Circumstantial evidence alone argues that the establishment and maintenance of sex differences in the brain depend on epigenetic modifications of chromatin structure. More direct evidence has recently been obtained from two types of studies: those manipulating a particular epigenetic mechanism, and those examining the genome-wide distribution of specific epigenetic marks. The manipulation of histone acetylation or DNA methylation disrupts the development of several neural sex differences in rodents. Taken together, however, the evidence suggests there is unlikely to be a simple formula for masculine or feminine development of the brain and behaviour; instead, underlying epigenetic mechanisms may vary by brain region or even by dependent variable within a region. Whole-genome studies related to sex differences in the brain have only very recently been reported, but suggest that males and females may use different combinations of epigenetic modifications to control gene expression, even in cases where gene expression does not differ between the sexes. Finally, recent findings are discussed that are likely to direct future studies on the role of epigenetic mechanisms in sexual differentiation of the brain and behaviour.

1. Sexual differentiation and epigenetics

Mammalian sex determination relies on sex chromosome complement (XX versus XY), so male and female mammals are not genetically equivalent. It is worth remembering, however, that many species that come in two sexes do not have sex chromosomes, and sex is instead determined by environmental signals. For example, in many reptiles and some fish the ambient temperature during incubation determines the sex of the individual [1,2]. In other words, perfectly good male or female brains and bodies can develop from an identical genome, based on differences in the epigenetic regulation of that genome [3,4]. Similarly, although there are genetic differences between male and female mammals, many of the sex differences in mammalian brains and behaviour are likely epigenetic in origin.

There is not a single definition of epigenetic regulation, but its essence is the modulation of gene activity through changes in chromatin structure. The fundamental unit of chromatin, the nucleosome, comprises approximately 150 base pairs of DNA that wraps around an octamer of histone proteins. Adjacent nucleosomes are further packaged to different degrees, with epigenetic modifications determining the extent of compaction, accessibility to transcription factors, and rate of gene transcription [5,6]. The two best-studied types of epigenetic modifications are DNA cytosine methylation and the covalent modifications of histone tails, both of which have been linked to sexual differentiation of the brain and are the focus of this review.

Although the first studies on specific epigenetic mechanisms in brain sexual differentiation appeared less than 10 years ago [7–10], circumstantial evidence that epigenetics plays a role was available much earlier. For example, most known sex differences in the brain depend on gonadal steroid hormones, acting during a critical developmental window [11]. Soon after the testes differentiate they begin secreting testosterone. In rodents, testosterone frequently acts after being converted in the brain to an oestrogen (oestradiol) in a process known as aromatization [11]. There is often a delay between the testosterone or oestradiol exposure and emergence of a sexually dimorphic trait, suggesting
BHSTp is not known, however. In adults, exactly how testosterone regulates cell death in the BNSTp is not known, however. It causes the differences in volume and cell number seen in sex difference in cell death in the BNSTp several days later,\textsuperscript{[26]} Thus, exposure to gonadal hormones at birth results in the size of the BNSTp in adulthood can be masculinized in females. This size difference in cell death in the BNSTp and male sexual behaviour. The principal nucleus of the bed nucleus of the stria terminalis (BNSTp) of mice. Males, females and females treated with testosterone propionate (TP) received VPA or saline (sal) at birth and cell counts were made at weaning. (a) VPA reduced cell number in the BNSTp of males and females + TP but did not affect cell number in the BNSTp of females. (b) VPA did not affect cell number in the suprachiasmatic nucleus (SCN) of any group. Adapted with permission from \textsuperscript{[9]}. a ‘memory’ for the hormone exposure; epigenetic modifications, which can be long-lived, are likely candidates for this kind of cellular memory. Second, the normal mode of sex steroid action involves epigenetic events: after binding their ligands, steroid hormone receptors recruit co-activators that themselves can modify histones (e.g. by acetylation) or that attract other proteins with histone modifying activity [12–17]. With 20/20 hindsight, it therefore seems almost inevitable that hormone-dependent sex differences in the brain would involve modifications to the epigenome, and direct tests have borne this out.

2. Role of histone acetylation in differentiation of the BNSTp and male sexual behaviour

The principal nucleus of the bed nucleus of the stria terminalis (BNSTp) and the medial preoptic area (mPOA) of the hypothalamus have been the focus of several studies on the role of epigenetic mechanisms in the development of brain sex differences. These interconnected brain regions are involved in the processing of olfactory cues and the regulation of male sexual behaviour \textsuperscript{[18–20]; the BNSTp is also an important node in the processing of stress and anxiety \textsuperscript{[21,22]}. The BNSTp is larger in males than in females in adults of many species, including rats, mice, guinea pigs and humans \textsuperscript{[23–26]}. In rodents, this sex difference depends on the perinatal actions of gonadal steroids and is due to differential cell death. BNSTp volume and cell number are equivalent in rats and mice of both sexes at birth \textsuperscript{[27,28]}. Females have more dying cells than males during the latter part of the first postnatal week \textsuperscript{[27–29]}, and the volume differences emerge thereafter. The size of the BNSTp in adulthood can be masculinized in females treated with testosterone on the day of birth; oestradiol is similarly effective, suggesting that testosterone normally acts after conversion to an oestrogen \textsuperscript{[24,30]}. Finally, the sex differences in BNSTp volume and cell number are eliminated in mice lacking Bax, a gene required for the death of developing neurons \textsuperscript{[26]}. Thus, exposure to gonadal hormones at birth results in a sex difference in cell death in the BNSTp several days later, which causes the differences in volume and cell number seen in adults. Exactly how testosterone regulates cell death in the BNSTp is not known, however.

Given the role of histone acetylation in steroid hormone action, we hypothesized that this epigenetic mark might be important for sexually dimorphic BNST development. Histone acetylation is usually associated with transcriptional activation and is controlled by histone acetyltransferases and histone deacetylases (HDACs) that, respectively, add or remove acetyl groups from lysine residues of histone tails \textsuperscript{[31]}. When newborn mice were treated with the HDAC inhibitor, valproic acid (VPA), histone acetylation in the brain was transiently increased \textsuperscript{[9]}. Neonatal VPA treatment also prevented masculinization of BNSTp volume and cell number in both control males and testosterone-treated females (figure 1). We could exclude a non-specific effect on cell survival, because VPA did not affect cell number in the BNSTp of females, or in control brain regions \textsuperscript{[9]}. Although these findings supported a role for histone deacetylation in brain masculinization, VPA, like other HDAC inhibitors, has effects independent of HDAC inhibition. Work by Matsuda \textit{et al.} \textsuperscript{[32]} was therefore important in taking things several steps further. These investigators gave newborn male rats intracerebroventricular (icv) injections of the HDAC inhibitor trichostatin A, and also included groups treated with antisense oligonucleotides to block the \textit{endogenous} production of specific HDACs. When compared to animals receiving control treatments, those receiving either the HDAC inhibitor or antisense oligonucleotides to HDACs showed marked impairments in male sexual behaviour in adulthood \textsuperscript{[32]}. Thus, this and the previous study \textsuperscript{[9]} are consistent in demonstrating that HDAC activity is required for masculinization, in one case of brain morphology and in the other of behaviour. Because HDACs decrease histone acetylation, which in turn would be expected to decrease gene expression, on the surface these findings suggest that masculinization requires the suppression of one or more gene(s). Neither study identified the specific gene targets, however and, as we shall see, a simplistic formula such as ‘masculinization requires gene suppression’ does not hold up.

3. DNA methylation and sexual differentiation of the brain and behaviour

The covalent addition of methyl groups to cytosine residues of DNA is catalysed by a family of enzymes known as DNA methylation.
methyltransferases (DNMTs). Methylated cytosines then attract methyl-binding partners, such as methyl-CpG-binding protein 2 (MeCP2), which in turn recruit other proteins, such as HDACs, that generally act to repress transcription [33,34]. In one of the first studies to examine effects of manipulating a specific epigenetic mechanism on sexual differentiation, Kurian et al. [35] used small interfering RNAs to decrease MeCP2 expression in the amygdala of neonatal rats and measured several behaviours at weaning. Juvenile male rats normally play more than females, but neonatal treatment with MeCP2 siRNA reduced social play behaviour specifically in males, thereby eliminating the sex difference [35]. Sociability and anxiety were not affected, but also did not show sex differences. This suggests that preventing the sequelae of DNA methylation in the amygdala prevents the masculinization of play behaviour.

In the most comprehensive study on the topic to date, however, Nugent et al. [36] found a role for greater DNA methylation in females in normal, sexually differentiated development of the rat mPOA. A number of sex differences have been described in the mPOA, including the existence of a cell group that is several times larger in males, greater complexity of astrocytes in males and greater dendritic spine density in males [37–39]. All of these features are masculinized in females treated with testosterone, or an aromatized product of testosterone such as oestradiol, during a critical neonatal period; male sexual behaviour is also masculinized in such hormone-treated females [40]. In punches of the mPOA of neonatal female rats, Nugent et al. found greater DNMT activity and more fully methylated sites (90–100% of cytosines methylated) genome-wide in females than in males. Both of these sex differences were eliminated by treating newborn females with oestradiol [36], illustrating their hormone dependence.

To determine whether sex differences in DNA methylation may be required for sexual differentiation of the mPOA, the investigators then administered the DNMT inhibitors, zebularine or RG108, icv to newborn rats. Neonatal DNMT inhibition masculinized dendritic spine density and copulatory behaviour of females in adulthood, with little to no effect in males (figure 2) [36]. Remarkably, DNMT inhibition on postnatal days 10 and 11 (i.e. after the critical period for sexual differentiation) also masculinized dendritic spines and at least some features of male copulatory behaviour [36]. Since DNA methylation is usually associated with transcriptional repression, this suggests that normal female brain morphology and behaviour involve the active repression of masculinization, an interesting twist on the traditional view that female development is the ‘passive,’ or default, mode.

4. Do the studies to date contradict each other?

The histone acetylation and DNA methylation studies described above seem to give conflicting messages about whether repression or activation of gene expression is associated with masculinization of the brain and behaviour. If we accept at face value that both HDAC inhibition and DNMT inhibition increase gene expression, then findings could be summarized as in table 1. It is possible, of course, that effects of a specific epigenetic manipulation may vary by age, species or brain region. Recent data from our laboratory suggest that such effects even vary by dependent measure within a brain region. For example, in the mPOA of mice, males have more cells expressing the calcium-binding protein calbindin, but females have more cells expressing oestrogen receptor α [42,43]. We treated newborn mice, icv, with the DNMT inhibitor zebularine or vehicle, and examined effects on expression of these two proteins after weaning. Our preliminary findings [41] indicate that neonatal treatment with zebularine increases both the number of calbindin cells and oestrogen receptor α cells in the mPOA (figure 3) [41]. While the changes are in the expected direction for a DNMT inhibitor (decreased DNA methylation leading to increased gene expression), expression is pushed in the ‘masculine’ direction in one case (calbindin) and in the ‘feminine’ direction in the other.

More to the point, however, it is probably too simplistic to think of histone deacetylation and DNA methylation as repressing transcription, or HDAC inhibitors and DNMT inhibitors as increasing transcription. First, there are many examples where DNA methylation or histone deacetylation of a specific
gene increases transcription [44–46]. Second, in contrast to what one might think, treatment of normal cells with an HDAC or DNMT inhibitor alters the expression of only a few per cent of all genes [36,47,48], with expression of some going up and others going down. In other words, these are not sledgehammer approaches that globally increase all gene expression. Although it is not known what makes a given gene susceptible to an HDAC or DNMT inhibitor, one suggestion is that genes actively undergoing regulation may be particularly affected [48,49].

Thus, the foregoing studies [9,32,35,36] are important in showing a requirement for epigenetic modifications in sexually differentiated development of brain and behaviour. However, we may not know what needs to be repressed or activated until specific gene cascades are identified and, even then, the answer is likely to be complex and region- or even variable-specific. In a complementary—in some ways opposite—approach, several recent studies have taken a broad view by conducting whole-genome analyses of epigenetic marks in the male and female brain.

### 5. Whole-genome studies of sex differences in epigenetic mechanisms

The past 2 years have seen the publication of at least five genome-wide studies of sex differences in specific epigenetic marks in the brain [36,50–53].

Ghahramani et al. [50] used reduced representation bisulfite sequencing to examine the programming (or organizational) effects of neonatal testosterone on the DNA methyolme and transcriptome of the mouse brain. Brains were collected on postnatal day 4 or in adulthood from control males, control females or females treated with testosterone on the day of birth, and the BNST/mPOA and striatum were microdissected from all animals. To ensure that group differences were not due
to different levels of circulating hormones at the time of sacrifice, mice killed as adults were gonadectomized prior to puberty and given capsules designed to produce equivalent, male-like levels of testosterone in all animals. The ambitious design of this study generated a large amount of data, with at least four interesting take-home messages.

First, effects of neonatal testosterone on the methylome were late emerging. At P4, there were very few genes (fewer than 70) with an effect of neonatal testosterone exposure (females versus females plus testosterone) or with a sex difference (males versus females in DNA methylation. By adulthood, however, 700–1400 genes had significant testosterone- or sex-dependent differences in methylation in the striatum and BNST/mPOA (figure 4) [50]. Thus, hormone exposure at birth led to differences in DNA methylation, most of which emerged well after birth.

A second finding from this study was that the differentially methylated genes were unevenly distributed: between 85 and 90% of the genes that were differentially methylated in females versus testosterone-treated female mice had less methylation in the female group (figure 4). This was true at both ages and in both brain regions, and was also seen in the female versus male comparison (i.e. most genes with a sex difference were hypomethylated in females) [50]. Similarly, a study of the DNA methylome during human fetal brain development identified about 60 genes, or genomic sites, with a significant sex-by-age interaction in DNA methylation; of these, most sites showed progressive hypomethylation in females beginning at approximately 100 days gestation [51]. Because the prenatal testosterone surge in humans occurs for about 250 genes. Of these, the majority (approx. 75%) had greater H3K4me3 in females [52]. Similarly, a study of the DNA methylome during human fetal brain development identified about 60 genes, or genomic sites, with a significant sex-by-age interaction in DNA methylation; of these, most sites showed progressive hypomethylation in females beginning at approximately 100 days gestation [51]. Because the prenatal testosterone surge in humans occurs from about 70–125 days gestation [54,55], the relative hypo-methylation in females coincides with the differential exposure to testosterone in males and females.

A third major message from the Ghahramani et al. [50] study was that the striatum and BNST/mPOA showed similar patterns and, if anything, there were more sex differences in DNA methylation in the striatum. Although sex differences have been described in the striatum, it is not as overtly dimorphic as the BNST/mPOA. This suggests that at the level of control of gene expression, sex differences are not limited to brain regions we traditionally think of as very dimorphic. This may be related to the final take-home message, which is that most genes with a sex difference in DNA methylation were not differentially expressed [50]. In some ways, this may actually be the most interesting outcome, especially when combined with the sex bias in differentially methylated genes because it suggests that males and females may use different epigenetic mechanisms to achieve the same outcome in terms of gene expression.

A similar conclusion was reached by our recent study using chromatin immunoprecipitation followed by deep sequencing (ChIP-Seq) to examine the genome-wide distribution of H3K4me3 in males and females in the striatum and BNST/mPOA of adult mice [52]. This histone modification is of particular interest because it is highly enriched at transcription start sites of active genes, or of genes poised for activity [56–58]. While the large majority of genes had very similar H3K4me3 profiles in males and females, profiles differed significantly for about 250 genes. Of these, the majority (approx. 75%) had greater H3K4me3 in females, and this was seen whether the sex chromosomes or autosomes were considered (figure 5). Also, as in [50], sex differences in H3K4me3 did not map closely onto gene expression [52]. Genes with greater H3K4me3 in females were significantly associated with synapse structure and function; the expression of such genes is presumably tightly regulated in both sexes, but males and females may do it differently.
Taken together, the findings suggest sex biases in the use of epigenetic marks to control gene expression. Such biases could come about if, for example, gonadal steroids control the expression of enzymes involved in placing or removing particular epigenetic marks, as was seen for DNMT activity in the mPOA in the study mentioned above [36]. Predispositions to use particular epigenetic mechanisms could also result from sex chromosome complement. In every cell of females, and in no cells of males, one X chromosome is inactivated. This occurs via countless epigenetic modifications of the silenced chromosome that must be continually maintained [59,60]. If any of the epigenetic machinery involved in X chromosome inactivation is rate-limiting, this creates an uneven playing field for regulating the expression of autosomal genes [61,62]. Thus, for any given epigenetic modification, the majority of differences between the sexes genome-wide may actually serve a compensatory role, i.e. to prevent differences in expression that would occur otherwise [63]. Sex biases in the use of epigenetic modifications could be clinically important because they would render the sexes differentially vulnerable to drugs or diseases that disrupt a particular epigenetic mechanism.

6. New rules for studies of epigenetic mechanisms in brain sexual differentiation

In the past few years, research findings have forced revisions to several cherished epigenetic concepts that are likely to impact the design of future studies on the epigenetics of sexual differentiation.

(a) It is not just CpG any more

Until recently, the dogma held that DNA methylation in mammals is restricted to cytosine nucleotides adjacent to a guanine nucleotide (CpGs). As such, investigators typically restricted their analyses to CpG sites, and often to a subset of selected CpGs (e.g. in promoter regions). It is now clear, however, that cytosines followed by A, C or T nucleotides can also be methylated in mammalian cells, and that this non-CpG methylation correlates with gene repression [64]. This finding is particularly relevant to neuroscience, because neurons show the highest levels of non-CpG methylation of any known mammalian cell type: roughly 50% of all DNA methylation in adult human neurons and 25% in adult mouse neurons is non-CpG [64–66]. Future studies on the epigenetics of brain sexual differentiation will undoubtedly have to take this into account.

(b) Mechanisms for demethylation are now established

Originally, DNA methylation was viewed as a permanent event. Methyl marks can indeed be long lasting, which accounts for the stable differentiation of cell types, but it has been clear for some time that DNA methylation (as well as histone modifications) in the brain dynamically change with age, learning and other factors [67–69]. The mechanism for such changes has been somewhat mysterious because there was no known demethylase, but recent work convincingly describes a mechanism for DNA demethylation involving hydroxymethylation of cytosine residues as an intermediary [70]. Interestingly, hydroxymethylation is more prevalent in the brain than in any other tissue [66,70]. As yet, we are not aware of any studies of sex differences in brain hydroxymethylation, but this is surely just around the corner.

(c) The question of site-by-site fidelity

Cells of the same type do not all have exactly the same pattern of DNA methylation. Under steady-state conditions, a population of cells may maintain average per cent methylation at a given gene region, but exactly which cytosines are methylated changes through an apparently stochastic process [71]. Indeed, it has been argued that no known DNMT has sufficient specificity to maintain site-by-site fidelity in methylation [72]. Many of us in the field of behavioural neuroscience first became aware of epigenetics through landmark papers showing DNA methylation changes to single CpG sites in a specific gene that could account for a behavioural and neuroendocrine phenotype [73]. This is likely to be the exception rather than the rule. Evidence suggests that only rarely do differences in DNA methylation at a single site relate to measureable differences in gene expression. In a recent whole-genome study of sex differences in DNA methylation in the human prefrontal cortex, for example, about 6% of the CpG sites that were differentially methylated in men and women correlated with a sex difference in transcription [53]. The odds get a little better going in the other direction: starting with a significant sex difference in gene transcription, a significant difference in DNA methylation was found about 35% of the time (but even then, only three-fourths of those were in the predicted direction) [53]. Taken together, the data suggest that rather than a focus on individual methylation events, it may be useful to think about ‘methyl tone’ or ‘acetyl tone’ in a given cell or gene region, much as we would use terms such as ‘GABAergic tone’ or ‘glutamatergic tone.’

(d) Accounting for cellular heterogeneity

Most of the sex differences in epigenetic marks found in the brain so far have been relatively subtle (i.e. 20% or fewer differences between males and females for individual genes) [36,50,52,67]. One reason for this is almost certainly the reliance on brain homogenates, which contain a multitude of cell types. Sex differences in epigenetic regulation that are cell type-specific will be masked in studies relying on homogenates (essentially, all studies to date), owing to the absence of a difference in other cells comprising the sample. A few studies have surveyed epigenetic marks in the brain following cell sorting to separate neurons from non-neurons; as might be predicted, very different patterns of age-related epigenetic changes are seen in neuronal versus non-neuronal cells (e.g. [74,75]). This is a relatively crude division, however. More refined analyses in which different subtypes of neurons are compared in the male and female brain have not yet been performed, in large part because of the amount of tissue required (the cell sorting papers cited above were conducted on human cortex, which permits a large volume of starting material). The development of techniques for quantifying the use of specific epigenetic marks, by specific genes, within specific cell types would do for the study of brain epigenetics what immunocytochemistry and in situ hybridization did for studies of gene expression in the brain. Until such techniques are available, we are likely to continue to see sex differences of underwhelming magnitude, and the interpretation of every
study on sex differences in brain epigenetics must take this limitation into account.

7. What will the future bring?

Sixteen years ago, Strahl & Allis [76] coined the term ‘histone code’ to capture the idea that multiple histone modifications act in combination to regulate chromatin structure and gene transcription. Inherent in the concept was the suggestion that if we just learned to decipher the code, we could ‘read out’ expression levels for a given gene region. However, each year brings the identification of new epigenetic modification sites, and new molecules interacting with these sites. The ‘epigenetic code’ becomes more and more complex, and it sometimes seems that we are farther than ever from a fluent reading of it. In addition, the brain may be thought of as a mosaic of sex differences and sex similarities [77], and there is likely to be a mosaic of mechanisms, including epigenetic mechanisms, underlying sexual differentiation of neural structures.

Given all of the complexity in both fields, what will the future bring? Although it is hard to know, I feel fairly confident making two predictions. First, more investigators will be studying sex differences in the brain. The new National Institutes of Health initiative to balance the sex of animals and cells in preclinical research (NIH Notice: NOT-OD-15-102) will lead to the identification of new brain sex differences along with an influx of new investigators with fresh perspectives. Second, it seems inevitable that computational and statistical methods will play an ever-increasing role in those studies with an epigenetic component. The new field of computational epigenetics [78] holds some promise for dealing with the enormous combinatorial complexity of epigenetic mechanisms; the hope is that these tools may point us to new, testable hypotheses and allow for progress not possible using traditional approaches alone.

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