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Epigenetics and Sex Differences in the Brain: A Genome-Wide Comparison of Histone-3 Lysine-4 Trimethylation (H3K4me3) in Male and Female Mice

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Abstract

Many neurological and psychiatric disorders exhibit gender disparities, and sex differences in the brain likely explain some of these effects. Recent work in rodents points to a role for epigenetics in the development or maintenance of neural sex differences, although genome-wide studies have so far been lacking. Here we review the existing literature on epigenetics and brain sexual differentiation and present preliminary analyses on the genome-wide distribution of histone-3 lysine-4 trimethylation in a sexually dimorphic brain region in male and female mice. H3K4me3 is a histone mark primarily organized as ‘peaks’ surrounding the transcription start site of active genes. We microdissected the bed nucleus of the stria terminalis and preoptic area (BNST/POA) in adult male and female mice and used ChIP-Seq to compare the distribution of H3K4me3 throughout the genome. We found 248 genes and loci with a significant sex difference in H3K4me3. Of these, the majority (71%) had larger H3K4me3 peaks in females. Comparisons with existing databases indicate that genes and loci with increased H3K4me3 in females are associated with synaptic function and with expression atlases from related brain areas. Based on RT-PCR,

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Link to deposited data: The H3K4me3 data discussed in this publication have been deposited in the BioProject database (BioProject ID: PRJNA256195) and can be accessed at <http://www.ncbi.nlm.nih.gov/bioproject/256195>.

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only a minority of genes with a sex difference in H3K4me3 has detectable sex differences in expression at baseline conditions. Together with previous findings, our data suggest there may be sex biases in the use of epigenetic marks. Such biases could underlie sex differences in vulnerabilities to drugs or diseases that disrupt specific epigenetic processes.

Keywords

histone; sex difference; methylation; bed nucleus of the stria terminalis; preoptic area; Chip-Seq

INTRODUCTION

Many psychiatric disorders and neurological diseases exhibit sex differences in incidence, severity, or response to treatment (Kornstein, 1997; Seeman, 1997; Weinstock, 1999). This is especially true of neurodevelopmental disorders (Zahn-Waxler et al., 2008; Martel et al., 2009), which presumably reflects underlying sex differences in brain development. Chromosomal differences between males and females (XY in males and XX in females) could cause sex differences in the brain (Chen et al., 2009; Arnold, 2012), but most effects of the sex chromosomes on sexual differentiation are thought to be indirect and mediated by gonadal steroid hormones (reviewed in McCarthy et al., 2009a). Sexual differentiation has long been considered “epigenetic,” in reference to the indirect role that sex chromosomes play in hormone-dependent sex differences. More recently, the term “epigenetics” has re-entered the sexual differentiation field, but now to refer much more specifically to changes in chromatin that lead to long-term changes in gene expression without any change in the underlying DNA sequence (McCarthy et al., 2009b; Qureshi and Mehler, 2010; Auger and Auger, 2011; Xu and Andreassi, 2011).

The fundamental structural unit of chromatin is the nucleosome, comprised of about 146 base pairs of DNA wrapped around an octamer of histone proteins. The nucleosomes are further packaged and condensed to different degrees; in general, loose packaging is associated with increased gene expression, whereas more compact states are associated with reduced gene expression. DNA cytosine methylation and hydroxymethylation, and chemical modifications of the nucleosomal histones define chromatin structure and affect gene expression (Felsenfeld and Groudine, 2003; Jiang et al., 2008). For example, DNA cytosine methylation around transcription start sites and promoter elements is typically associated with gene repression. The majority of functionally relevant histone modifications are thought to reside at the flexible N-terminal tails that protrude from the nucleosome. These histone tails undergo a diverse array of covalent modifications such as acetylation, phosphorylation, ADP ribosylation, methylation, and ubiquitination, which correlate with specific transcriptional states (Jenuwein and Allis, 2001; Fischle et al., 2003; Iizuka and Smith, 2003).

Circumstantial evidence suggested that epigenetic modifications might be important for sexual differentiation. For example, a brief exposure to gonadal steroid hormones can have long-lasting or even permanent effects on gene expression, suggesting a cellular memory that is consistent with alterations in the epigenome. In addition, the classical actions of

gonadal steroids are mediated by intracellular receptors that recruit co-activators or co-repressors to steroid responsive genes. Many of these co-factors either directly or indirectly cause changes in nearby histone proteins by, for example, increasing or decreasing the acetylation or methylation of histone tails (Spencer et al., 1997; Kim et al., 2001; Stallcup et al., 2003; Privalsky, 2004; Kishimoto et al., 2006; Kininis et al., 2007). An important mechanism by which gonadal steroid hormones activate gene expression may therefore involve a relaxation of chromatin structure following the binding of a receptor–co-activator complex. One steroid receptor co-activator, SRC-1, was linked to sexual differentiation over 10 years ago when it was demonstrated that reducing SRC-1 protein in the developing rat brain interfered with the development of sex differences in behavior and brain morphology (Auger et al., 2000).

Although still in its infancy, the role of epigenetics in sexual differentiation of the brain has recently become an active line of research, with much of the focus on a group of interconnected brain regions including the amygdala, preoptic area of the hypothalamus (POA), and bed nucleus of the stria terminalis (BNST). These are important, steroid-sensitive nodes within neural circuits controlling sexual behavior, the modulation of stress and anxiety, and the processing of olfactory cues, among other functions (Simerly, 2002; Toufexis, 2007). Evidence for both DNA methylation and nucleosomal histone modifications in sexual differentiation of these and other brain areas has been found.

DNA Methylation

Schwarz and colleagues (2010) used pyrosequencing to examine DNA methylation in the promoter regions of three genes—estrogen receptor alpha (ER α), ER β , and the progesterone receptor—that are important for hormone-mediated sex differences in the brain. They found several age- and brain region-specific sex differences at individual CpG sites; some of these were reversed by treating females with estradiol at birth, indicating that they were due to perinatal steroid exposure (Schwartz et al., 2010). The group differences in DNA methylation were relatively small, and it is not known whether they relate to changes in gene expression. However, the findings are important because they were the first to document sex differences in DNA methylation in the brain and to demonstrate that this methylation may be dynamically regulated during development.

DNA methylation is controlled by a family of DNA methyl transferases (DNMTs), two of which (DNMT1 and DNMT3a) are abundantly expressed in the brain. Methylated cytosine recruits proteins, such as methyl CpG binding protein 2 (MeCP2) and nuclear receptor co-repressor (nCOR), which in turn cause chromatin changes that may alter gene expression. Auger and colleagues found that female rats express more DNMT3a, MeCP2 and nCOR than males in the neonatal amygdala (Kolodkin and Auger, 2011; Auger et al., 2011). Moreover, the sex differences in MeCP2 and nCOR were functionally linked to sex differences in juvenile play behavior (Auger et al., 2011). Taken together, several proteins related to DNA methylation are expressed in a sexually dimorphic manner in the rodent amygdala during the critical period for sexual differentiation.

Histone Modifications

The best understood of the histone modifications are acetylation and methylation. Histone acetyltransferases add acetyl groups to histone tails, opening the chromatin structure and increasing access for transcription factors, whereas histone deacetylation is catalyzed by histone deacetylases (HDACs) and is generally associated with reduced transcription (Grunstein, 1997; Cosgrove and Wolberger, 2005). The first study to compare histone modifications in the brains of males and females used immunoblotting to examine histone 3 lysine 9/14 acetylation (H3K9/14Ac) in several brain regions of perinatal mice. While no difference was seen in the highly hormone sensitive POA/hypothalamus, males had greater H3K9/14Ac in the cortex/hippocampus (Tsai et al., 2009). Treating newborn females with testosterone increased (masculinized) H3K9/14AC in the cortex/hippocampus, suggesting that the sex difference is due to gonadal steroids.

To more directly test whether changes in histone acetylation are required for hormone-dependent sexual differentiation, Murray et al. (2009) treated mice with an HDAC inhibitor during the critical neonatal period. Newborn male, female, and androgenized female mice were given saline or valproic acid and effects on the principal nucleus of the BNST (BNSTp) were determined at weaning (Murray et al., 2009). The BNSTp exhibits a number of morphological and neurochemical sex differences in rodents and humans (Guillamon et al., 1988; Hines et al., 1985; Forger et al., 2004; Allen & Gorski, 1990), and in mice these differences can be eliminated by giving females a single injection of testosterone propionate on the day of birth (Hisasue et al., 2010). Valproic acid treatment transiently increased histone acetylation in the brain and prevented masculinization of BNSTp cell number in both males and testosterone-treated females (Murray et al., 2009). There was no effect on the BNSTp of control females or on two non-sexually dimorphic brain regions, suggesting that a transient blockade of histone deacetylation prevented the masculinizing actions of testosterone without a generalized effect in non-dimorphic brain regions.

Matsuda et al. (2011) used a similar approach to ask whether the masculinization of sexual behavior in rats requires alterations in histone acetylation. When the HDAC inhibitor, trichostatin A, was infused into the cerebral ventricles of newborn males, these animals showed impairments in sexual behavior in adulthood. Similar behavioral effects were seen when antisense oligonucleotides to specific HDACs were infused neonatally, supporting the conclusion that the effects seen after trichostatin A administration were due to effects on HDAC activity (Matsuda et al., 2011). Taken together, the studies by Murray et al. (2009) and Matsuda et al. (2011) suggest that masculinization of neuroanatomy and behavior in rodents normally requires hormone-dependent reductions in histone acetylation. Because reductions in histone acetylation are generally associated with reduced gene expression, some genes may need to be silenced in males for normal masculinization, although specific gene targets underlying the effects of the HDAC inhibitors are not known.

The addition of methyl groups to lysine or arginine residues of histone tails is another well-studied epigenetic modification. Histone methylation can repress or activate transcription depending on the position of the modification and the number of methyl groups added at a given site (mono-, di- or tri-methylation). To date, the only study we are aware of comparing histone methylation in the brains of males and females used immunoblotting to examine the

overall level of trimethylated histone 3 at lysine 9 (H3K9me3) in several brain regions of neonatal mice and found higher levels in the cortex/hippocampus of males than of females (Tsai et al., 2009).

The trimethylation of histone 3 at lysine 4 (H3K4me3) has not yet been compared in males and females and is of particular interest because this modification is enriched at transcriptional start sites of genes and, on a genome-wide scale, broadly correlates with active transcription (Santos-Rosa et al., 2002; Bernstein et al., 2005; Berger 2007). Chromatin immunoprecipitation followed by deep sequencing (ChIP-Seq) has successfully been used to examine the genome-wide distribution of H3K4me3 in different cell types of the human and non-human primate brain (Cheung et al., 2010). Furthermore, in the rodent forebrain, H3K4me3 is subject to dynamic changes in the context of hippocampal learning and memory (Gupta et al., 2010) and exposure to dopaminergic drugs (Aguilar-Valles et al., 2013; Huang et al., 2007). To the best of our knowledge, sex-specific regulation of H3K4me3 and two related marks, mono- and di-methyl H3K4 (H3K4me1/H3K4me2), has not yet been explored on a genome-wide scale. This is surprising given that subtle sex-specific differences in the expression of H3K4- methyltransferase and demethylase enzymes have been reported for various brain regions. For example, in adult human cerebral cortex, expression of MLL1/KMT2A methyltransferase is higher in females as compared to males (Huang et al. 2007), while other genes, including *KDM5C/SMCX/JARID1C* encoding an H3K4-specific demethylase, are X-linked genes that escape X-inactivation in some brain regions and therefore may also be expressed at higher levels in females (Xu et al., 2008a; Yang et al., 2010). Here, we report initial analyses of a ChIP-Seq experiment designed to identify genome-wide sex differences in H3K4me3. We focus on the BNST and POA, two forebrain structures that show sex-specific differences in mammals, including humans (McCarthy et al., 2009a; Forger, 2009).

METHODS

Animals

All experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committees (IACUC) of the participating institutions. C57BL/6J mice were housed in a 12:12 light/dark cycle with food and water supplied ad libitum. All mice were adults (70–103 days of age) and were gonadally intact in order to identify sex differences that exist under normal, physiological conditions.

Brain Dissections

Mice were sacrificed by CO₂ inhalation and the brain rapidly removed. Regions of interest were dissected under a microscope on an ice-cold slide. Two coronal cuts were made to obtain the BNST/POA: one just anterior to the optic chiasm and the second at the point where the optic tract enters the brain. Within this slab the BNST/POA was defined as the region just ventral to the lateral ventricle to about 1 mm ventral of the anterior commissure, bounded laterally by the medial edge of the internal capsule. For prefrontal cortex, samples were collected from a 2 mm thick transverse slab immediately caudal to the olfactory bulbs and rostral to the genu of the corpus callosum by dissecting the tissue between the left and

right forceps minor of the corpus callosum. Dissected tissue was immediately placed on dry ice and stored at -80°C until processing.

Chromatin Immunoprecipitation and Deep Sequencing (ChIP-seq)

To prepare the chromatin-immunoprecipitation (ChIP) library, three male and three female samples were processed; each sample was an independent replicate, comprised of the BNST/POA pooled from four same-sex animals. Samples were homogenized in ice-cold douncing buffer (DB) [10 mM Tris-Cl [pH 7.5]/4 mM MgCl_2 /1 mM CaCl_2], then digested into mononucleosomes by incubation with 5 μl of micrococcal nuclease (MNase) (Sigma-Aldrich, St. Louis, MO, USA) for 7 min at 37°C . Reaction was stopped with 30 μl of 0.5 M EDTA (pH 8). Nuclei were lysed after the addition of hypotonic buffer (0.2 mM EDTA, 0.1 mM Benzamidine, 0.1 mM PMSF, and 1 mM DTT). Samples were pre-cleared with protein G agarose, and incubated with anti-H3K4me3 antibody (rabbit polyclonal, Millipore, #07-473) and ChIP dilution buffer (50 mM EDTA, 200 mM Tris and 500 mM NaCl) for 16 h at 4°C under rotation. The antibody-DNA complexes were bound to protein G agarose beads, and the beads were washed with low salt, high salt, lithium chloride, and TE buffers. The antibody-DNA complexes were eluted from the beads, and incubated with Proteinase K for protein digestion. DNA was then eluted and purified by phenol-chloroform extraction.

The ChIP-seq library was prepared as previously (Cheung et al., 2010). The ChIPed DNA was end-blunted using the End-it DNA Repair kit (Epicentre) and A-tailed by using the exo-Klenow DNA polymerase (Epicentre). Genomic adaptor oligos were added to the ChIPed DNA by using the Fast-Link kit (Epicentre), and the products were PCR amplified. A region of ~250 bps containing the expected DNA fragment of 150 bp mononucleosomal ChIPed-DNA and 100 bp adaptors was gel-extracted and DNA purified using PCR purification kits (Qiagen). Libraries were sequenced using Genome Analyzer II (Illumina).

Bioinformatical Analyses

Quality control on the raw sequence reads was performed using FastQC software v0.10.0 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Single end sequencing reads (36 bp) generated from ChIP-seq experiments were aligned to the July 2007 assembly of the mouse genome (UCSC version mm9, NCBI37) using Bowtie (version 0.12.8) (Langmead et al., 2009) to determine the total number of reads per library, as well as the number of uniquely-mappable reads. At most two mismatches in the seed sequence were allowed. Only uniquely mapped reads were kept for the subsequent peak-calling step. For one of the ChIPed female samples, the number of reads was significantly lower than for all others and this sample was excluded from the further analysis. An average of 97.94% of the reads were aligned with the five raw read sequences files F1, F2, M1, M2, M3. Detailed statistics are presented in Table 1.

Enriched methylation signals of H3K4me3 were analyzed using the R statistical programming environment (R Core Team, 2012). Significant peaks of tag binding were determined using the iSeq program (Mo, 2012) and differential binding analysis between male and female samples was carried out with the DESeq Bioconductor package (Anders

and Huber, 2010). Genes/loci of interest were initially identified as those with at least a 1.2-fold sex difference in H3K4me3 at any location throughout the gene length. Separate analyses were then run further restricting this list based on a range of p-value thresholds ($p < 0.1$; $p < 0.05$; $p < 0.01$) for individual genes. The pattern of results was very similar regardless of p-value cutoff and the data presented below are presented for $p < 0.01$.

For the graphical presentation of composite male and female peaks as in Figure 1, the two female and three male libraries were merged, and “bedGraph” files were generated (via BEDtools, version 2.17.0). This allowed visualization of the genomic distribution of H3K4me3 peaks in BedGraph format, averaged across all male and all female samples.

Real-time RT-PCR

For gene expression analysis, BNST/POA and prefrontal cortex samples were obtained from a new cohort of animals. Dissections from four animals were pooled for each sample. Total RNA was extracted using RNeasy Lipid Tissue Mini Kit (QIAGEN, #74804). RNA was reverse-transcribed to cDNA using iScript cDNA Synthesis Kit. cDNA was then amplified using Power SYBR Green PCR Master Mix (Applied Biosystems). Primer sequences used are given in Table 2 and analysis and quantification were performed as in Huang et al., 2006. Expression for each group was calculated relative to male cortex, which was arbitrarily assigned a value of 1.0.

Western blotting

To compare global H3K4me3 in males and females, dissected BNST/POA regions were directly homogenized in 700 μ L 2x Laemmli buffer containing 10% 10x protease inhibitor cocktail (Roche, Indianapolis, IN). Samples were rotated at 37 °C for 20 min and spun at 12,000 rpm at 25 °C for 1 min. Supernatants were collected and boiled for 10 min at 95% after the additional of 10% of 10x NuPAGE Sample Reducing Agent (Novex). Samples were run on 4–20% Tris-Glycine Gels (Thermo Scientific) and blotted onto nitrocellulose membranes. Rabbit polyclonal anti-H3K4me3 primary antibody (1:1000; Millipore, #17–614), anti-histone H3 Pan primary antibody (1:40,000; Millipore, #07–690), and anti-rabbit HRP conjugated secondary antibody (1:5000; Sigma, #A9169) were used. Signals were developed with ECL Western Blotting Detection Reagent (anti-H3 Pan; GE Healthcare) or with Supersignal West Dura Extended Duration Substrate (anti-H3K4me3; Thermo Scientific) and were captured on X-ray film (Kodak). Labeled bands were densitometrically analyzed using Image J (National Institute of Health). H3K4me3 bands were normalized for sample loading differences using the H3 Pan band.

RESULTS

Overall Similarity in H3K4me3 in males and Females

H3K4me3 peaks were similar between males and females across most genes (Supplementary Figure 1). Consistent with H3K4me3 landscapes in other brain regions (Cheung et al., 2010; Shulha et al. 2012), H3K4me3 profiles in the BNST/POA appeared mainly as relatively sharp peaks, extending 1–2Kb around the vicinity of the transcription start sites and other regulatory sequences. The shape and position of the peak at a specific

gene transcription start site was often remarkably similar between samples, suggesting that the genomic profiles were highly reproducible (Figure 1 and Supplementary Figure 2).

For all analyses we excluded Y chromosome genes since the Y is absent in females. Analyses were performed both with X chromosome genes/loci included and for autosomal genes only, as described below.

Sex Differences in H3K4me3

We identified 248 loci associated with specific genes that showed a sex difference in H3K4me3 (216 autosomal and 32 X chromosome genes; Supplementary Tables 1 and 2). The average sex difference in peak size among these genes was 83% (53% for autosomes only). Figure 1 depicts composite peaks for males and females of several genes depicting a range of sex differences.

The H3K4me3 peak associated with *Xist*, a gene exclusively expressed in females, was about 70 fold larger in female than in male samples (Figure 1, bottom trace), supporting the validity of our Chip-Seq analysis. Interestingly, the 248 genes/loci exhibiting a sex difference in H3K4me3 were not equally distributed: 177 had greater H3K4me3 in females (referred to here as F > M genes; Supplementary Table 1) and only 71 had greater H3K4me3 in males (M > F genes; Supplementary Table 2; Chi squared = 45.3; $p < 0.0001$). This was not due to a difference in X chromosome gene dosage. Activating histone marks such as H3K4me3 are uncommon on the inactivated X chromosome (Khalil and Driscoll, 2007), and when only autosomal genes were considered a sex bias remained (151 versus 65 for genes/loci with more H3K4me3 in females or in males, respectively; Chi squared = 34.2; $p < 0.0001$; Figure 2).

We used the open source gene list analysis tool, Enrichr (Chen et al., 2013; <http://amp.pharm.mssm.edu/Enrichr/> accessed 6/2/2014), to analyze the F > M and M > F gene lists. Specifically, MGI Mammalian Phenotype was used to identify predicted phenotypes resulting from mutations in genes in our lists, and Mouse Gene Atlas was used to correlate our gene lists with previous gene expression studies. MGI Mammalian Phenotype indicated that mutations of genes in our F > M list were associated with nervous system disorders, with the most significant associations for the terms *seizures*; *synaptic transmission*; *emotion/affective behavior*; *learning/memory/conditioning*; and *nervous system*, whether analysis was restricted to autosomal genes only or when all genes were considered (Table 3 and Supplementary Table 3). In addition, when the F > M genes were correlated with existing mouse gene expression atlases significant associations were seen for *hypothalamus*, *nucleus accumbens*, and *amygdala* (all p 's < 0.05). Our dissections included a portion of the hypothalamus (preoptic area) and the BNST is often considered part of the “extended amygdala,” based on similarities in connectivity and neurochemistry (Alheid and Heimer, 1988). Taken together, our F > M gene list was highly enriched in genes important in brain function and correlated with actively expressed genes in related brain regions.

The top phenotypes associated with the M > F gene included terms related to embryogenesis and the nervous system (Table 3 and Supplementary Table 3). There was no statistically significant overlap with any brain region gene expression atlas in the Enrichr library of

atlases. This could be related to both the smaller number of genes/loci and the smaller peak sizes in the M > F list: average H3K4me3 peak size was 259.3 reads for genes/loci in the M > F list and 444.2 in the F > M list. Since H3K4me3 is an activating histone mark, genes in the F > M list may be more likely to be actively transcribed.

We also used DAVID (Database for Annotation, Visualization, and Integrated Discovery NIAID; <http://david.abcc.ncifcrf.gov>; Huang et al., 2009a, b) for functional annotation analyses of our gene lists. For the F > M gene list, terms related to synapses showed the greatest fold enrichment (with *synapse* and *synapse part* statistically significant after a Benjamini correction for multiple comparisons when all genes were considered, and *synapse*, *synapse part*, and *neuron projection* significant when only autosomal genes were considered; Supplementary Table 4). No functional categories survived the Benjamini correction in the M > F gene list whether or not X chromosome genes were included.

Several genes are expressed in a sexually dimorphic pattern in both the BNSTp and POA, including *Esr1*, *Greb1* and *Ecell1* (Xu et al., 2012). Of these, *Greb1* had 1.52-fold greater H3K4me3 peaks in males than in females, in the direction predicted by expression data (Supplementary Figure 3; Xu et al., 2012). We did not find similar differences in H3K4me3 for *Esr1* or *Ecell1*, although expression differences for these genes are smaller than for *Greb1* (Xu et al., 2012).

Gene Expression

On a genome-wide scale, the H3K4me3 mark broadly correlates with RNA polymerase II occupancy at sites of active gene expression (Guenther et al., 2005) and is thought to provide an additional layer of transcriptional regulation (Shilatifard, 2008). However, recent findings in differentiating stem cells with genetic ablation of the *Mll1* and *Mll2* H3K4 methyltransferase genes suggest that only 10–15% of H3K4me3 peaks that became dysregulated in the mutant cells are associated with altered expression of the corresponding transcripts (Denissov et al., 2014). This suggests that the mark is less predictive for the expression of individual genes, at least under baseline conditions. Our preliminary analyses suggest that many of the sex-specific H3K4me3 differences are not associated with detectable gene expression changes.

Dissections of the BNST/POA were obtained from a new cohort of male and female mice, and expression determined by RT-PCR for 13 genes from our gene lists (*Usf2*, *Narf*, *Syt4*, *Rgs8*, *Bbs1*, *Kdm5c*, *Kdm6a*, *Ank3*, *Eif2s3x*, *Wbp2*, *Spag9*, *Myb*, and *Lrch*). We also collected a prefrontal cortex sample from each animal in order to determine whether any changes in gene expression were region-specific. *Esr1* was included as a positive control in this experiment because *Esr1* mRNA is higher in the BNST/POA of gonadally intact females than of males (Xu et al., 2012; Kelly et al., 2013), and that difference was confirmed here (Figure 3). We also found a significant sex difference in expression of three other genes (*Kdm5c*, *Eif2s3x* and *Rgs8*), in each case in the direction predicted by H3K4me3 peak sizes. No significant difference was seen for expression of the remaining 10 genes in the BNST/POA and none of the 13 genes showed a significant sex difference in expression in cortex (Figure 3).

We also used immunoblotting to examine total H3K4me3 protein in BNST/POA samples from a small number of males and females, and did not find a significant difference overall (Supplementary Figure 4). This analysis detects H3K4me3 not only within gene regions, but at all chromosomal locations. We conclude that global H3K4me3 across the genome is similar in males and females.

DISCUSSION

The “sexome” is a recently articulated framework for understanding sex differences in any tissue, and is defined as the sum of all sex biased effects on gene networks or cells (Arnold and Lusis, 2012). The idea is that although sex differences may on average be small, in the aggregate these differences can cause functional effects. The sexome concept thus shifts attention from sex differences in single genes to whole-genome or gene network approaches (Arnold, 2014). Few such studies, however, have been conducted to date. Here we have reported preliminary analyses of sex differences in the genome-wide distribution of H3K4me3, a histone mark generally associated with active transcription.

We found that the majority of genes/loci throughout the genome exhibited very similar H3K4me3 peaks in males and females. However, for over 200 loci there was a > 1.2 fold and statistically significant sex difference in peak size. The distribution of genes with a sex difference in H3K4me3 peaks was non-random, with the majority showing larger peaks in females; this was true whether or not X chromosome genes were included in the analyses. Genes/loci with greater H3K4me3 peaks in females were related to brain function and mapped on to genes expressed in related brain areas. This suggests that these genes are likely to be important for normal function, and hence carefully regulated, in both sexes.

Sex differences in H3K4me3 peak size were relatively small, with the large majority under two-fold. This may be related to the heterogeneous nature of brain tissue, which inevitably comprises multiple diverse cell types. It is possible, for example, that there are large sex differences in H3K4me3 peak size at some genes within specific cell types, but that these are obscured in the overall sample. The BNST and POA exhibit sex differences in cell numbers due to differential developmental cell death in males and females (Forger et al., 2004; Holmes et al., 2009), and some sex differences in H3K4me3 peaks may be due to differences in cell type distribution. However the BNST/POA also exhibits sex differences in gene expression unrelated to cell death (De Vries et al., 2008; Xu et al., 2012; Kelly et al., 2013), which likely have epigenetic underpinnings.

Because H3K4me3 is generally associated with gene activation (Bernstein et al., 2002; Santos-Rosa et al., 2002), we used RT-PCR to evaluate whether sex differences in H3K4me3 peaks correlate with differences in expression. For three of the 13 genes examined, we found a significant sex difference in expression in the direction predicted by the H3K4me3 mark. In the majority of cases, however, we did not detect differential expression. It is possible that our RT-PCR experiments did not have the statistical power to detect small differences in expression or that the sex differences in H3K4me3 peak sizes observed here (which ranged from 1.28- to 1.98-fold for the genes for which expression was examined) may not impact expression. We also examined gene expression and H3K4me3

status under steady state conditions and sex differences in H3K4me3 peaks may poise genes for differential responses to specific signals. In pluripotent cells, for example, H3K4me3 is often seen in combination with the (usually repressive) H3K27me3 mark, a combination thought to identify genes that are currently silenced but primed for transcriptional activation (Azuara et al., 2006).

However, H3K4me3 is just one of many histone modifications and multiple histone marks in combination with DNA methylation state and other factors interact to determine levels of gene expression. We suggest that for genes in which H3K4me3 is greater in females, a different epigenetic modification may in many cases compensate for this sex difference (i.e., a counter-acting repressive mark in females or a different activating mark in males). By analogy, the most dramatic epigenetic sex difference known, namely the inactivation of one X chromosome in every female cell, operates largely to makes gene expression similar in males and females.

Two of the three genes with confirmed expression differences (*Kdm5c* and *Eif2s3x*) had the largest fold-difference in H3K4me3 of the genes we tested. These genes also are X-linked and escape X-inactivation in some tissues, so the increased H3K4me3 associated with these genes is likely due to a double dose of active X genes in females. Regions of the X chromosome that escape inactivation are enriched in H3K4me3 relative to the rest of the chromosome (Khalil and Driscoll, 2007), although specific genes were not examined in that study. We note that we did not find greater H3K4me3 peaks in females for all genes thought to escape X inactivation in mice (Yang et al., 2010): five X chromosome genes/loci that escape inactivation had at least 1.2-fold greater H3K4me3 in females (*Kdm5c*, *Kdm6a*, *Eif2s3x*, *Ddx3x* and *2610029G23Rik*), whereas four others did not (*Mid1*, *Shroom4*, *Car5b*, and *Bgn*).

The greater expression of *Kdm5c* and *Eif2s3x* in the BNST/POA of females is consistent with previous findings (Xu et al., 2008; Bonthius and Rissman, 2013). *Kdm5c* is a lysine demethylase that itself might alter the epigenome, and *Eif2x3x* encodes a subunit of eukaryotic translation initiation factor 2, which regulates the rate of protein translation. Sex differences in the expression of either of these genes could cause differences in brain function. No sex difference was detected in expression of either *Kdm5c* or *Eif2x3x* in prefrontal cortex, however, and it would be interesting to know whether this correlates with absence of a sex difference in the H3K4me3 peaks associated with these genes. The escape from X inactivation has been reported to vary by tissue type and between brain regions (Xu et al., 2006; 2008b).

Sex differences are generally attributed to one of three causes: sex chromosomal genes, gonadal steroid hormones acting early in life, or gonadal steroids acting in adulthood. All three mechanisms contribute to sex differences in the BNST (De Vries et al., 2002; Hisasue et al., 2010; Xu et al., 2012; Kelly et al., 2013) and could underlie sex differences in H3K4me3. Mice in our study were gonadally intact, so activational effects of steroids are likely. A genome wide study in liver, for example, found that most of the sex differences in gene expression seen in intact mice were eliminated by adult castration (van Nas et al., 2009). It would be of interest to follow up the observations here with a study of H3K4me3

in animals that were gonadectomized in adulthood, to identify genes at which H3K4me3 is regulated by circulating gonadal steroids.

A substantial literature links estrogen action in various tissues to epigenetic changes at target genes. One of the earliest demonstrations of a role for DNA methylation in the control of gene expression involved the regulation of the estrogen-inducible vitellogenin II gene in avian liver (Saluz et al., 1986). Since then, estradiol has been shown to alter histone acetylation and methylation in promoter regions of estrogen target genes in mammalian cell lines (e.g., Metievier et al., 2003; Liu et al., 2005) and, more recently, in the brain. For example, Gagnidze and colleagues found that global H3K4me3, and H3K4me3 at two estrogen target genes, were transiently elevated in the ventromedial hypothalamus of adult female mice following acute estrogen treatment (Gagnidze et al., 2013). Estradiol also increases DNMT expression and decreases HDAC2 protein levels in the hippocampus of adult female rats (Zhao et al., 2010) and these changes appear to be required for estradiol's effects on memory enhancement. Interestingly, there is a sex difference in estrogen receptor mRNA and protein in the BNSTp of adult mice (female > male; Xu et al., 2012; Kelly et al., 2013), which may confer greater estrogen sensitivity to females.

Androgen receptors may also mediate changes in the epigenetic landscape, although the evidence in brain is scant thus far. For example, vasopressin expression in the BNST is eliminated following adult castration of male rats (De Vries et al., 1994). This correlates with increased DNA methylation in a regulatory region of the vasopressin gene (Auger et al., 2011) and both the decrease in vasopressin and the increase in DNA methylation can be prevented by treating castrates with testosterone. It is not clear whether this is an example of androgen receptor-dependent modulation of the brain epigenome, however, because testosterone can be aromatized to estrogenic metabolites, which are actually more potent than androgens in maintaining BNST vasopressin expression (De Vries et al., 1994).

Any sex differences in H3K4me3 seen in the current study could also be due to early organizational effects of gonadal steroids. In the mouse uterus, neonatal estrogen treatment causes acute changes in the expression of histone methyltransferases; moreover, several histone modifications associated with active transcription, including H3K4me3, are enriched at promoter regions of target genes whose expression is permanently enhanced by the early estrogen treatment (Jefferson et al., 2013). As mentioned above, neonatal estradiol treatment leads to DNA methylation changes in adulthood at single CpG sites on steroid receptor genes in the hypothalamus of rats (Schwarz et al., 2010) and a recent, genome-wide study of DNA methylation in the mouse brain identified late-emerging effects of neonatal exposure to gonadal hormones (Ghahramani et al., 2014).

Our findings suggest that more genes show a larger H3K4me3 peak in females versus males than genes that show the opposite pattern. Similarly, a sex bias in differentially methylated genes was found in the study by Ghahramani and colleagues (2014): over 80% of the genes found to exhibit a sex difference in DNA methylation in the adult mouse brain were more methylated in males. Moreover, as in the current study, sex differences in DNA methylation did not correlate well with sex differences in gene expression (Ghahramani et al., 2014). This suggests that there may be sex differences in the epigenetic underpinnings of gene

expression control, even when expression itself is not dimorphic. If so, then drugs, hormones, environmental factors, or disease states that selectively perturb specific epigenetic processes might affect one sex more than the other, leading to sex-specific vulnerability to disease.

Epigenetic modifications of chromatin have been discovered in the brains of patients with schizophrenia and bipolar disorder, and most of these modifications are sex specific (Mill et al., 2008; Connor and Akbarian, 2008). Moreover, peaks of susceptibility to major psychosis often coincide with hormonal transitions (e.g., puberty, menopause; Petronis, 2004), suggesting that sex differences in susceptibility to disease may be mediated by gonadal hormone-induced differences in the epigenetic regulation of key genes (Kaminsky et al., 2006). Similarly, and in line with the sexome concept described above, Hodes (2013) recently proposed that sex differences in stress-related disorders may be due to myriad small changes in gene networks due to epigenetic processes. Given the key role of the BNST in the control of stress and anxiety (Toufexis, 2007), the findings reported here suggest a starting point in identifying the relevant genes and epigenetic marks that may underlie well established sex differences in stress-related and other disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We review recent work on the role of epigenetics in brain sexual differentiation.
- A hormone-sensitive brain region was dissected from adult male and female mice.
- ChIP-Seq was used to examine the distribution of H3K4me3 throughout the genome.
- H3K4me3 varied by sex in 248 genes; for 70% of these, peaks were larger in females.
- Genes/loci with more H3K4me3 in females were associated with synaptic transmission.

Figure 1.

H3K4me3 profiles for autosomal (*Creb1*, *Syt4*) and X-linked (*Kdm5c*, *Eif2sx*, *Xist*) genes shown separately for female and male BNST/POA, as indicated. UCSC genome browser tracks for female and male samples were averaged to create a composite peak within each sex. Note the sharp regulation of H3K4me3 around gene transcription start sites.

Figure 2.

Pie chart showing the distribution of the chromosomal location (autosomal vs. X-linked) and the direction of sex differences (M > F vs. F > M) of the genes/loci that were identified as showing a sex difference in H3K4me3 (defined as 1.2-fold difference between M and F and $p \leq 0.01$).

Figure 3.

Sex differences ($F > M$) in gene expression were found for *Esr1* (positive control), *Kdm5c*, *Eif2s3x*, and *Rgs8* in the BNST/POA (* $p < 0.05$; *** $p < 0.001$). The fold-difference between males and females in H3K4me3 for these genes was 1.82 (*Kdm5c*), 1.94 (*Eif2s3x*), and 1.32 (*Rgs8*). No expression differences were found in the cortex for any of the genes.

Table 1

Sequencing statistics for samples used in the current study

Sample ID	Total Reads	Unique Reads	Alignment Percentage	Number of Peaks Returned by Peak Caller
F1	16741602	16458175	98.31%	22431
F2	20416450	20037137	98.14%	24726
M1	23778978	23042614	96.90%	26463
M2	18474257	17971304	97.28%	24023
M3	23863392	22868641	95.83%	25921

Table 2

RT-PCR primers

Gene	Name	Forward	Reverse	Amplicon Size [bp]
Usl2	Upstream Transcription Factor 2	CTGCCCTCTGTACCCACAGGT	GCCTGTAGGCTCTGGTCTGT	187
Narf	Nuclear Prelamin A Recognition Factor	ACCACITTTGTGGAGGTGCTC	AGTACCCCTCAGCCACTCCT	191
Syf4	Synaptotagmin 4	CGCTCACTGTGGTGGTCTTA	CTTGGCATGGTACAGGTTCA	98
Rgs8	Regulator Of G-Protein Signaling 8	CCTGCCGAGGAGTTCAA GAAG	CTGTGGACITTTTCCCTGAGC	193
Bbs1	Bardet-Biedl Syndrome 1	AGAGTCTGCATGGCTTCACC	AGGGCCTTGTCTCGGTAAAT	163
Kdm5c	Lysine (K)-Specific Demethylase 5C	GAGAAAGGAGCTGGGGTTGTA	CGAAGCTGCAGTATCCCTTC	137
Kdm6a	Lysine (K)-Specific Demethylase 6A	GCTGGAACAGCTGGAAAGTC	GAGTCAAACTGTTGGCCCAAT	111
Ank3	Ankyrin 3	TGCTTTCACTTGCAGGTTGG	TTCTCCCGTGAGGTATGAGG	152
Esr1	Estrogen Receptor 1	CAGGTGCCCTACTACCTGGA	AGGCATAGTCATTGCACACG	199
Eif2s3x	Eukaryotic Translation Initiation Factor 2, subunit 3	TGGAAITTTCTTTTACTAGCCTAGGGGT	TTTGTCTGTGTGTGTGTGCTACTT	308
Wbp2	WW Domain Binding Protein 2	GCTGCCAGTGCCTATTAC	CTACTGGGCTCTCTTGTCTTC	108
Spag9	Sperm Associated Antigen 9	AGGTTGCCCAAGAGACTA	GGGAGTGTGAAACCTTTAT	140
Myb	V-Myb Avian Myeloblastosis Viral Oncogene	GAAAAGTGTGAACCCCTGAA	CCGACACTGCTTTTCCAAT	145
Lrch4	Leucine-Rich Repeats and Calponin	TGTGAGCAGCAATGAGC	ATGCGGTTACAGGAGAAAATC	153
18S RNA	18S Ribosomal RNA	GTAACCCCGTTGAACCCCAAT	CCATCCAATCGGTAGTAGCG	151

Table 3

Summary of MGI Mammalian Phenotype analysis (Enrichr) which identifies abnormal phenotypes associated with mutations in genes of interest. The most statistically significant terms are listed for gene lists with greater H3K4me3 in females (F>M) or males (M>F).¹

Term	# Genes	Adj. p- value ²
F>M		
seizures	9	0.0047
synaptic transmission	9	0.0266
emotion/affective behavior	8	0.0266
learning/memory/conditioning	8	0.0329
nervous system	6	0.0329
M>F		
embryonic tissue	11	0.0005
nervous system	11	0.0007
eyelid morphology	5	0.0068
embryogenesis development	7	0.0068
embryogenesis phenotype	7	0.0068
brain morphology	11	0.0068

¹ See Supplementary Table 3 for additional terms and details of this analysis.

² P-values listed are following adjustment for multiple comparisons.