although the SDN and AVPV have different responses to estrogens, precedent exists showing that estrogens can up- or down- regulate gene expression in a tissue-specific manner [64]. Clearly, estradiol's role as an anti-apoptotic or apoptotic factor depends on cell type and possibly other downstream targets such as calbindin. It may be that calbindin facilitates estrogens role as neuroprotective in the SDN of mice where it is expressed at PND 0 versus an estrogenic apoptotic role in the AVPV due to calbindin's absence [39].

3.3 Sex difference in TH expression in midbrain

Interestingly, the sex difference in the dopaminergic neurons in the midbrain appears to be independent of steroidal hormones, but it is affected by the origin of the cell populations in the midbrain [66–68]. In cell cultures extracted from the mesencephalic region of the midbrain at E14, the uptake of [3H]dopamine, which designates functional maturation in aminergic neurons, was higher in male as opposed to female cells, yet females showed significantly more TH positive neurons in these cultures than males. In contrast, cells from female diencephalic regions of the midbrain showed significantly more [3H] dopamine uptake and endogenous dopamine, but smaller TH cell size than males. These sex differences all occurred at a time point before the surge in embryonic male testosterone, and are therefore independent of gonadal hormones [66–68]. Hormonal independence of these sex differences has been demonstrated in different strains of mice that were exposed to long-term steroid treatments [69, 70]. These results suggest that sex differences in rodent dopaminergic cell number arises in the absence of gonadal hormones in a region specific manner and may depend on genetic sex (XX or XY) for their differentiation [67]. Cell cultures derived from the mesencephalon of E14 FCG mice definitively demonstrated the effect of genetic sex on these sex differences. XY males and XY females had more TH-cells than XX cultures from either sex [71]. Interestingly, Dewing and co-workers [72] showed that *Sry*, the Y-chromosome testes determining gene, is co-expressed with TH in neurons in the substantia nigra of adult male rats and down-regulation of this gene results in a decrease of TH in neurons. One speculation then is that in the normal XY male midbrain neurons, *Sry* expression during embryonic development increases TH expression.

4 Vasopressin

Vasopressin (AVP) is an early evolved peptide with several forms, one of which is expressed in nearly all vertebrates [73]. The peptide is produced in two major neural subpopulations. The AVP-producing neurons in the supraoptic and paraventricular regions primarily project to the posterior pituitary where they release peptide directly into the capillary bed surrounding the pituitary gland. In the periphery AVP plays important roles in regulation of osmolarity, blood pressure, and stress [74–76]. In addition to these hypothalamic neurons, AVP is produced in cells within the medial amygdala, bed nucleus of the stria terminalis (BNST), SCN and the POA [77, 78]. These cells project to other brain sites and it is here AVP acts as a neuropeptide.

The list of actions attributed to AVP in the brain seems to grow on a daily basis. One of the first neural functions attributed to AVP was the ability to modulate social memory. [79–82]. This neuropeptide is also involved in circadian rhythms, body temperature regulation, parental behavior, sex behavior, aggression, anxiety and scent marking [83–88]. While several AVP producing cell populations are sexually dimorphic, the majority of the work describing this marker has been conducted on mRNA in AVP producing cells in the BNST and protein expression in the fiber projections from these cells which project to the lateral septum (LS) [89].

4.1 Organization of the BNST AVP-producing neurons in rats and voles

In the BNST of adult rats, *in situ* hybridization first revealed sex differences in AVP cell numbers and mRNA for the vasopressin precursor, propressophysin, [90, 91]. Males have more AVP producing cells and more vasopressin precursor mRNA, quantified by the numbers of radioactive grains per cell, in all species examined to date. Male rats castrated on the day of birth had fewer cells containing mRNA for propressophysin, which could be restored to normal levels in castrated rats simultaneously treated with testosterone [90]. More elegant manipulations of gonadal status in neonatal rats have demonstrated that an ER

likely controls the sex difference in vasopressin expression. Neonatal gonadectomy followed by DHT replacement on PN1, 3 and 5, only produced a partial increase in cell numbers, but full restoration to normal male cell numbers was achieved by either estradiol alone or estradiol plus DHT [92]. These treatments were capable of enhancing AVP mRNA in adults of both sexes. In other species a much more complex story is still evolving.

Sex differences (males>females) are found in adult prairie voles both in immunoreactive AVP fiber density in the LS and cells that contain AVP mRNA in the BNST [93]. Castration reduces both components in adult males. To block testosterone's actions and the production of estradiol pregnant dams received both flutamide and the aromatase inhibitor, ATD. The male pups were next castrated at birth and in adulthood AVP mRNA containing cells in the BNST were reduced to levels comparable to females. This demonstrates that neonatal steroids organize the sex difference. Pinpointing the critical period(s) and which steroid(s) are essential has remained elusive in this animal. In the same study testosterone was administered during the neonatal period to reverse the affects of gestational anti-hormone treatment and castration. Several doses given at different time intervals largely failed to maintain masculine AVP cell numbers and fibers. The reverse, masculinization of the female AVP system was also unsuccessful and large doses of testosterone given for variable intervals failed to increase AVP cells or immunoreactive fibers. Better success was noted when the estrogen antagonist tamoxifen was given to males during the week after birth which lead to reductions in the AVP system. In a reciprocal study, administration of the potent estrogen analog DES to females, increased AVP fibers in the LS. Taken together it is possible that prairie voles only utilize estrogens for sexual differentiation of the AVP system, and/or there are other factors involved.

These findings highlight potential species differences in the AVP system. Experiential factors, likely acting via epigenetic modulation of AVP, may also play a role and may also contribute to species differences. For example in the prairie vole, the number of male siblings affects female AVP responses to neonatal estrogen treatment [93]. In rats, maternal separation reduces the AVP cell numbers in the male paraventricular region to that of females. This is likely caused by AVP interactions with the HPA axis [94]. Male California mice fostered to white footed mice as pups had lower levels of AVP immunoreactivity in the BNST as compared with controls raised by California mice [95]. In addition to these neonatal experiences, it is possible that sex chromosome complement has an impact on the AVP system in some of these species.

4.2 Sexual differentiation of the laboratory mouse AVP system

A comprehensive developmental study using exogenous administration of steroid agonists, antagonists and gonadectomy has not yet been done in the laboratory mouse. Instead, a number of studies on KO mice report that in WT littermates a sex difference in AVP immunoreactive fibers in the LS and immunoreactive density in the medial amygdala match those found in rats (males>females) [12, 78, 89, 96]. Cells containing AVP mRNA in BNST are also sexually dimorphic in mice with males having more cells and females expressing more mRNA (quantified as grains) per cell [78]. Using Tfm and ER α KO mice, along with littermate males with both the mutant AR and null for ER α (double knockouts), our group showed that in the LS AVP, immunoreactivity was not impacted by the AR mutation alone, while the loss of functional ER α reduced AVP immunoreactivity. This was significant when WT males were compared to double mutants (lacking functional ER α and AR) [96]. No changes in immunoreactive cell numbers in the BNST were found, but in the medial amygdala the double mutants had less immunoreactivity than any other group. This suggests that both AR and ER α are important for the sex differences in the mouse AVP fibers in the LS and AVP immunoreactive cell numbers in the medial amygdala. A more descriptive

study of the entire extra-hypothalamic AVP system revealed no influence of the progesterin receptor on these sex differences [78].

Several studies with KO mice demonstrate that estradiol may only have an activational role on AVP containing fibers in the LS. In the aromatase enzyme KO mouse AVP immunoreactivity was compared in males that were gonad intact and less was noted in the KO males than the WT males [97]. However, the design of this study cannot distinguish between activational and organizational roles for estradiol since KO males lack estradiol throughout development and as adults. Pierman and colleagues demonstrated that when both estradiol and DHT are given to adult aromatase KO males after castration AVP-immunoreactivity in the LS is as dense as that noted in WT males [98]. In alpha fetal-protein KO mouse both males and females are exposed to high levels of estradiol during gestation. Females that received over-exposure to estrogens did not have male-like levels of AVP and the sex difference was maintained when adults were examined [99]. These studies suggest that some factor other than estrogens may organize the LS sex difference in laboratory mice. Two final studies in two different mouse strains of the FCG mice have demonstrated a sex chromosome affects on AVP immunoreactivity in the LS [12, 100]. In sum these studies suggest that sex chromosome genes, and perhaps the androgen receptor, are important for the sexually differentiation of the AVP extra-hypothalamic system in mice.

5 Speculations and future directions

5.1 Location

One trend that is evident from this review (Table 1) is that sex differences in and outside of the MPOA are regulated by different mechanisms. Sex chromosome effects are present in calbindin in the cerebellum and frontal cortex, AVP in the lateral septum and TH in the midbrain. In addition, cell cultures from male rat hippocampal neurons contain about two-fold more GABA-immunoreactive cells than those from females and this sex difference is independent of gonadal steroids [101]. Another confirmed effect of sex chromosomes is the expression of precursors for dynorphin (XX>XY) in the mouse striatum [102]. Sex chromosome mechanisms are not implicated in the AVPV and have yet to be examined in the SDN-MPOA. This is not to say that sex chromosomes are acting in a vacuum, in fact, in most of the regions listed both steroid receptors and sex chromosome complements are operational. Moreover, all of the regions we have discussed express ERs and/or AR. One of the challenges to be tackled is how and when steroids and sex chromosome genes interact to affect neural development.

5.2 Timing

To date, the cerebellum and the midbrain TH cells are the only populations in which steroid hormones have yet to be implicated in sexual dimorphisms. Interestingly, although Purkinje cells arise at E15 in rats and E11-13 in mice, their differentiation proceeds postnatally at a gradual pace with full maturity not realized until about PN40 [103–105]. This is far past the critical period of testicular steroid production in the neonatal male, which starts at E17 and ends a few hours after birth [41–43]. Calbindin expression in the cerebellum first appears at E11-14 in rodents, and steadily increases from birth with peak expression occurring between postnatal weeks 2–4 coincident with Purkinje cells synaptogenesis and the approaching full maturation of these cells [106, 107]. Thus maturation is completed outside the classical critical period for sexual differentiation. Like calbindin, TH is produced in the midbrain before the testosterone surge in rats and mice and the sex difference between males and females is independent of gonadal hormones [67, 71]. Thus, the timing of differentiation in a population of neurons may influence whether sex differences in those cells arise from hormones or sex chromosomes, the latter favoring neurons that develop outside the

developmental organizational window of gonadal surge. However another aspect that needs to be considered is that in rodents, some regions, such as the cerebellum, produce neurosteroids *de novo* and thus the gonads are not the only source of steroids [108, 109].

5.3 ER/AR synergy

Another distinct subpopulation that can be identified includes neurons that make calbindin in the MPOA and AVP in the BNST. The sex differences in these regions require both AR and ER α , and perhaps ER β , which has not yet been directly examined. The MPOA and BNST are also regions that produce large amounts of aromatase enzyme, particularly in the developing brain [110, 111]. Thus this synergy may reflect the requirement for AR to enhance aromatase transcription which in turn is needed to convert the available androgens to estrogens [112]. Another facet of this synergism to consider is that in some cells ER, in addition to, or instead of AR may regulate aromatase gene expression. Recent *in vitro* studies with hypothalamic mouse cell lines have shown that estradiol feeds back on the aromatase promoter to enhance aromatase activity in one line but not in another [113]. Interestingly both the AVP and calbindin promoters contain estrogen responsive elements (ERE) [64, 114]. ER α likely regulates calbindin and AVP gene expression in both sexes via an ERE in the promoter region. No experimental work has been done to explore the possibility of an ARE in either gene.

5.4 Epigenetics

Regardless of which mechanisms are operational, steroid receptors and/or sex chromosome genes, there are many ways that the environment can modify brain sexual dimorphisms. After birth, hypomethylation of the AVP promoter can be induced by early life stress (*i.e.* maternal separation; [115]). The effects have been examined in the “stress-related” AVP neurons in the periventricular region (PVN). However, some of these cells project to the LS where they may contribute to that sexual dimorphism. In addition, no one has systematically asked if the same environmental stress that affects AVP in PVN also affects BNST or medial amygdala. Maternal choline, a methyl donor, is important for neonatal development and neurogenesis. In rats, choline deprivation affects many behavioral outcomes [116, 117] and affects global methylation of the histone H3 in mouse brain [118]. Interestingly, maternal choline deprivation on embryonic days E12-17, decreases H3K9me1,2, both markers of gene repression, in an *in vitro* model of hippocampal, fetal mouse neural progenitor cells [118, 119]. This histone modification was in part responsible for an increase in calbindin gene expression also observed in this model [118]. We speculate that cerebellar sex differences we now attribute to sex chromosome complement may ultimately be traced to histone demethylation. Two of the X-chromosome genes, which escape X-inactivation in brain, *Kdm5c* and *Kdm6a*, code for histone demethylase enzymes and are expressed in the cerebellum [120, 121].

Sex differences in histone modifications occur in the cortex and hippocampus of the embryonic mouse [122]. Sex differences (males>females) were noted on PN0 for two marks; H3K9Me3 and H3K9/14Ac. One mark, H3K9/14Ac was also dimorphic on E18 and the other was dimorphic on PN6 suggesting that the timing of these two sex differences were not identical. More importantly, the acetylation mark was modified in females by administration of testosterone to the dams and the trimethylation mark was unaffected.

In sum, the combination of genetic and hormone manipulations can be employed to precisely uncover the genes that are responsible for the regulation of sex differences in the brain. We have reviewed here what is known to date for just a handful of sexually dichotomous proteins and it is already clear that sex chromosome genes are involved in sexual differentiation of the mouse brain. Second, it is obvious that these genes interact with

steroid receptors. The manner in which these genes interact may be epigenetic. In fact, in general we predict that sex differences are strongly influenced by epigenetic modifications. Some of the variability between individuals must be caused by differences in the neonatal environment, whether the environmental factor is in uterine position and/or grooming and licking by dams, something else, or a combination is not yet known. In rats, stroking of neonatal pups to simulate licking and grooming alters the methylation of the ER α and this environmental modification can eliminate sex differences in ER expression [123]. Of course each subpopulation of sexually dimorphic cells has different phenotypes, which includes an array of different steroid co-activators and repressors. Many of these (CBP, NCor, p300 etc.) are histone modifying enzymes. In turn, several X-chromosome genes that escape X-inactivation, code for similar histone-related enzymes. The cross talk between steroid and sex chromosome genes may in fact be carried on at the level of chromatin modification. We hope to pioneer this new avenue of investigation and inspire others to join with us in these exciting endeavors.

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Fig. 1.

Mean+SEM Calbindin mRNA levels in frontal cortex (**a,b**) and cerebellum. (**c,d**) of FCG mice (**a,c**) and estrogen receptor alpha (ERαKO) mice (**b,d**). In the frontal cortex of FCG mice (**a**) and ERαKO mice (**b**), * Significantly different from all other groups ($p<0.05$). In the cerebellum of FCG mice (**c**) * XX mice had significantly more Calbindin mRNA than XY mice ($p<0.0001$). In the cerebellum of ERαKO mice (**d**) ** Wild type (WT) littermates had significantly more Calbindin mRNA than their ERαKO littermates ($p<0.007$). *** Females had greater concentrations of Calbindin mRNA than males ($p<0.015$). $n=5-6$ mice per group. WT=wild type, KO=estrogen receptor KO

Table 1

Protein and regional sex differences in mouse brain. Distribution of calbindin, tyrosine hydroxylase and vasopressin in different areas of mouse brain as a function of sex differences; Males (M), female (F), cerebellum (CB), frontal cortex (FC), medial preoptic area (MPOA), anteroventral periventricular nucleus (AVPV), lateral septum (LS), bed nucleus of the stria terminalis (BNST). Sex differences are regulated by estrogen receptor α (ER α), estrogen receptor β (ER β), androgen receptor (AR) and/or sex chromosomes. Regulatory factors that have not been examined in a specific brain region (nd)

Sex dimorphism regulated by	Calbindin		Tyrosine hydroxylase			Vasopressin		
	CB F>M	FC F>M	MPOA M>F	AVPV F>M	Midbrain M>F	LS M>F	BNST M>F	BNST M>F
ER α	no	yes	yes	yes	no	yes	yes	yes
ER β	nd	nd	nd	yes	no	nd	yes	yes
AR	nd	nd	yes	no	no	yes	nd	nd
Sex chromosomes	yes	yes	nd	no	yes	yes	yes	nd