

# Epigenetic Underpinnings of Developmental Sex Differences in the Brain

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## Key Words

Sexual differentiation · Sexual dimorphism · Epigenetics · DNA methylation · Histone acetylation

## Abstract

Sexual differentiation of the brain is a crucial developmental process that enables the lifelong expression of sexually dimorphic behaviors, including those necessary for successful reproduction. During a perinatal sensitive period, gonadal hormones defeminize and masculinize the male brain, and a lack of gonadal steroids allows for feminization in the female. This hormonally-induced differentiation permanently alters neural structures, creating highly dimorphic brain regions; however, the mechanism by which hormones exert their long-lasting effects are still largely unknown. Epigenetic processes such as DNA methylation and histone modifications serve as an interface for environmental stimuli to exert control over the genome. These modifications have the capacity to activate or repress gene expression, thereby shaping the developmental outcomes of cells, circuits, and structures in the brain. Sex differences in methylation, methyl-binding proteins, and chromatin modifications indicate that epigenetic mechanism may be important for sexual differentiation of the brain. The data outlined in this review provide evidence that gonadal hormones in the neonatal brain

influence epigenetic processes such as DNA methylation and histone acetylation, but also emphasize the primitive status of our current understanding of epigenetics and sexual differentiation and the brain.

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## Sexual Differentiation of the Brain

Sexual differentiation of the brain is a hormonally-driven process involving steroids synthesized by the recently differentiated gonads in the developing embryo. Sexual differentiation is distinguishable by sex differences, which may occur in the adult and be the product of sex-specific gonadal steroids, or the result of genes on the X or Y chromosome [1, 2]. Adult sex differences established by hormones should disappear following gonadectomy, whereas those endpoints that are truly sexually differentiated will endure. Whether genetics contributes only to sex differences or also impacts sexual differentiation remains to be determined. Hormonally-mediated sexual differentiation consists of two active processes: defeminization and masculinization. Defeminization involves the permanent elimination of the female brain phenotype controlling sexual behavior and therefore a loss of the ability to express female sexual behavior. Mas-

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culinization is the active development of the male-typical neural anatomy and the capacity to express male sexual behavior in adulthood. Together, these two processes differentiate the male brain from the female, the development of which is defined as feminization [3].

In the rodent there is a carefully defined critical period for brain sexual differentiation that occurs between embryonic day 18 (E18) and approximately postnatal day 10 (PN10) [4, 5]. The onset of this critical period coincides with the beginning of testosterone production by the testes in genetic males, while the offset is defined as the point after which females are no longer masculinized by exogenous steroid treatment. In male fetuses high levels of testosterone synthesized by the testis reach the brain where a portion is converted to estradiol by the enzyme p450 aromatase [6, 7]. Estradiol acts via its nuclear receptors, estrogen receptors  $\alpha$  (ER $\alpha$ ) and  $\beta$  (ER $\beta$ ) to initiate defeminization and masculinization [8, 9]. When bound by ligand, both ER isoforms can act as transcription factors, thereby influencing gene expression [10]. Administering testosterone or estradiol to a genetic female during the critical period for sexual differentiation of the brain leads to a defeminized and masculinized neural phenotype, rendering this female anovulatory and therefore sterile [11], and capable of male-typical behavior in adulthood if treated with testosterone [12]. Conversely, blocking aromatization of testosterone to estradiol or antagonizing ER binding during the critical period impedes organization of the male brain [13, 14]. Importantly, these manipulations have no impact on the determination of brain sex if made before or after the critical period, indicating a highly regulated and tightly orchestrated timeframe for these crucial organizational events. If one considers the importance of matching brain and body sex for successful reproduction and species propagation, it seems logical to have a highly controlled critical period for sexual differentiation of the brain. However, the question of how such fleeting hormone exposure can have enduring, lifelong consequences on the nervous system, and ultimately reproductive fitness, remains to be determined.

### Epigenetic Processes

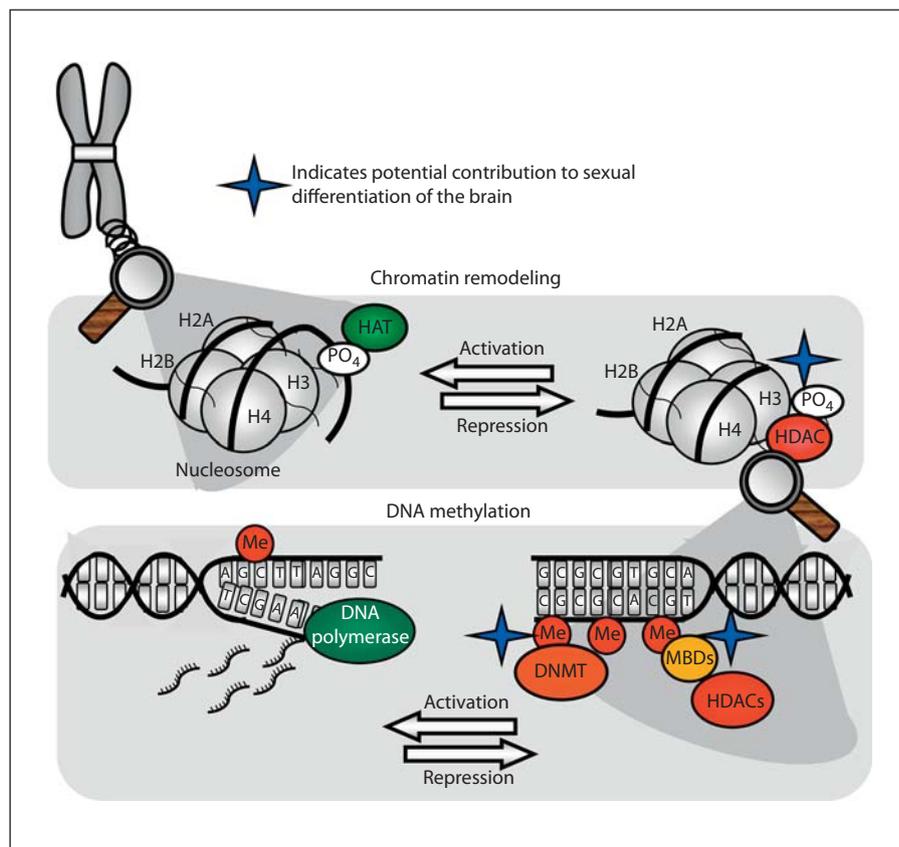
How does brief hormone exposure during early development permanently organize sex differences in the brain? Emerging interest in the field of epigenetics has provided potential answers to this question. The term *epigenetics*, literally meaning ‘above genetics’, refers to modifications made to the genome that can impact gene

expression without affecting the underlying DNA sequence. Epigenetic processes are important for coordinating the impact of environmental factors during developmental sensitive periods [reviewed in 15]. Steroid hormones are a component of the internal environment that vary between males and females, and steroid receptors associate directly with DNA and enzymes that mediate some forms of epigenetic changes, making them ideal candidates to exert a lasting effect on the epigenome, or the overall epigenetic state of a cell.

The genome is contained within chromosomes, which are comprised of a highly organized mix of DNA and histone proteins called chromatin. Chromatin’s specialized structure ensures that lengthy stretches of DNA are efficiently packaged within the confines of a cell’s nucleus in addition to aiding mitosis, meiosis, and regulating gene expression [16]. Two copies of each core histone protein (H2A, H2B, H3, H4) form a histone octamer around which 147 DNA base pairs wrap, forming a nucleosome, the basic building block of chromatin structure [17]. Chromatin in its loosened state, referred to as euchromatin, is often associated with active gene transcription. Genes are typically thought to be in a silent state when chromatin is in its tightly condensed heterochromatin state. The N-terminal tail of each histone protein protrudes from the nucleosome allowing for posttranslational modifications to alter chromatin structure and ultimately gene expression (fig. 1). Modifications made to histones affect gene transcription in two ways: (1) by affecting electrostatic links between DNA and histone proteins causing chromatin loosening and thereby enhancing access of transcriptional machinery to the DNA, and (2) by changing histone-histone interactions [18, 19], which impacts chromatin reassembly following transcription [20]. Such modifications include histone acetylation, methylation, phosphorylation, ubiquitination, and sumoylation.

The impact of posttranslational modifications on gene expression depends on which modification is made, as well as which histone tail and specific amino acid residue is modified [21]. Because of its location within the nucleosome structure, histone H3 is the most highly modified histone protein and has received the most focus for its role in epigenetic control of gene expression [22]. Histone phosphorylation and acetylation release DNA from histones enhancing gene transcription [23, 24]. In the case of acetylation, histone acetyltransferase (HAT) enzymes covalently attach a negatively charged acetyl group to a histone tail, causing repulsion of the negatively charged phosphate backbone of DNA. In this state, acetylation al-

**Fig. 1.** Epigenetic control of gene expression. Chromosomes have complex higher order structures, the basic unit of which is called the nucleosome. Nucleosomes consist of segments of DNA wrapped around core histone proteins with protruding N-terminal tails. Histone acetyltransferase enzymes (HATs) acetylate histone protein tails, opening chromatin structure and activating transcription. Removal of acetyl groups by histone deacetylase enzymes (HDACs) results in chromatin condensing and transcriptional repression. Interference with HDAC activity can interfere with masculinization of the neonatal brain. Chromatin in a repressed state is usually highly methylated by DNA methyltransferase enzymes (DNMTs). Methylation at CpG dinucleotides attracts methyl-binding proteins (MBDs), which recruit chromatin remodeling proteins such as HDACs. Sex differences in DNA methylation and MBD expression in sexually dimorphic brain regions during the critical period suggest an involvement of DNA methylation in brain organization. Promoters with low levels of DNA methylation are typically associated with active transcription, but many empirical findings challenge this dogma.



lows for DNA to separate from the nucleosome and to be accessed by transcriptional machinery. Removal of the acetyl group by a histone deacetylase (HDAC) enzyme restores the nucleosome's tightly wound structure [25] (fig. 1). Other modifications, such as histone methylation and ubiquitination, which do not impact electromagnetic charges within the nucleosome, have various outcomes based on their titer and placement. For instance, methylation of lysine 9 on histone H3 is associated with active gene transcription, whereas di- or trimethylation of the same residue causes chromatin condensing [26]. Reversible, protein mono-ubiquitination functions as a signaling mechanism at a given substrate [27], and can be coupled to other histone modifications such as methylation [28]. Although less is known about the function of histone ubiquitination, it has been shown that ubiquitination of histone H2A results in gene repression, whereas ubiquitination of H2B can cause transcriptional activation or silencing [29]. Sumoylation is a negative regulator of transcriptional activity, which has been shown to block chromatin remodeling and gene expression induced by acetylation and ubiquitination [30]. Histone alterations

induced by small ubiquitin-related modifier (SUMO) are associated with heterochromatin, and SUMO has been shown to interact with HDACs and other members of repressor complexes [31].

In addition to histone protein modifications, direct DNA methylation often has a profound epigenetic impact. DNA methyltransferase (DNMT) enzymes covalently affix a methyl group to the cytosine residue within a CpG dinucleotide (referred to as a CpG site). CpG sites are commonly methylated throughout the genome, however CpG sites are typically unmethylated within so-called CpG islands, which are regions containing large quantities of CpGs in the 5' regulatory region, or promoter, of a gene [32–34]. Low baseline methylation levels in regulatory regions enable small changes in methylation at specific CpG sites within a gene's promoter to have a significant effect on gene expression.

The prevailing dogma is that methylation of cytosine residues is permanent, irreversible, and consistently results in transcriptional repression. This view has proved true for some biological systems, although recently large differences in DNA methylation were found in embry-

onic and fetal cells during cellular differentiation, revealing that cytosine methylation is a highly dynamic process [35]. Within the central nervous system, DNA methylation can be transient and is not necessarily associated with decreased gene expression. DNA methylation impacts gene expression and chromatin structure in several ways. Most notably, methylated cytosines can be used as docking sites for methyl-binding proteins which then recruit chromatin remodeling complexes, typically containing an HDAC enzyme, resulting in tightening of the chromatin structure. Proteins containing methyl-binding domains (MBD) such as MeCP2 and MBD1, 2, 3, and 4 all bind to methylated CpG sites but exert distinct outcomes [36]. MeCP2 is critical for neurological development and is typically associated with gene silencing through its recruitment of HDACs [37, 38]. However, recent evidence indicates MeCP2 actually enhances expression of 85% of its effector genes [39]. In fact, the majority of MeCP2-binding sites are outside of genes, associated with active transcription, and only a small percentage of these sites are methylated cytosines [39], suggesting MeCP2 plays opposing roles in regulating gene transcription in addition to having other functions. Other methyl-binding proteins such as MBD2 and MBD4 are primarily associated with transcriptional silencing, yet both have been linked to hypomethylation [40], and may be involved in demethylation [41] and DNA repair [42].

In addition to recruiting methyl-binding proteins, DNA methylation can directly block transcriptional machinery from accessing binding sites within a gene's promoter [43]. In genes with multiple promoters, DNA methylation can dictate promoter utilization [44]. As our knowledge of epigenetic processes expands and the dogma regarding DNA methylation unravels, we will likely learn that DNA methylation serves other as yet unknown functions.

### Epigenetic Consequences of Early Life Experiences

Much of our knowledge of epigenetic processes stems from research on cell fate determination during embryogenesis and tumorigenesis. More recently, epigenetic modifications have been observed in the brain in response to various exogenous stimuli such as maternal behavior, drugs, and learning [45–47]. The work that fueled an interest in epigenetics among neuroendocrinologists comes from Michael Meaney and colleagues who discovered that a seminal link between maternal care and adult hypothalamic-pituitary-adrenal-axis (HPAA) function

involves epigenetic alterations of the glucocorticoid receptor [48]. Meaney's model revealed that rat pups reared by less attentive, low licking/grooming (low LG) dams have lower levels of acetylation at lysine 9 of histone H3 (a site synonymous with active chromatin [49]), increased methylation of a critical region of the glucocorticoid receptor promoter, and decreased transcription factor binding. Importantly, changes in epigenetic control of the glucocorticoid receptor are established neonatally but persist into adulthood, wherein the animals exhibit impaired HPAA function. Moreover, reduced maternal care increases methylation of the ER $\alpha$ -1b promoter resulting in decreased ER $\alpha$  expression in the female preoptic area (POA) [49, 50]. Conversely, reduced maternal care increased ER $\alpha$  expression in other regions, such as the anteroventral periventricular nucleus of the hypothalamus (AVPV) and ventromedial nucleus of the hypothalamus (VMN), two areas critical for the control of gonadotropin release, estrous cyclicity, and female sexual behavior [51].

### Sex-Specific DNA Methylation during Development and Beyond

DNA methylation directs gonadal sex determination by controlling the timing and spatial expression of the *sry* gene in genetic males [52] and may influence offspring sex ratio [53]. Similarly, DNA methylation is emerging as a critical component of steroid hormone-mediated organization of the brain. During sexual differentiation of the brain, estradiol derived from testosterone causes permanent structural and behavioral masculinization and defeminization via activation of ER $\alpha$  and ER $\beta$ , respectively [54, 55]. Because these receptors are critical in organizing sex differences in the brain, their promoters have been thoroughly examined as potential targets of epigenetic control via DNA methylation.

In the rat, five alternative promoters control ER $\alpha$  gene expression. A system of untranslated exons and regulatory elements determine ER $\alpha$  transcription initiation [56–58]. Estrogens are known to regulate the selective usage of these promoter systems [59] and although it has not been explicitly tested, sex differences in ER $\alpha$  promoter methylation may be related to alternative promoter selection and thereby involved in sexual differentiation of the brain. The POA is a highly sexually dimorphic region necessary for male sex behavior [60, 61]. An analysis of ER $\alpha$  alternative promoter usage in the POA following exposure to endocrine disrupting chemicals revealed that sex and exposure to estrogenic compounds determines

the methylation status and usage of alternative promoters. These differences are dependent on both developmental timepoint and compound dosage [62].

More recent studies have examined the impact of physiologically relevant masculinizing doses of estradiol. At the close of the sensitive period (PN10), males have higher levels of methylation at two CpG sites on the non-coding exon 1b of the ER $\alpha$  promoter within the POA compared to females. Treatment of female pups with estradiol to mimic the hormonal milieu of a male results in increased methylation at one site [63]. However, just upstream of exon 1b, in intron 1, a region containing high sequence homology to the intron 1 enhancer region in humans [64, 65], the methylation pattern is markedly different. Newborn females have significantly higher levels of methylation at two CpG sites compared to males, and again, treating females with estradiol decreases methylation levels at these sites to that of males [66]. Interestingly, these differences are no longer present by around 3 weeks of age, a timepoint after the close of the sensitive period but before the onset of adult levels of circulating hormone. Even more surprising, a sex difference in CpG methylation reappears in the POA in adulthood in gonadally intact animals. A different pattern of methylation emerges in a CpG island located just upstream of ER $\beta$  exon 2. There are no sex differences present at birth but instead they appear at 3 weeks and 60 days of age (fig. 2). Thus within the POA the methylation status of ER regulatory regions can be organized with neonatal hormones and these differences may become apparent only in adulthood when hormones are present, a classic example of how early hormones organize the ability for adult hormones to activate sex differences in the brain. Within the medial basal hypothalamus, another region differentiated by neonatal estradiol exposure, newborn females have higher methylation of ER $\alpha$  intron 1 compared to males at two different CpG sites. This sex difference appears to be a transient developmental difference as methylation levels are equal between the sexes at later timepoints [66].

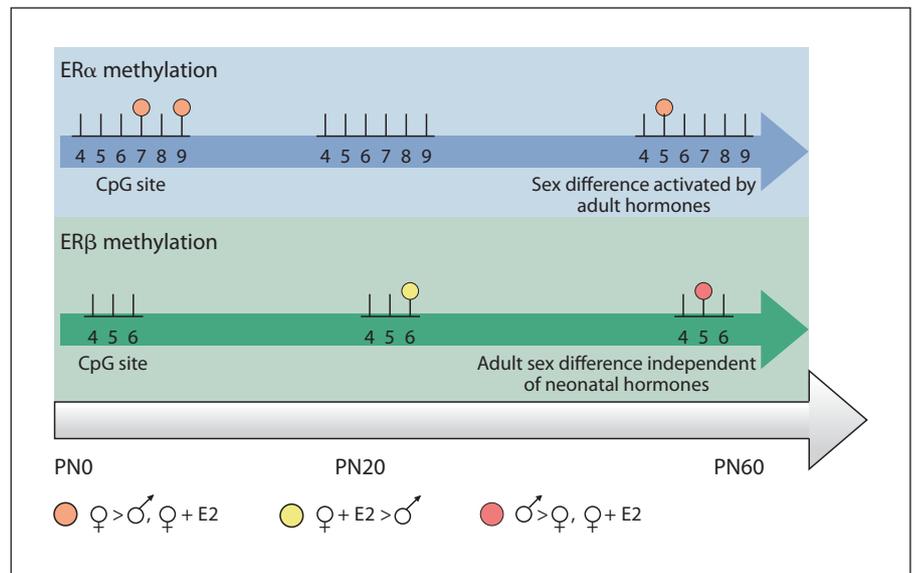
In addition to sexually differentiating the brain, estrogens are important for normal development of many regions not typically considered highly sexually dimorphic [67]. ER $\alpha$  is highly expressed in the neonatal cortex, but its expression declines by around 10 days of life and is nonexistent in the adult [68, 69]. The suppression of ER $\alpha$  expression in the adult cortex is the result of increased DNA methylation at specific areas of the ER $\alpha$  5' untranslated region, and coincides with higher expression of DNMT1, the methyltransferase isoform responsible for maintenance of DNA methylation [70].

ER $\beta$  is highly expressed in the hippocampus [71] and has been implicated in controlling stress and learned-helplessness-related behaviors associated with depression [72–74]. In newborn and 3-week-old rats, sex and neonatal estradiol treatment have no impact on methylation status of the ER $\beta$  exon 2 promoter, whereas neonatal estradiol treatment causes a significant increase in methylation at one CpG site in adults (for detailed methods see Schwarz et al. [66]; fig. 3). This finding supports the idea that early hormone exposure can impact an animal's epigenome across its lifespan. In adulthood, acute estradiol is known to enhance memory consolidation [75]. Infusing estradiol into the dorsal hippocampus alters both DNMT and HDAC protein levels, and inhibition of DNMT activity blocks estradiol's enhancement of memory, indicating that estradiol works through epigenetic mechanisms to boost memory consolidation in adult animals [76].

The progesterone receptor (PR) is an additional target for neonatal hormones that may be important in sexual differentiation of the brain. Estradiol induces PR expression in the neonatal brain [77], and newborn males have significantly higher levels of PR in subregions of the POA [78]. While the functional relevance of this sex difference is unknown, blocking PR activity attenuates estrogen-induced increases in POA subnucleus volume [79]. Although estradiol is known to enhance PR expression in the POA, estradiol treatment at birth significantly increases methylation within exon 1 of the PR promoter in the neonatal POA [66]. This unexpected finding provides another example of how gene methylation does not always correspond to gene expression and further supports the view of new roles for DNA methylation in brain organization.

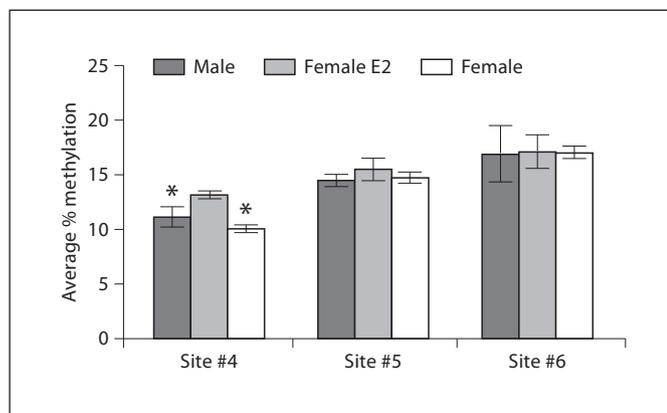
A sex difference in levels of MeCP2 provides further evidence for a role of DNA methylation in sexual differentiation of the brain. Females have more MeCP2 in the amygdala and hypothalamus during the critical period than do males, but expression levels are equal between the sexes by day 10 [80]. Females also express higher levels of nuclear receptor corepressor (NCoR) in the developing amygdala, and estradiol treatment reduces NCoR levels to that of males [81]. NCoR interacts with ER $\alpha$  [82] as well as various methyl-binding proteins, such as MeCP2, and forms corepressor complexes with HDAC enzymes [83].

Although fewer studies of sex differences in DNA methylation have been reported in the adult brain, a study profiling changes in the human epigenome associated with major psychosis revealed sex-specific methylation



**Fig. 2.** Dynamic methylation of ER regulatory regions. Schematic representation of the methylation patterns seen on ER $\alpha$  intron 1, and ER $\beta$  exon 2 within the POA across the lifespan following neonatal estradiol treatment ( $\text{♀} + \text{E2}$ ). CpG methylation on both promoters is transient and affected by both neonatal hormonal milieu and adult hormone status. In the neonatal POA, females have higher methylation at two sites on ER $\alpha$  intron 1 compared to males and estradiol-treated females; however, this difference is not permanently organized because it disappears by PN20. With

the activation by adult circulating hormones at PN60, the same trend (females having higher methylation compared to males and estradiol-treated females) reemerges. Conversely, the early hormonal milieu does not appear to play a role in the methylation patterns seen on ER $\beta$  exon 2, since treating females with a masculinizing dose of estradiol during development does not significantly alter methylation levels at any timepoint compared to vehicle-treated females.



**Fig. 3.** ER $\beta$  promoter methylation in the hippocampus. Female rat pups were treated with a masculinizing dose of estradiol on the day of birth, and levels of CpG methylation were assessed in males, females, and estradiol-treated females on P1, PN20, and PN60. There was no developmental impact of hormone treatment on CpG methylation of ER $\beta$  in the hippocampus (data not shown), but a significant difference in methylation at CpG site 4 of ER $\beta$  intron 1 emerged in adulthood (ANOVA  $F_{2,13} = 6.4$ ,  $p < 0.05$ ; \*  $p < 0.05$  compared to estradiol-treated female).

patterns in genes associated with bipolar disorder and schizophrenia [84], two diseases with sex differences in prevalence, symptomology, and age of onset [85, 86]. It remains to be seen if the differential gene expression found in this study is the result of hormonal or genetic sex difference and whether developmental factors are at play.

### Hormonal Modulation of Chromatin Structure

Within the perinatal cortex and hippocampus, males have higher levels of acetylated and trimethylated histone H3 than females. The sex difference in H3 acetylation appears to be hormonally dependent as testosterone treatment administered to rat dams during their final days of gestation raises H3 acetylation in the cortex and hippocampus of neonatal females [87]. However, testosterone treatment fails to alter methylation of histone H3, suggesting this sex difference is not organized by early hormone exposure and may be the result of other genomic differences.

The principle nucleus of the bed nucleus of the stria terminalis (BNSTp) is a forebrain structure organized by perinatal hormone exposure that is larger and contains more neurons in males than females [88]. During the critical period for sexual differentiation of the brain, Bax-dependent apoptotic cell death kills cells in the female BNSTp, while gonadal steroids protect males and hormone-treated females from cell loss in this region [89, 90]. Interestingly, the protective effect of hormones is not evident until about 5 days after hormone treatment, leading Forger, de Vries and colleagues [91] to investigate epigenetic mechanisms controlling this cellular memory. They found that inhibition of histone deacetylation with the anticonvulsant drug, valproic acid (VPA), significantly impairs masculinization of BNSTp volume and neuron number in genetic males and testosterone-treated females, suggesting hormones influence chromatin conformation during sexual differentiation.

Histone modifications also appear to play a role in sexual differentiation of the POA. During the sensitive period males have higher levels of acetylation of histone H3 and H4 around the ER $\alpha$  1b and aromatase promoters compared to females, suggesting males may have higher levels of transcriptional activity at these genes during the critical period [92]. HDAC protein levels are equal in both sexes neonatally, but males have greater levels of HDAC2 and HDAC4 bound to the ER $\alpha$  1b and aromatase promoters. These sex differences appear to be critical for neonatal masculinization of the brain because inhibiting HDAC activity with TSA or antisense oligonucleotides directed against HDAC2 and HDAC4 impairs male sexual behavior performance in adulthood.

## Conclusions

As interest in epigenetic processes grows, the importance of these genomic modifications in normal and pathological biological systems is becoming apparent. The currently unknown mechanisms for permanent sexual differentiation of the neonatal brain are beginning to be addressed through the study of epigenetics. As outlined above, recent findings suggest that epigenetic processes such as DNA methylation and histone acetylation may play an important role in the creation and maintenance of sexual dimorphisms in the brain. Sex differences in the epigenome are prevalent in the developing brain in regions classically considered sexually dimorphic as well as other brain regions known to exhibit sex differences but not necessarily of the same magnitude or obvi-

ous functional significance. Importantly, many of the observed epigenetic changes are mediated by hormones and are only apparent during the critical period for sexual differentiation of the brain, lending credence to the hypothesis that hormone exposure during the critical period organizes the bipotential brain via epigenomic alterations. It is well established that epigenetic processes are integral to determining cell fate in the developing brain [93]. Likewise, gonadal steroids exert ubiquitous actions on the developing brain to determine cell fate and neuronal anatomy, the question remains as to how, when, and how much hormonally-induced epigenetic mechanisms contribute. For instance, do estradiol-induced patterns of gene methylation determine sex difference in the distribution of different cell types by controlling gene expression, or does estradiol differentiate cellular fate which in turn causes sex differences in epigenetic markers? The study of the involvement of epigenetic mechanisms in sexual differentiation of the brain is still in its infancy. A better understanding of the mechanisms by which hormones alter the epigenome and a more comprehensive analysis of the genes impacted by epigenetic regulation during sexual differentiation is necessary to fully understand the importance of these processes in the establishment of the sexually dimorphic brain.

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