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Gender Differences in Neurodevelopment and Epigenetics

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Summary

The concept that the brain differs in make-up between males and females is not new. For example, it is well-established that anatomists in the nineteenth century found sex differences in human brain weight. The importance of sex differences in the organization of the brain cannot be overstated as they may directly affect cognitive functions, such as verbal skills and visio-spatial tasks in a sex-dependent fashion. Moreover, the incidence of neurological and psychiatric diseases is also highly dependent on sex. These clinical observations reiterate the importance that gender must be taken into account as a relevant possible contributing factor in order to understand the pathogenesis of neurological and psychiatric disorders. Gender-dependent differentiation of the brain has been detected at every levels of organization: morphological, neurochemical, and functional, and have been shown to be primarily controlled by sex differences in gonadal steroid hormone levels during perinatal development. In this review, we discuss how the gonadal steroid hormone testosterone and its metabolites, affect downstream signaling cascades, including gonadal steroid receptor activation, and epigenetic events in order to differentiate the brain in a gender-dependent fashion.

Introduction

The importance of gonadal steroid hormones for behavioral regulation was shown early on in Berthold's experiments conducted in 1849 [23], who showed that male-typical behaviors in roosters, such as crowing, aggression, and male sexual behavior, disappeared after castration, whereas replacement of the missing gonads restored the male-typical behaviors. However, it was not until Phoenix and colleagues (1959) who demonstrated that testosterone administration in pregnant guinea pigs caused the female offspring to display male sexual behavior as adults, that the idea of gonadal steroid hormone-dependent sexual differentiation of the brain itself was put forward [133]. More importantly, these studies led to the pivotal hypothesis that gonadal steroid hormones actions on the mammalian brain can be categorized as organizational *versus* activational. In general, organizational effects of gonadal steroid hormones during perinatal development are thought to be permanent, whereas activational effects are transient, and mainly restricted to adulthood.

In the early 1970's it was finally confirmed that the central nervous system (CNS) itself contains specific regions that differ between males and females at the neuronal and synaptic level [32,136]. A further landmark discovery was that the medial preoptic nucleus (MPN) is 2–6 larger in males than in females [64]. These studies also confirmed the organizational

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versus activational hypothesis at the level of the brain, as changes in gonadal steroid hormone levels had no effect on the size of the adult rat MPN [64]. In contrast, perinatal castration of male rat pups resulted in a female-sized MPN, whereas neonatal female rats injected with testosterone showed a malesized MPN in adulthood [64]. These initial reports and others solidified the idea that the vertebrate brain is organized in a sex-dependent fashion under the control of perinatal gonadal steroid hormones (i.e., testosterone) [10,114,21]. In this review, we will discuss the role(s) of the gonadal steroid hormone system, and its interaction with epigenetic events to cause brain sexual differentiation.

Sex Determination

The fundamental fact is that brain sexual differentiation cannot begin without the initiation of normal sex determination of the fetal gonads under influence of genetic sex. In early fetal development, the gonads (i.e., primary source of plasma gonadal steroid hormones) do not differ between males and females, and have therefore been called bi-potential gonads. Differentiation of the male fetal gonads into testes is in essence controlled by the sex determining region-Y chromosome (SRY) protein, which is encoded by the sex determining region-Y chromosome (*Sry*) gene located on the short arm of the Y chromosome [153,85,22,92]. The SRY protein, a member of the SOX protein family of DNA transcription factors, differentiates pre-Sertoli cells to Sertoli cells in the testis cords [134]. In turn, Sertoli cells secrete Desert Hedgehog protein, which causes the differentiation and expansion of the nearby located testosterone-synthesizing Leydig cells [177]. In the absence of *Sry*, as is the case in the female fetus, the bi-potential gonads differentiate into ovaries.

Inherent to testes formation, overall testosterone levels are higher in males than in females during mammalian fetal and perinatal development [42,52,171]. Interestingly, circulating levels of testosterone are markedly increased in males at specific time points in development. In male perinatal rats, circulating testosterone levels peak around embryonic day 18 and 19, which is followed by a smaller peak in testosterone levels just hours after birth [41,42,171]. Studies in rats showed that this sex difference in circulating levels of testosterone only has a small developmental window of opportunity to cause the organizational (permanent) sex-dependent changes in mammalian brain morphology and function. In rats, this so-called “critical period”, in which testosterone can program permanent sex-dependent central changes to the morphology and neurochemical phenotype of the brain has been pinpointed to start between embryonic day 18 and approximately end 10 days after birth, which coincides with the perinatal sex differences in circulating levels of testosterone in the rat [45]. In humans, circulating testosterone levels in the male fetus are also much higher than in the female fetus. Specifically, testosterone production in the male human fetus start and rises during the second month of the first trimester and reach its highest levels in the second trimester, which are maintained until late gestation (i.e., third trimester) when testosterone are only slightly higher in males than in females at the time of birth. In the first neonatal year, a second surge in testosterone plasma levels has been observed, which subsides until the onset of puberty [1,41]. Therefore, the sex difference in testosterone levels is, as in rodents, the primary signal that initiates human brain sexual differentiation.

Observations of Morphological and Neurochemical Brain Sex Differences

The concept that the brain differs in make-up between males and females is, of course, not new. For example, it is well-established that anatomists in the nineteenth century found sex differences in human brain weight [157]. These early weight measures are in line with more recent studies showing that the total volume and number of neurons of the human neocortex is about 10–15% larger in men than in women [129].

The importance of sex differences in the organization of the brain cannot be overstated as they may directly affect cognitive functions, such as verbal skills and visio-spatial tasks in a gender-dependent fashion see for reviews [21,155,159]. Moreover, the incidence of human neurological and psychiatric disorders is highly dependent on gender. For instance, the incidence of anorexia nervosa and bulimia is much higher in women than in men, whereas the opposite is true for dyslexia, sleep apnea and Gilles de la Tourette [159]. The incidence of gender identity disorders depends on sex as well. For instance, in the Netherlands, there are about three times fewer male-to-female transsexuals than female-to-male transsexuals [166]. These clinical observations reiterate the importance that sex must be taken into account as an important and relevant contributing factor when considering the possible mechanisms that result in the onset of neurological and psychiatric disorders.

Sex differences in the CNS have been found at every level of brain organization: brain area volume, cell number, cell cytoarchitecture, cell activity, synaptic connectivity and neurochemical content, and in a large number of organisms, such as fish, lizards, songbirds, rodents and primates including humans [15,21,19]. For example, the thoracolumbar intermediolateral nucleus of the spinal cord in male cats was shown to contain more sympathetic motoneurons than in the female cat [32]. In the same year, Raisman and Field (1971) reported that the preoptic area in rats contained more synapses from non-amygdaloid origin in females than in males in adulthood [136]. This was of particular interest, because they also demonstrated that a single injection with testosterone in newborn female rats decreased the number of synaptic contacts, whereas neonatal castration increased the number of synaptic contacts [136,137]. Similar sex differences in synaptic wiring were also found in other hypothalamic and limbic regions of the rodent brain [120,106]. Soon after the findings of Raisman and Field, much more dramatic gonadal steroid-responsive sex differences were found in other vertebrate species, such as in song birds. For instance, song-regulating brain areas in canaries and zebra finches are 6 times larger in males than in females [122]. In the next sections, we will highlight the most prominent sexually dimorphic areas that have been discovered to date in the preoptic/hypothalamic and limbic brain regions.

Medial Preoptic Nucleus

The rat sexually dimorphic medial preoptic nucleus (MPN) is anatomically larger and contains more cells in males than in females [150,64,125,30], and has been found in the preoptic area of the ferret, gerbil, guinea pig and hamster brain [27,40,72,161]. The divergence of MPN between males and females becomes measurable around birth and is completed within the first 10 days after birth [45,64,82,39]. Lesion studies in rats showed that the MPN may be involved in the regulation of sexual behavior [8,46]. The first study to report that the human preoptic/hypothalamic region also contains a sexually dimorphic nucleus was published by Swaab and Fliers in 1985, who showed that the human interstitial nucleus of the anterior hypothalamus 1 (INAH-1) is about two times larger, and contains more cells in young adult men than in young adult women [156,158,74]. Since then, other studies reported additional sexually dimorphic nuclei in the human preoptic/hypothalamic region. Notably, INAH-2 and INAH-3 were shown to be also larger in men than in women [5,99,31].

The MPN is also sexually dimorphic in terms of neurochemistry. For example, the male rodent MPN contains more galanin expressing cells and is more heavily innervated with serotonin fibers than the female MPN [28,150]. On the other hand, the female's rat MPN is heavily interconnected with brain areas that are involved in gonadotropin release, and female copulatory behavior, such as the anteroventral periventricular nucleus (AVPv), bed nucleus of the stria terminalis (BST), amygdaloid nucleus and ventral medial hypothalamus (VMH). Therefore, it may also contribute significantly to the regulation of female sexual

behavior [66,77,50]. In humans, it is at present not known whether the MPN is involved in similar behaviors. The presence of morphological and neurochemical sex differences in the MPN is indicative for the possibility of sexual differences in brain functions. On the other hand, it has been proposed that brain sexual differentiation may be an adaptation in order for the male and female brain to function similarly [50].

Bed Nucleus of the Stria Terminalis

This limbic forebrain region is part of a continuum of columns of sublenticular cell groups traversing the basal forebrain and cell groups that accompany the stria terminalis, which is developmentally closely related to the amygdaloid nucleus [3,144]. The rodent principal nucleus of the BST (BSTp) is about two times larger and contains more cells in males than in females [72,51,67,88,39], whereas the opposite is true for the lateral anterior BST and medial anterior BST [51, 67]. Similar to the MPN, the sexual differentiation of the BSTp occurs during the first 10 days after birth [39,51,67]. The human BST also contains several subdivisions that differ in volume between men and women. Both the darkly staining posteromedial component of the BST and the central nucleus of the BST (BSTc) are larger in men than in women in adulthood [180,4,5,36], both of which have been shown to be observable after ten years of age or in adulthood only, respectively [36,4]. These data demonstrate that the period of organizational sexual differentiation of the human brain occur over a much more protracted period of time than found in the rodent brain. Consequently, illustrating the importance that data from animal studies must complement with human brain studies in order to understand the sexual differentiation of the human brain itself.

The rodent and human BST also exhibits prominent sex differences in neurochemistry. For example, the number of vasopressin, substance P, and cholecystokinin producing cells in the rat BST is larger in male than in female rats [47,48,50,113]. Relatively, recent studies showed that the human male BSTc contains more vasoactive intestinal polypeptide fibers and somatostatin neuronal staining than in the female BSTc [94,180,36]. These data have helped us to better understand the inherent functions of this region in the human brain. Functionally, the BST has been shown to be involved in the regulation of a number of behaviors, such as reproduction, aggression, addictions, parental behavior and stress [59,57,2,69,101,163, 169, 20]. In humans, the BSTc has been shown to develop differentially in people with a gender identity disorder called transsexuality, in which subjects express the strong feeling of being born in the wrong body. Indeed, these studies showed that the size of the BSTc in male-to-female transsexuals is similar to that found in control women, whereas in the only female-to-male transsexual studied so far the BSTc size was similar to that found in men [94,180]. Therefore, it might be that the human BSTc has a role in human gender identity, however, it must be noted that this is merely correlational, and require far more studies to clearly elucidate the functions of the human BSTc.

Anteroventral Periventricular Nucleus

Some brain regions are larger in females than in males. For example, the rat, mouse, hamster and gerbil AVPv was shown to be larger in females than in males [27,121], and is heavily interconnected with other brain areas, such as the BST, organum vasculosum laminae terminalis (OVLT) and arcuate nucleus [77].

The rat AVPv also contains neurochemical sex differences that are biased in a female direction. For instance, more dopaminergic cells are found in the female AVPv than in the male AVPv [66]. Interestingly, the AVPv is more heavily innervated by the BST and MPN in males than in females [77]. The ascending AVPv projections terminate, in part, close to the OVLT where gonadotropin-releasing hormone containing cells have been observed. Because descending AVPv fibers terminate in the periventricular nucleus and arcuate

nucleus, it has been suggested that the AVPV may function as a nodal point in the regulation of gonadotropin secretion [174,56]. At present, it is not known whether the human brain contains a similar analogous AVPV brain region.

Ventromedial Hypothalamic Nucleus

The VMH in the rat brain is another prominent region that is larger in males than in females, which is first detectable ten days after birth [105,36]. Further analysis showed that the number of synaptic contacts is higher in males than in females [106,135]. Tracing experiments showed that the VMH projects to many sexually dimorphic and non-sexually dimorphic brain areas, such as the MPN, lateral septum, BST and paraventricular nucleus [33]. Presently, it is unknown whether the VMH in the human brain is sexually dimorphic in volume. However, studies showed that metabolic activity in the human VMH seemed to be higher in young women than in young men. As the metabolic activity of the VMH appears to increase with age in men, it has been proposed that androgens may inhibit metabolic activity in the VMH [80].

The VMH has been implicated in the regulation of feeding behavior, and also plays a central role in the regulation of male and female reproductive behavior [131,132,123]. For instance, neurons residing in the lateral ventral portion of the VMH have been implicated in the regulation of lordosis after appropriate priming with estradiol and progesterone [26,11]. The VMH in the human brain may be involved in the sexually dimorphic integration of pheromonal input. Positron emission tomography scan studies in humans showed that an androgen-like compound activated the female hypothalamus centering on the VMH, while in males the activation of the hypothalamus by an estrogen-like substance was centered on the PVN and dorsomedial hypothalamus [143].

Testosterone Metabolites Facilitate Neuronal Sexual Differentiation

Evidence from studies investigating the development of sex differences in animals showed that testosterone's effect on brain sexual differentiation are mediated by its metabolite estradiol, a conversion process facilitated by neuronal aromatase [110,102]. In general, rodent studies showed that perinatal estradiol treatment can reproduce the masculinizing effects of testosterone on brain organization and behavior in gonadectomized animals [19,15,38]. For example, although the neonatal MPN showed the ability to bind testosterone or its metabolites estradiol and dihydrotestosterone (DHT), it was only estradiol treatment in female and castrated male rat pups that was able to increase the size of the MPN [64,81]. Moreover, newborn brain areas that contain anatomical and neurochemical sex differences in adulthood, such as the preoptic area, hypothalamus and limbic system contain high aromatase expression [86,118,142]. Masculinizing effects of testosterone brain development and function were also effectively blocked by estrogen antagonists and aromatase inhibitors [109,110,168,16–18].

On the other hand, perinatal treatment with the non-aromatizable androgenic metabolite of testosterone, DHT, showed little to no ability to affect the MPN in a testosterone-like manner [15,21,19]. However, DHT's role in sexual differentiation cannot be fully discounted. Indeed, the sexually dimorphic vasopressinergic system in the BST (m > f) requires the presence of both estradiol and DHT to be fully maintained [47,48,128]. The ability of DHT to partially maintain this particular sexual dimorphism may be due to its intracellular conversion to its metabolite 3 β -diol, which has a relatively high affinity for estrogen receptors (ER) [128]. Although DHT's role cannot be discounted completely during brain sexual differentiation, it has been shown beyond doubt that the testosterone causes sex-dependent brain development, primarily through its intracellular aromatase-dependent conversion to estradiol.

Estrogen Receptors

Actions of gonadal steroid hormones are classically described as being mediated through their specific receptors, which are part of a large family of nuclear steroid hormone receptors [60,141]. Despite the large diversity in steroid hormone receptors, they are highly conserved transcription factors, which have variations of a general basic modular organization. They contain a ligand binding domain, DNA binding domain and transactivation domain. The ligand - and DNA binding domain of the steroid receptor conveys specificity for ligands and binding to hormone response elements on DNA, respectively. Classical activation of steroid hormone receptors through a specific ligand(s) causes homo- or heterodimerization and translocation of the ligand-receptor complex to the nucleus, which in turn will interact through the DNA binding domain with its specific hormone response element on DNA in order to regulate gene transcription. Classical activation of steroid receptors is not the solitary mode of cellular signaling, but may include the activation of so-called non-genomic second messenger pathways. Moreover, several studies showed that many of these steroid hormone receptors, such as ER's are expressed as mRNA splicing variants or membrane receptors throughout the body, including the brain, and has specific transcriptional and epigenetic consequences [37,116,147,172,173,58, 68, 53, 79].

Estrogen receptors were the first nuclear receptors to be discovered [162]. Several laboratories have found two subtypes of ERs in the mouse, rat, and human brain [95,115,164], which have been referred to as ER α and ER β . The two receptors are encoded by separate genes that are located on different chromosomes [95]. ER α and ER β expression in the rat brain shows considerable overlap in brain areas, such as the MPN, BST, amygdala, lateral habenula, and midbrain regions [149,146]. On the other hand, the VMH and subfornical organ contain almost exclusively ER α , while neurons of the olfactory bulb, supraoptic nucleus, PVN, suprachiasmatic nucleus, zona incerta, ventral tegmental area, cerebellum, and pineal gland among other areas are exclusively ER β positive [146].

ER α and ER β distribution has also been studied in the human brain, and is largely consistent with the results from animal studies. Examples of human brain areas that contain ER α and ER β expressing cells are the diagonal band of Broca, nucleus basalis of Meynert, interstitial nucleus of the anterior hypothalamus, BST, amygdala, PVN, supraoptic nucleus, arcuate nucleus, hippocampus, and cerebral cortex [54,63,127,70]. Postmortem human brain studies further showed that both ER α and ER β differ in expression in the INAH-1 and BST between men and women in adulthood [93].

Expression of ERs in rodents and primates is regulated by circulating levels of gonadal steroid hormones. Indeed, removal of circulating levels of testosterone or its metabolite estradiol increases expression of ER α in the rat sexually differentiated brain region, such as the AVPv, MPN, BST, and VMH [151,100,55]. ER β expression in the PVN of the rat brain was shown to be decreased by estradiol, while estradiol had no effect on ER β expression in the MPN or BST [130,154]. On the other hand, estradiol seems to up-regulate ER β expression in the arcuate nucleus of the rat brain [126]. In humans, ER β expression may be affected by circulating levels of gonadal steroid hormones. For example, ER β expression was higher in the SON of young women as compared to postmenopausal women, while ER α expression was lower in young women than in postmenopausal women [78]. Together these data are indicative of a gonadal steroid hormone system that is dynamic in nature and responsive to ever changing central and peripheral cues.

How Do Gonadal Steroid Hormones Cause Sexual Differentiation?

Gonadal steroid hormones acting through their specific receptors regulate sexual differentiation of the mammalian brain by affecting one or more of four major

developmental processes: neurogenesis, neuronal migration, apoptosis and/or differentiation of cell phenotype. To date no studies have found strong evidence for the direct involvement of gonadal steroid hormone-dependent neurogenesis or neuronal migration in the sexually dimorphic organization of the MPN or BST [83,84,82].

Apoptosis is a highly regulated distinct form of cell death that histologically is characterized by shrinkage of cell cytoplasm, condensation of chromatin, blebbing of cell membrane and formation of membrane bound apoptotic bodies containing intact organelles and condensed chromatin [89]. Biochemical analysis indicated that DNA fragmentation during apoptosis occurs in multiples of 180–200 base pairs [9]. Many studies showed that apoptosis is a widespread phenomenon during early brain development, which is required to remove brain cells that do not migrate, differentiate and/or form appropriate neuronal circuits in a given developmental time period [43,124]. Examples of apoptotic cell death during brain development have been described early on, for instance in the rodent striatum, hippocampus, amygdala and cerebellum [111,87]. The presence of apoptotic cell death has also been documented in a number of studies investigating fetal human brain [35,152,138].

Apoptosis has been observed during sexual differentiation of a number of regions in the preoptic area in the rat brain. For example, the incidence of apoptosis in the perinatal AVPV was higher in males than in females, while the incidence of apoptosis in the postnatal MPN was higher in females than in males [6,7,44,39]. Moreover, these studies showed that testosterone or its metabolite estradiol increased the incidence of apoptosis in the perinatal rat AVPV, whereas testosterone or its metabolite estradiol decreased the incidence of apoptosis in the developing rat MPN [39,44]. These studies suggest that gonadal steroid hormones control the sexual differentiation of the vertebrate brain through the context-dependent induction or prevention of apoptotic cell death.

The importance of apoptosis during sexual differentiation of the BST was inferred from earlier studies. One of the most prominent cytoarchitectural sex difference in the rat is the BSTp, which is larger and contains more cells in males than in females [51,67,71,39]. Sexual differentiation of the BSTp is controlled by the sex difference in early circulating levels of testosterone [51,67,39]. Our studies showed that the incidence of apoptosis in the BSTp during the first postnatal week was much higher in females than in males. Moreover, the size of the BSTp became larger in males than in females only after the sex difference in apoptosis [39]. In addition, the incidence of apoptosis was higher in animals devoid of testosterone than in animals with testosterone [39]. These results strongly suggest that sex differences in developmental apoptosis are prerequisite for the gonadal steroid hormone-dependent sexual differentiation of the BSTp in the rat brain.

Gonadal Steroid Hormone Regulation of Apoptosis

Although testosterone was shown to be effective in protecting BST and MPN cells against apoptosis; this effect is facilitated by its estrogenic metabolite acting on ERs. Estrogen-bound ERs form homodimers or heterodimers and translocate to the cell nucleus to associate with specific estrogen response elements (EREs) on DNA [91,148,49], which are palindromic enhancer sequences located in promoter regions to modulate the transcriptional activity of genes involved during apoptosis. For example, members of the *Bcl-2* gene family, including the anti apoptotic *Bcl-2* and *Bcl-XL* have the putative EREs in their promoter regions supporting the idea that the presence of testosterone-derived estradiol may directly modulate the transcriptional activity of genes that favor cell survival [49,175]. Indeed, estradiol increased *Bcl-2* and *Bcl-XL* expression in neuronal cell lines [175,49,108], while decreasing the expression of *Bad* mRNA, a proapoptotic *Bcl-2* family member [107]. Estrogens also decrease the expression of cellular factors, such as *Bnip-2* mRNA which in

turn down-regulate *Bcl-2* expression [76]. Conversely, estradiol removal increased mRNA expression of two proteolytic so-called initiator *Caspases* (i.e., 1 and 2) in chick oviduct studies, while at the same time activating the executioner proenzymes, caspase-3 and caspase-6 [75]. More recently, the sex difference in BSTp apoptosis was shown to be dependent on *Bax* function [39,104]. Interestingly, testosterone's ability to prevent BSTp apoptosis could be recapitulated with ER and ER selective agonists [73]. Together these studies suggest that estrogen-bound ERs oppose apoptosis by genomically acting on the molecular mechanisms that control cell survival.

Gonadal steroid hormones may regulate cell survival by acting on the transcription level of neurotrophic factors. Indeed, the gene encoding for brain-derived neurotrophic factor (BDNF) contains a putative ERE. Moreover, estrogen increased mRNA levels of BDNF in the rat cerebral cortex and olfactory bulb [147]. Similarly, androgens rescue motoneurons in the spinal nucleus of the bulbocavernosus androgens is facilitated by ciliary neurotrophic factor (CNTF) expressed in the perineal muscles, which act on CNTF receptors located on the motoneurons [173]. These studies indicate that gonadal steroid hormone regulate cell survival not only directly, by targeting the expression of specific components of the apoptotic cell death mechanisms, but also indirectly by modulating the expression of neurotrophic factors.

In vitro studies strongly suggest that gonadal steroid hormones can also regulate apoptosis through non-genomic pathways. In particular, estrogen-bound ERs interact directly with phosphatidylinositol-3 kinase (PI3K) through protein:protein binding, which in turn phosphorylates the downstream effector AKT to rescue cortical neurons [75,76]. Estrogen-dependent rescue through the PI-3K/AKT pathway was prevented by ICI 182,780, a selective ER antagonist or LY 294002, a selective PI3K inhibitor [76]. *In vitro* studies also showed that PI3K/AKT phosphorylation of the AR in similar prostate cancer cells may inhibit apoptosis [145]. In addition, PI3K/AKT was shown to increase AR mRNA expression [104]. These studies suggest that apoptosis-dependent sexual differentiation of the vertebrate brain may be mediated by the PI3K/AKT signaling pathway.

Epigenetics: A Further Layer of Complexity

Research from the last few decades has uncovered a wealth of information about the vertebrate brain sexual differentiation. More recent studies have focused on the field of epigenetics, which has been defined in a multitude ways, ranging from those that include a heritable component in gene function to those simply state that epigenetic changes are molecular events that remodel chromatin without altering the underlying DNA code [24]. Much of our fundamental knowledge is derived from studies examining the epigenetic regulation of steroid hormone receptors, in particular glucocorticoid receptors and ER's [170,34,97]. In this review, we minimally defined epigenetics as a change to chromatin without changing the underlying DNA code, similar to Adrian Bird's definition [24]. Therefore, the heritability of epigenetic remodeling is irrelevant for this definition. Furthermore, it should be noted that an epigenetic change does not always result in immediate alteration of gene transcription, but it may alter the genes response to future signaling events. This inherently complicates our task to relate epigenetic remodeling of DNA to disease and mental health, because epigenetic changes of a particular gene(s) may directly participate in neurological pathogenesis, or merely alter the probability or severity of a disorder in response to additional gene or environmental challenges.

Steroid Receptor Co-Regulators

Most of what we know about early gonadal steroid hormone action in the brain is due to its activational effects (i.e., increase) on gene transcription, which generally involves the

recruitment of coactivator complexes, such as steroid receptor coactivator-1 (SRC-1) and cAMP response element binding protein-binding protein (CPB) [160]. These coactivator complexes can directly alter histone acetylation through their own histone acetyltransferase (HAT) activity or indirectly through their association with other complexes exhibiting HAT activity. The primary consequence of histone acetylation is to reduce the affinity between histones, resulting in the unwinding of chromatin structure, and consequently in heightened gene transcription efficiency.

Both SRC-1 and CBP have been shown to be critical for brain sexual differentiation. Indeed, reduced SRC-1 expression in the developing hypothalamus prevented sexual differentiation of adult sexual behavior in males, and the androgenization of sexual behavior in females [13]. These data indicate that gonadal steroid hormone actions in the developing brain require the increase of histone acetylation and unwinding of the chromatin structure [12]. This idea is supported by recent studies showing that males have higher levels of acetylated histone H3 as compared to females during neonatal brain development [165].

Recent findings indicate that some molecules associated with gene repression are also important in mediating brain sexual differentiation. For instance, DNA methylation can initiate a cascade of events leading to gene repression, and occurs when a methyl group is attached to cytosine within a 5'-CpN-3' dinucleotide, an enzymatic reaction catalyzed by DNA cytosine-5-methyltransferases (DNMTs) primarily at CpG sites [65,139]. The act of CpG site methylation in itself typically does not have a direct impact on gene transcription rates. Rather inhibition of gene transcription occurs when methyl-CpG-binding proteins (MBPs) are bound to the methylated DNA, which recruits co-repressor complexes as opposed to coactivators. The histones deacetylase (HDAC) activity from these co-repressor complexes will deacetylate histones resulting in chromatin condensation, and consequently gene repression [179,25,90].

An very exciting line of research showed that relatively subtle changes in maternal care resulted in the significant modification of the DNA methylation patterns of steroid hormone receptor genes, such as ER and glucocorticoid receptors, within the developing offspring brain [170,34]. These data illustrate the plasticity of DNA methylation patterning within the developing postnatal brain. This is further reiterated in our recent assessment of the tactile components of maternal care on ER α expression, and its promoter methylation by simulating maternal grooming (SMG).

During brain development and in adulthood ER α expression within the rodent MPN is higher in males than in females [55,176,178,29,98,103]. In line with these observations, we found that the 5' flanking region of ER α exon 1b promoter region in males had higher levels of CpG methylation than females, which may be the underlying cause of reduced ER α expression [97]. Moreover, in our studies we were able to use SMG to not only decrease ER α expression, but also increase ER α promoter methylation in females to male-like levels [97]. These data indicate that programming of a critical signaling pathway for brain sexual differentiation, ER α expression, during brain development is not only influenced by internal changes in gonadal steroid hormone environment, but also neonatal social (i.e., external) cues. Interestingly, the programming of ER α expression appears to be associated with gene repression in males. Also intriguing, is that estradiol treatment caused increased ER α promoter methylation in females. Together, these data suggest that sexual differentiation of the brain may require the interplay of both activation and repression of gene expression.

As stated above, DNA methylation does not directly alter gene transcription rates; rather it is methyl-binding proteins binding to methylated CpG sites that lead to gene repression. Therefore, we transiently reduced the expression of methyl CpG binding protein 2 (MeCP2)

during amygdala development, and assessed whether this intervention could impact the sexual differentiation of juvenile social play behavior. Remarkably, our studies showed that a relatively subtle reduction in neonatal amygdaloid MeCP2 expression was able to block the masculinization of juvenile social play behavior in males [96,61] without disrupting juvenile sociability. Together these data suggest that the differentiation of the male brain can necessitate increased methylation of some genes during development, and consequently require methyl-binding protein function (i.e. MeCP2) to fully differentiate the male brain. These data further support the idea that some genes need to be repressed for appropriate masculinization of the brain. Further evidence for this concept comes from experiments that disrupt HDAC function during early brain development. Specifically, treatment of neonatal males with valproic acid (HDAC inhibitor) disrupted BST masculinization [117], and was shown to increase histone H3 acetylation. A likely interpretation of these data is that masculinization of the brain requires the inhibition of some genes. Of course, one could then hypothesize that these genes may be involved in feminizing the brain, and therefore need to be turned OFF for masculinization of the male brain to proceed properly.

Epigenetics is a further regulatory layer that can explain how cells with the same genetic material produce different phenotypes [119]. The general consensus is that methylation of cytosine into 5-methylcytosine is relatively stable, and is maintained through DNA replication. However, it was also suggested over 30 years ago that there may be plasticity (i.e. "shuffling") in DNA methylation patterns throughout cellular differentiation [140]. This was recently confirmed in a study that showed that methylation patterns of the estrogen-responsive pS2/TFF1 gene can undergo cyclical changes within minutes to hours [112]. Similar findings have been reported to occur in regulation of the gonadal steroid hormone-dependent vasopressinergic system in the rat BST [14]. Based on these dynamic experiments, it has been conjectured that cyclic pattern of methylation is required for gene transcription, and disrupting this cycle may ultimately block gene transcription. These findings challenge and seriously question the validity of the concept that methylation patterns are always stable, and support the idea that there are both stable pattern and highly dynamic methylation patterns. Previous research has hypothesized that there are gradual shifts in human DNA methylation patterns during ageing. Sometimes these inappropriate shifts in DNA methylation patterns can be observed as aberrant methylation patterns in some cancer cells or in some neurological diseases later in life [167]. Moreover, studies in twins revealed their DNA methylation patterns to be very similar during early childhood, but go on to be very different in adulthood [62]. These epigenetic studies are in line with this reviews common theme that the cellular and molecular mechanisms of brain sexual differentiation are far from being static, but show a high level of temporal and spatial plasticity.

In Conclusion

The wealth of information that has been generated over the last several decades has revealed that brain sexual differentiation is a highly regulated "event" that occurs throughout life, and has been shown to include a multitude of molecular, cellular and epigenetic mechanisms, which can even be detected through gross measures, such as brain area size or cell number. However, there are still many important questions that remain to be studied. For instance, how does testosterone cause male-biased cell death in the AVPv, while this is female-biased in the MPN and BST? In other words, what are the molecular mechanisms that underlie and specify region-specific sexual differentiation? Similarly, can sex-dependent epigenetic changes on the DNA during early brain development be reversed in adulthood, and would this cause changes in established behaviors? Notwithstanding the importance of these immediate questions, there is need to include gender in the analysis of the pathogenesis of neurological and psychiatric disorders, especially given the fact many of these disorders are

distributed in a gender-dependent fashion. However, what does this mean? Does it mean that gender increases the vulnerability and predisposes the brain to a particular neurological or psychiatric disorder? Or could it be that the cause(s) of a disorder is similar in males and females, but that the pathogenesis process is more or less 'effective' in males *versus* females. To better understand this concept, further analyses are necessary to examine the true relationship between the sexual differentiation process of human brain and the gender-dependent incidence of neurological and psychiatric disorders.

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