Insights & Perspectives

Histone modifications proposed to regulate sexual differentiation of brain and behavior

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Expression of sexually dimorphic behaviors critical for reproduction depends on the organizational actions of steroid hormones on the developing brain. We offer the new hypothesis that transcriptional activities in brain regions executing these sexually dimorphic behaviors are modulated by estrogen-induced modifications of histone proteins. Specifically, in preoptic nerve cells responsible for facilitating male sexual behavior in rodents, gene expression is fostered by increased histone acetylation and reduced methylation (Me), and, that the opposite set of histone modifications will be found in females. Conversely, in ventromedial hypothalamic neurons that are responsible for coordinating female sexual behavior, transcriptional activities in genetic females are fostered by increased histone acetylation and reduced Me, and, further, that the opposite set of histone modifications will be found in males. Thus, these epigenetic events will guarantee that effects of sex hormone exposure during the neonatal critical period will be translated into lasting sex differences in adult reproductive behaviors.

Keywords:
- androgens; chromatin remodeling; estrogens; histone modification; hypothalamus; sex behavior

Introduction

Our hypothesis, newly presented here, that during development estrogen-bound estrogen receptors (ERs) induce sexually dimorphic covalent modifications of histone tails, is based on broad knowledge of ERs and their transcriptional activity, which involves modifications of chromatin structure through association with coregulator proteins with histone acetyltransferase (HAT) or histone methyltransferase (HMT) activities. By inducing differential histone modifications in a region- and sex-dependent manner, estrogen-bound ERs may be able to create a specific “histone code” for the set of genes that facilitate development of male sexual behaviors in medial preoptic area (MPOA) neurons and another “code” for the genes necessary for the expression of female sexual behaviors in ventromedial hypothalamic (VMH) neurons.

Historical background

The sexual differentiation of a mammalian embryo starts with the expression of the SRY gene, which is located on the sex-determining region of the Y chromosome and drives the formation of testes from the bipotential gonads [1]. The testes in turn produce a peptide, anti-Mullerian hormone (AMH) that causes the atrophy of the female gonads and accessory reproductive tissues. In the absence of the SRY gene and AMH, XX females develop ovaries and female
sexual characteristics [2]. In the last days of gestation in rodents the testes begin to produce testosterone [3], which stimulates the formation of the internal and external organs of reproduction [4], and at the same time has a profound effect on the development of sexually dimorphic brain.

During the perinatal period the hormonal milieu of male and female brains differ significantly. In developing male rodents, testosterone secreted from the testes around the time of birth, freely enters the brain [5]. In contrast, female brains are relatively devoid of sex steroid influence during this period due to quiescent ovaries and high levels of circulating α-fetoprotein, which is produced by the embryo and potentially binds and sequesters maternal estradiol (E2) in the fetal circulation [6]. The organizational/activational hypothesis, proposed by Phoenix et al. [7] in the landmark 1959 paper, states that during development testosterone and its metabolites act to permanently establish (i.e. "organize") male neural circuits, which are then activated by gonadal steroids in adulthood to produce male-specific behaviors [7]. On the other hand, in the absence of hormonal influence a female-typical brain is developed. Later it was shown that E2, one of the metabolites of testosterone, could masculinize the brain to a similar extent as testosterone [8, 9], leading to the aromatization hypothesis [10]. The discovery that sexually dimorphic brain regions express high levels of P450 aromatase [11], the enzyme that converts testosterone to E2, and high concentrations of ERs supported this hypothesis [12, 13].

Estrogens are gonadal steroids that exert their biological effects by binding to intracellular receptors that are members of the nuclear receptor superfamily of transcription factors [14, 15]. There are two isoforms of the ER (ERα [16] and ERβ [17]), both of which can bind estrogens with high affinity [17, 18]. ERs bound to estrogens can dimerize [19, 20] and translocate to the nucleus where they bind to hormone-responsive elements (e.g. the high affinity estrogen-responsive element) in the promoter regions of target genes and alter the rate of transcription [21]. Transcriptional coregulators, which physically associate with the receptors [22, 23], control the interaction between nuclear hormone receptors and the basic transcriptional machinery. In addition, nuclear receptor coregulators can induce chromatin remodeling through their intrinsic enzymatic activity by placing or removing acetyl and methyl groups on histone proteins, major constituents of chromatin [23–25].

The basic structural unit of chromatin is the nucleosome, which comprises 147 base pairs of DNA wrapped around a core of eight histones (two H2A, H2B, H3, and H4 histones). The N-terminal tails of core histones protrude from the nucleosomes and are subject to a wide range of post-translational modifications of specific amino acid side chains [26], including acetylation of lysines, methylation (Me) of lysines and arginines, and phosphorylation of serines and threonines. These modifications of histone tails disrupt or strengthen interactions between the nucleosome and DNA, thus regulating the access of transcription factors, such as ERs, to the cis-acting elements on the target gene promoters [27]. It is now well accepted that hyperacetylated histones H3 and H4 are mostly associated with activated gene transcription, while deacetylation results in gene repression [28, 29]. On the other hand, histone Me appears to have multiple, sometimes opposing effects on chromatin function. Among these, Me of H3K9 and H3K27 is mostly associated with repressed chromatin and gene silencing, whereas Me of H3K4 often leads to permissive chromatin structure [30]. Compared with other post-translational modifications, lysine Me of histone tails is also considered a long-lasting mark [31] found on chromatin regions that are silenced over the long term, inactive X chromosome being one example.

Recently it has been proposed that distinct stimuli (or combination of stimuli) may elicit specific sequences and combinations of histone modifications, establishing the so-called "histone code", which in turn determines the transcriptional profile of response genes associated with it [32, 33]. Multiple studies have demonstrated that proper epigenetic control of gene expression requires the cooperation of histone modifications and disruption of these processes can lead to abnormal gene expression profiles seen in human cancers.

Hypotheses

Based on these data we hypothesize that to support the development of dimorphic sexual behavior, ER signaling may induce differential chromatin remodeling in the brain areas that are involved in the expression of these behaviors, i.e. MPOA in males and VMH in females (Fig. 1). Specifically, by inducing histone acetylation at the promoters of target genes in male rodent brain, estrogen can increase the expression of genes that facilitate development of male sexual behavior, while Me of histones at the promoters of genes critical for female-type behaviors will lead to their suppression. These molecular events will result in the fine-tuning of neural circuits in MPOA necessary for the expression of male sexual behaviors. In the absence of estrogen in the female rodent brain the pattern of histone modifications is most likely the opposite of that found in male brain. Promoters of genes in the VMH involved in the expression of female sexual behaviors will be associated with acetylated histones and exhibit increased transcriptional activity. On the other hand, genes necessary for masculinization of behaviors will be turned off as a result of association with methylated histones.

These differential processes are presumably induced by association of ligand-bound ERs with various transcriptional coregulators that can catalyze covalent histone modifications on the promoters of target genes and alter their rate of transcription. Such mechanisms of transcriptional regulation employed by ERs have been described in neuronal as well as non-neuronal systems [34–37], and a large number of cofactors involved in ER-mediated transactivation have already been identified [22–24, 38–40]. Among them, nuclear receptor coactivators, SRC and CPB, as well as the corepressor NCoR exhibit sexually dimorphic pattern of expression [41–43], and are necessary for the development of sexually dimorphic behaviors in adulthood [42–44]. These data directly support our hypothesis and emphasize the importance of chromatin remodeling in the development of dimorphic brain and behavior.

The most exquisite test of our hypothesis would be to use chromatin immunoprecipitation (ChIP) for specific,
behaviorally relevant genes in the VMH and in the MPOA (Fig. 2). For example, histones governing access to the progesterone receptor (PR) promoter in the VMH of the female brain should be more heavily acetylated and less heavily methylated than in the VMH of the male brain. This is because estrogens induce PR and, as a result, the crucial, lordotic sex behavior in the female but not in the male rodents. Conversely, MPOA neurons must be turned on by dopamine (DA) in the male rodent brain for male sex behavior to occur. Thus, we would predict that in POA neurons of the male brain, histones governing the promoter of the D1 DA receptor would be more heavily acetylated and less heavily methylated, than would be true in the female brain.

Our hypothesis suggests that taking into account the number of nuclear receptor coregulators and the number of possible histone modifications that they can induce, estrogen binding to its receptor may result in a nearly limitless combination of activated or repressed genes, creating the developmental framework that will lead to the expression of complex mammalian behaviors in the adulthood in a sex-specific manner.

**Figure 1.** Cartoon sketched to illustrate in the simplest possible terms the types of histone modifications we hypothesize. For clarity, they are illustrated in their most extreme possible form. Top panel: In the MPOA of the male, we predict that a larger number of histone tails would be acetylated (Ac) and methylation (Me) would be minimized. As a result, behaviorally relevant transcriptional activities would be heightened, and the initiation of male-typical behaviors would be facilitated. By comparison, in the VMH, Me would predominate, and female-typical behavior would be suppressed. Bottom panel: In contrast, in the female, in the MPOA, Me would be widespread with the consequence of suppressing transcription-dependent male behavior. In the VMH, acetylation would predominate, thus facilitating a range of behaviorally relevant transcriptional events and permitting the initiation of female-typical behaviors.

**Appropriate histone modifications result in sexually dimorphic behaviors**

Perinatal exposure to steroid hormones has profound effects on the expression of sexually dimorphic behaviors in adulthood. Behaviors that serve a reproductive function are considered the best examples of sexually dimorphic behaviors and include preceptive and receptive behaviors in female rodents as well as appetitive and copulatory behaviors for male rodents. The neural substrates that are important for the execution of these behaviors have been mapped out and include the MPOA, a hypothalamic nucleus that is critical for the expression of male sexual behavior, and the VMH, a nucleus that is essential for the display of estrogen-induced lordosis, the receptive posture of female rodents [45, 46]. In both sexes, the expression of reproductive behaviors is hormone dependent and can be abolished by gonadectomy in adulthood [7, 47–49]. Although there are some emerging data that describe the importance of genetic sex in the development of sex differences in brain morphology and behavior [50], a large body of evidence supports the critical role of the hormonal milieu during the perinatal period of development in the expression of sexual behaviors in adulthood, particularly in mammals. In male rodents the perinatally released testosterone and its
metabolite-estrogen “masculinize” brain morphology and male-typical behaviors [51–53]. The time window during which steroid hormones are able to induce changes that lead to the sex differences in adulthood is very restricted and is termed a “critical period”. Manipulations of hormone levels during this time period by gonadectomy or exogenous hormone treatments as well as modulation of aromatase activity can reverse sex-typical phenotype and result in males that display lordosis or females that exhibit male-typical sex behaviors in adulthood [7, 47, 48, 53, 54].

Perinatal hormone exposure is necessary not only to “masculinize” but also to “defeminize,” i.e., to reduce the ability to display female sexual behavior. Rather than being a “default” state, defeminization is also believed to comprise active developmental processes that can be dissociated from “masculinization” at the cellular and molecular level [55]. This distinction became most evident from the analysis of sexual behaviors of ERα- and ERβ-knockout mice. Although these mice are not suitable for differentiating the “organizational” and “activational” effects of estrogen, comparison of male and female sexual behaviors in adulthood led to the distinction that ERα signaling is necessary for the masculinization and ERβ is necessary for the defeminization of behavior [56–59].

It is evident that the “organization” of sex-typical behaviors following hormonal exposure during development is not absolute and has variable effect on different components of male and female motivational and sexual behaviors, suggesting the complexity of the developmental programming. These complex outcomes and persistent effects of relatively brief hormonal exposure can be easily explained by epigenetic regulation of gene expression. This mechanism allows cells to maintain repressed or activated transcription states long after the initial signal is terminated by using acetylated or methylated histone proteins as “tags” for the association of transcriptional activators and repressors with the promoters of specific genes [60–62]. Thus, the genes that are necessary for the expression of various components of male sexual behaviors can be turned on and genes that foster the development of female sexual behaviors can be turned off in the brains of males as a result of a single brief exposure to E2 during development. Further, this pattern of gene expression may be able to persist through adulthood without any additional signal, as long as histone proteins associated with the regulatory regions of target genes retain post-translational modifications induced by E2.

Among these genes may be the classical target gene of ER–PR, which itself is a transcription factor of the nuclear receptor superfamily [15] and has been shown to be important for normal reproductive behavior in male rats [63] and the formation of the sexually dimorphic nucleus of the preoptic area (SDN-POA) [64]. Interestingly, the pattern of sex difference in the expression and transcriptional regulation of the PR in the MPOA and VMN during development...
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Period'' not only determines the expression of sexually dimorphic behaviors in adulthood but also permanently changes cytoarchitecture of the brain. Several dimorphic brain structures have been described in a number of species. Although sex differences are mostly found in nuclei that are involved in the execution of sexually dimorphic behaviors, such as MPOA and VMH, the direct link between the structure and function has not been clearly demonstrated [68].

The sex differences found in dimorphic brain structures are not uniform in nature: some result from disproportion in cell numbers and some arise from the differences in synaptic connections or neuropil density; dimorphisms in chemical characteristics of cells and fiber populations have also been described [52, 69–73]. Moreover, most dimorphic nuclei described in rodent species are larger in males with the exception of anteroventral periventricular nucleus (AVPV), which is larger in females [70]. Notably the difference in AVPV between the sexes is revealed in the density of cells as well as in their chemical characteristics: females have many more dopaminergic [74] and about ten times more kisspeptin-expressing neurons [71, 75], which project to and stimulate gonadotropin-releasing hormone (GnRH) neurons triggering the luteinizing hormone (LH) surge [76, 77]. Thus, the higher number of kisspeptin neurons in the female AVPV may have direct physiological consequences and explain the sex difference in the induction of LH surges, as males do not show this neuroendocrine response.

Regardless of the brain region and cellular compartment in which sex differences are found, they all result from perinatal exposure to the steroid hormones: testosterone converted into E2 and signaling through ERs drives the formation of sexually dimorphic brain structures. Experimental manipulations of hormone levels in neonatal animals, such as castration of males and treatment of females with E2 or testosterone propionate can eliminate these differences [69, 78–80]. Studies done using selective agonists of ERa or ERβ, as well as mice with genetic ablation of ERα and ERβ and the aromatase genes also confirmed that estrogen-mediated activation of ERs is a critical step for the dimorphic brain development [81, 82].

However, in male mice with a mutation that renders androgen receptors hypofunctional (Tfm mutation) the sex differences in the size of posterior bed nucleus of the stria terminalis (pBNST) is also reduced, suggesting that androgen receptor signaling itself is important for the development of sex difference in this nucleus [83].

It has been hypothesized that the control of cell numbers by apoptosis is the mechanism by which sex differences in brain morphology are achieved [84]. Supporting this hypothesis are the studies demonstrating dimorphism in the expression of pro- and anti-apoptotic proteins, Bax and Bcl-2, respectively, in structurally dimorphic brain regions [85, 86]. Moreover, both overexpression of anti-apoptotic proteins or deletion of pro-apoptotic genes in brain have been shown to eliminate structural sex differences [87, 88].

Sexually dimorphic expression of pro- and anti-apoptotic genes and apoptotic cell death is regulated by the same stimulus, E2, and evidence suggests that this regulation is on the transcriptional level [89]. In fact it has been demonstrated that the human Bcl-2 gene contains the sequence of estrogen-response elements, and that estrogen can inhibit apoptosis in human breast cancer MCF-7 cells by inducing transcription of Bcl-2 [90]. Thus, it is plausible that estrogen can directly regulate Bcl-2 transcription, and thereby also control cell death by apoptosis in neurons.

Paradoxically, estrogens regulate apoptotic genes with opposing patterns of expression in brain regions that exhibit opposing morphology between sexes, i.e. SDN-POA, which is larger in males, and AVPV that is larger in females. The question remains as to how this is achieved? One mechanism by which estrogens may be able to accomplish such dual regulation is through chromatin remodeling. By recruiting histone-modifying enzymes, which will place permissive acetyl groups or repressive methyl marks on the promoters of Bcl-2 and Bax, respectively, estrogen can selectively activate transcription of the former and repress the latter in the SDN-POA of male pups. On the other hand, if the histone modifications associated with pro- and anti-apoptotic gene promoters in AVPV are opposite to those found in SDN-POA.

In addition, preliminary results from our group indicate that neonatal expression of connexin-36, a major constituent of neuronal gap junctions, is sexually dimorphic, with higher levels of mRNA found in mediobasal and preoptic areas of hypothalamus and amygdala of male mice (Westberg, Devidze, and Pfaff, unpublished data). Although the importance of these differences in the development of sexually dimorphic behaviors has not been addressed, it is plausible that gap junction channels, which modulate neuronal activity by synchronizing large neuronal ensembles, exert profound effects on the development and fine-tuning of circuits underlying dimorphic sexual behaviors. Thus, the examination of the influence of hormonal exposure and the chromatin remodeling on the transcriptional regulation of Cnx-36 gene during critical period of development, is an interesting avenue of future research. Moreover, analyses of gene expression profiles following ChIP with antibodies for ERs, their coregulators and acetylated and methylated histones (ChIP on Chip) will provide valuable information about the molecular pathways employed by estrogen and identify largely unknown genes that are necessary for the development of sexually dimorphic brain and behaviors as well as help decipher the “histone code” associated with this process.

Same histone modifications result in sexually dimorphic brain morphology

Exposure to gonadal steroid hormones during the developmental “critical period” not only determines the expression of sexually dimorphic behaviors in...
Histone modifications are already associated with functional changes in the nervous system

Our hypothesis that implicates chemical modifications of histones in a set of central nervous system (CNS) processes has precedents. Although the cancer field has always been the stronghold of chromatin biology, in recent years a great deal of attention has been devoted to the study of epigenetics in the nervous system. Recent evidence indicates that within the CNS neurons employ epigenetic modifications to translate external stimuli into long-lasting functional and morphological changes that produce both physiological and pathological behaviors. For example, histone modifications, specifically histone acetylation, have been shown to play an important role in memory consolidation in a variety of paradigms as well as synaptic plasticity [91, 92]. Chromatin remodeling has also been implicated in the development of pathological conditions such as depression and addiction [93, 94] and has been shown to be induced by acute and chronic stress [95]. Furthermore, epigenetic dysregulation, specifically reduced histone acetylation, is a common theme in neurodegenerative and neurodevelopmental disorders [96, 97]. Based on these evidence, histone deacetylase (HDAC) inhibitors are now considered as valid drug targets for the treatment of a number of neurological and psychiatric disorders [96].

In line with our hypothesis that estrogen may induce sexually dimorphic changes in histone acetylation and Me, leading to the development of sexually dimorphic behaviors, is the recent study demonstrating sex differences in both acetylation (H3K9/14Ac) and Me (H3K9Me3) levels of histone H3 in cortex and hippocampus [98]. The authors also determined that H3 acetylation was regulated by prenatal exposure to testosterone; however, H3 Me was dimorphic regardless of hormonal milieu. Interestingly, these differences were only found in cortex/hippocampus and not in POA/hypothalamus, the area that exhibits dimorphic morphology and is involved in the control of dimorphic sex behaviors. In this regard, it needs to be considered that the authors only analyzed modifications affecting three residues on one of the histone proteins. It is plausible and expected that more complex combinatorial changes in chromatin structure will be necessary for the development of many components of sexually dimorphic behavior. It is also quite possible that estrogen induces significant changes in the acetylation and/or Me of histones associated with the promoters of specific genes without affecting the global levels of these modifications. If this is the case, the type of analyses described by Tsai et al. [98] is not sufficient to detect these changes and more detailed examination of target genes will be necessary.

Another piece of solid evidence that epigenetic regulation is important for the development of a sexually dimorphic bed nucleus of the stria terminalis (BNST) formation came from a study in which injection of HDAC inhibitor valproic acid (VPA) on the day of birth blocked masculinization of this nucleus [99] in males as well as in hormone-treated females. These data suggest that histone deacetylation is critical for the development of this sexually dimorphic brain region. However, the specific histone modifications that were affected by VPA treatment and/or specific genes regulated by these modifications were not described.

These studies, although preliminary, open a window onto the new emerging field of epigenetic regulation of neural functions and will certainly advance our understanding of developmental processes underlying brain formation and complex mammalian behaviors.

Outlook: Our new transcriptional interpretation of a classic hypothesis portends a new generation of experimental work

Few scientific hypotheses have stood the test of time as well as the “organizational/activational” hypothesis proposed by Phoenix and colleagues. Fifty years later, it is well established that the developing mammalian brain is a bipotential organ, which can assume either a male or female sex identity. The process that will determine the social and reproductive fate of the animal takes place within a very short developmental time period, generally around the time of birth, and is driven by estrogens, and to lesser extent androgens. During this developmental time window estrogen binding to its receptors initiates a cascade of molecular and cellular changes that permanently “organize” male brains and allows the expression of male-typical behaviors in adulthood, while suppressing female-specific behaviors. Very little, however, is known about the nature of the effector molecules and the downstream processes that underlie these permanent changes in the brain.

Testing our histone-code-based hypothesis will require systematic analysis of histone modifications in different regions of developing brain and, with the availability of new research tools, this is within reach. More important and challenging tasks would be identifying effector genes that are turned on and off by these epigenetic changes and elucidating downstream molecular and cellular mechanisms underlying hormone-induced sex differences.

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References
The biological clock that regulates sexual development is complex and involves multiple levels of regulation, from genomic to behavioral. This regulation is controlled by a variety of hormonal and environmental factors. 

1. The embryonic development of the gonads and the formation of the sex chromosomes (XY in males, XX in females) is the first step in sexual differentiation. 

2. During early development, the gonads produce sex steroids (testosterone in males, estrogen in females) that play a crucial role in sex determination. 

3. The sensitivity of the developing gonads to these steroids is determined by the presence or absence of specific receptors. 

4. The expression of these receptors is regulated by transcription factors that are activated by sex steroids. 

5. The transcription factors recruit coactivators and histone acetyltransferases to enhance transcription of target genes. 

6. The interaction between DNA, transcription factors, and coactivators is facilitated by chromatin remodeling complexes that remodel the chromatin structure. 

7. The chromatin structure affects the accessibility of transcription factors and coactivators to the DNA. 

8. The balance between euchromatin and heterochromatin can influence gene expression and sexual differentiation. 

9. The developmental program is set by the initial exposure to sex steroids and can be modified by subsequent hormonal exposure. 

10. Environmental factors, such as nutrition and temperature, can influence the expression of sex steroids and the development of sexual traits. 

11. The development of sexual behaviors is influenced by the environment, and these behaviors can be modified by social and learning experiences. 

12. The developmental program is modifiable throughout life, and interventions at different times can have different effects on sexual development. 


