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Neuroimmunology and neuroepigenetics in the establishment of sex differences in the brain

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Abstract

The study of sex differences in the brain is a topic of neuroscientific study that has broad reaching implications for culture, society and biomedical science. Recent research in rodent models has led to dramatic shifts in our views of the mechanisms underlying the sexual differentiation of the brain. These include the surprising discoveries of a role for immune cells and inflammatory mediators in brain masculinization and a role for epigenetic suppression in brain feminization. How and to what degree these findings will translate to human brain development will be questions of central importance in future research in this field.

Historically, the study of sex differences in brain and behaviour was not considered to be broadly important to neuroscience but was instead walled off under the rubric ‘reproductive endocrinology’. This niche area focused on the neurophysiology of ovulation and lactation and the essential behaviours of mating and parenting. Nevertheless, there were persistent reports of males and females differing in non-reproductive parameters, such as performance on learning tasks[1–4], and associated neurophysiological correlates like hippocampal long-term potentiation (LTP)[2] and cortical synapse number[5].

Parallel to animal studies was the advent of functional MRI, which allowed for assessment of human brain activity in real time and provided the opportunity to revisit the ageless question ‘do men and women think differently?’ Functional MRI studies revealing such differences have involved tasks assessing verbal recall, spatial learning, non-verbal reasoning, emotional face reading, fear and anxiety, and responses to sexually explicit stimuli, as well as the impact of stress on most of these tasks (for a review, see REF. 6).

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Competing interests statement

The authors declare no competing interests.

FURTHER INFORMATION

World Health Organization definition of gender: http://www.who.int/gender-equity-rights/understanding/gender-definition/en/

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Similarly, the emergence of diffusion tensor imaging revealed sex differences in the connectome: girls and young women were found to have stronger interhemispheric connections, whereas boys and young men had greater intrahemispheric connections[7]. In a follow-up study in a subset of individuals, a male bias in performance in sensorimotor tasks was found to correlate with a higher degree of connectivity between motor and sensory cortex nodes, whereas a female bias in performance in tasks involving social cognition and non-verbal reasoning corresponded to greater connectivity across the hemispheres of subcortical regions[8]. However, the authors of this study themselves noted the apparent 'playing to stereotype' of these findings, as have others[9], who have cautioned that a correlation in measures is not indicative of causation.

Arguments about the validity of using imaging to determine sex differences in humans continue to this day and are likely to persist for some time. This is particularly apparent when the confound of gender is considered[8,10]. Gender is a uniquely human construct that combines self and societal awareness of one's sex, thereby including the influence of cultural norms, implicit bias and parental expectations (according to the World Health Organization definition of gender; see Further Information). This makes it difficult, if not impossible, to isolate a purely biological contribution to sex differences in human brain and behaviour. Indeed, some would argue that we should not even try as there is potential to do real harm by lending scientific credence to well-entrenched stereotypes[10].

Despite these legitimate concerns, there are two reasons that an understanding of such a biological contribution continues to be sought. The first is that animal research unequivocally demonstrates that sex is a crucial variable that modulates fundamental neurobiological processes that range from rates of neurogenesis to synaptic physiology (TABLE 1). The second is that clinical research unequivocally demonstrates a gender bias in the relative frequency and severity of neuropsychiatric, neurological and neurodegenerative disorders (TABLE 2). This gender bias shifts markedly across the lifespan, with boys being much more likely to suffer adverse outcomes as a result of these disorders in early life than girls, whereas women disproportionately suffer in adulthood. This coherent pattern obliges us to explore how the biology of males and females contributes to disease risk based on gender, as well as the developmental events that lead to these biological differences.

The goal of this Review is to highlight recent findings that provide new investigative avenues for identifying the sources of gender-biased vulnerability and resilience. Sex differences in the brain are found at the macro and micro level: entire brain regions can vary in size according to sex, or a small subnucleus might exhibit a robust sex difference in the number and type of synapses found therein. The numbers of specific cell types can vary, as can the magnitude of projections between and within brain regions. Even the signal transduction pathways invoked following neuronal activation can be markedly different in males versus females (for a review, see REF. 11). Connecting these sex differences to specific behaviours and physiological responses is most easily achieved for those directly tied to reproduction, with the neural circuits controlling luteinizing hormone release from the anterior pituitary and sexual behaviour being the best characterized. Although these reproductive end points might seem far removed from neuropsychiatric, neurological and neurodegenerative disorders, we argue that discovery of the fundamental mechanisms establishing those sex

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differences are providing important insights into the sources of gender biases in vulnerability and resilience.

Mechanisms of sexual differentiation

Gonadal hormones and early life programming

The reproductive behaviour of adult males and females has long been known to be dependent on the gonadal steroid hormone milieu, which is unique to each sex. Males have higher circulating levels of testosterone derived from the testis, whereas females have a cyclic pattern of oestrogens and progestins arising from the ovary. In the ideal conditions of the laboratory (that is, in the presence of continuous food, water, light and shelter), males are always sexually active, but females will only readily engage in mating at or around the time of ovulation. What was not initially obvious is that the concordance of adult hormonal milieu and mating behaviour actually depends on early life programming, a concept that has been historically referred to as the organizational hypothesis[12].

In rodents, this programming is initiated prenatally in males when the fetal testes produce androgens that gain access to the brain, are converted to oestrogens in large amounts and initiate brain masculinization. Both male and female fetuses express high levels of the steroid-binding globulin α-fetoprotein, which sequesters the maternal oestrogens that are present in fetal circulation and thus protects the brain from the influence of maternal steroids. As the female fetal ovary remains quiescent, this sequestration of maternal oestrogens means that only males experience high intracerebral oestrogen levels (as a result of the aromatization of testicular androgens). If α-fetoprotein is ablated, maternal oestradiol penetrates the fetal brain during the critical period, and females are subsequently masculinized[13].

The processes of brain masculinization and feminization both occur during a critical period (FIG. 1). In rodents, the critical period begins prenatally at the time of the surge in male fetal androgen production. The end of the critical period is defined as the time at which the female phenotype can no longer be diverted to male by treatment with exogenous steroid hormones, which, in rats and mice, is one week to 10 days after birth. The existence of this critical window provides a tool for studying the process of masculinization. Injecting female rat or mouse pups with testosterone or its aromatized metabolite, oestradiol, for 1 or 2 days immediately after birth initiates many (although not necessarily all) of the same gene expression profiles and cell signalling cascades that occur normally in males *in utero* (for a review, see REF. 11). Although this is not a perfect model of masculinization, it provides an excellent means to interrogate both the short-term processes and long-term consequences of brain sexual differentiation by comparing males, females and masculinized females. There is an apparently separate sensitive period for rodent brain feminization that occurs weeks after birth; however, this remains less well characterized than the critical window for masculinization[14].

In addition to the adult consequences of the effects of gonadal hormones on brain development (known as ‘organizational’ effects), many sex differences in adult animals (and probably in humans too) are the result of the markedly different gonadal hormonal milieus

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that each experiences in adulthood. These are often referred to as ‘activational’ hormonal effects and may or may not depend on prior organizational hormonal effects.

Chromosome complement: XX versus XY matters too

Not all hormonal effects in adults are preordained developmentally, and not all sex differences are due to hormones. Besides its influence on gonadal differentiation and steroid hormone production, chromosome complement is an additional contributing variable to sex differences in brain and behaviour[15,16] (FIG. 2). The potential for direct sex chromosome effects was evident in a comparison of embryonic neural stem cells derived from male and female mouse embryos. When compared before the onset of gonadal steroid production, more than 100 transcripts were differentially expressed between female (XX) and male (XY) stem cells. Intriguingly, stem cells of both sexes were responsive to testosterone treatment but not in the same way: thousands more transcripts varied in response to testosterone in XX stem cells than in XY stem cells[17].

The *SRY* gene on the Y chromosome is best known for its role as the essential driver of testis development[18] but also promotes catecholamine production by dopaminergic neurons of the substantia nigra of the mouse[19] and by human NT2 cells differentiated to be dopaminergic. As only males carry the *SRY* gene, this is a true sex dimorphism and is speculated to contribute to the higher susceptibility of males to dopamine disorders such as Parkinson disease and schizophrenia[20].

A genetically modified mouse line in which *Sry* has been translocated from the Y chromosome to an autosome allows for generation of XX individuals with testis and XY individuals with ovaries, thereby separating sex genotype and gonadal phenotype[21]. This powerful tool is called the four-core genotype and has shown that multiple sex differences in brain and behaviour can be attributed to chromosome complement rather than gonadal phenotype. These include differences in the incidence of neural tube defects[22], pain perception[23] and many additional phenotypes (for a review, see REF. 24).

The four-core genotype was also exploited to dissect the sources of increased vulnerability of females to auto-immune disorders, focusing on multiple sclerosis (which is easily modelled in rodents in a paradigm referred to as experimental autoimmune encephalomyelitis). The severity of symptoms and degree of myelination were greater in XX mice compared with XY, regardless of gonadal phenotype[25], suggesting a genetic origin of disease vulnerability. However, it is important to note that the four-core genotype involves every cell in the body, preventing the dissociation of effects in the periphery from those in the CNS. This conundrum was addressed by the use of bone marrow transplantation to generate subjects with an XX nervous system and XY peripheral immune system and vice versa, thereby revealing that an XY nervous system was more sensitive to injury than an XX one[26]. The opposing effects of sex chromosome complement on the peripheral immune system and CNS, when combined with the sex-specific modulatory effects of gonadal steroids (for a review, see REF. 27), reveal the enormous complexity of gender bias in disease and the importance of determining the contribution of sex as a biological variable.

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Neuroimmunity and brain sex differences

Steroid hormones largely exert their effects by binding to intracellular receptors, which dimerize and dock at hormone response elements on gene promoters to regulate gene transcription[28–30]. Surprisingly, however, relatively few genes have been identified that are directly induced by steroid hormones and mediate masculinization. Instead, over the past decade, immune mediators have been strongly implicated as drivers of the masculinization process.

The brain is considered to be protected from systemic immune responses and pathogens by the blood–brain barrier. However, this does not mean that the brain is immunodeficient. Instead, the brain receives information from the periphery about immune status from various sources, including the vagal nerve, humoral signalling and cytokine transport into the brain, as well as meningeal and epithelial signalling across the blood–brain barrier[31–33]. The brain is also populated by resident immunocompetent cells, known as microglia, that engage in immune surveillance. These cells produce a host of neuroinflammatory mediators that are crucial regulators of brain development and homeostasis, including cytokines, prostanoids, purines and reactive oxygen species. Many of these neuroimmune mediators act in a manner that is indistinguishable from the functions of neuromodulators or neurohormones[34–36].

Microglia seed the brain early in fetal development, beginning at around 4.5 gestational weeks in humans and embryonic day 8–9.5 in mice and rats[37–40]. Microglia derive from yolk-sac progenitor cells, colonize the brain before the blood–brain barrier is formed and then locally proliferate across the remainder of fetal development and into the early postnatal period[41]. This process differs in males and females. On embryonic day 17, just before the fetal androgen surge in males, male and female rats have the same number of microglia in the brain. One week later, males have significantly more microglia than females in several brain regions, including the parietal cortex, hippocampus, amygdala and paraventricular nucleus of the hypothalamus[42]. Similarly, in the developing medial preoptic area (mPOA), male rats have significantly more microglia than females in the early postnatal period and twice as many microglia that are in an activated state (based on their ameboid-like morphology)[43]. Treating newborn females with a masculinizing dose of oestradiol increases both microglia number and activational state to male levels.

In recent years, the functional significance of these sex differences in neuroimmune signalling and microglial number has become apparent (FIG. 3). The mPOA is a crucial brain region for expression of male sexual behaviour. Olfactory cues from sexually receptive females are detected by the male olfactory system and projected to the amygdala, which in turn projects to the mPOA. From there, signals are integrated with the neural circuits of motivation and reward, as well as projected to the hypothalamus and midbrain for execution of mating behaviour[44]. Dendrites of mPOA neurons are studded with dendritic spines, and, in males, the density of these spines is twofold higher than in females, resulting in greater excitation in response to olfactory input[45]. A crucial molecular driver of the sex difference in the density of dendritic spine synapses in the rodent mPOA is the prostanoid prostaglandin E_2 (PGE_2). The surge in oestradiol levels in the male brain[46] during the critical period triggers an upregulation in the expression of cyclooxygenase 2 (COX2), the enzyme that leads to prostaglandin synthesis[47,48]. Elevated COX2 leads to increased PGE_2 in the mPOA,

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which is necessary for the induction of both dendritic spine patterning and male typical copulatory behaviour in adulthood[48]. The spinogenic effect of PGE_2 involves activation of the prostanoid receptors EP2 and EP4 (REF. 49), which are linked to protein kinase A activation[50], resulting in phosphorylation and mobilization of AMPA-type glutamate receptors to the membrane of both neurons and astrocytes[51], and glutamate-dependent formation of dendritic spines[50,51].

We now know that microglia are crucial for the PGE_2-mediated induction of male-typical synaptic patterning in the mPOA of rats and the resultant male- typical copulatory behaviour in adulthood. Treating females with oestradiol increases ameboid microglia numbers to male-typical levels, as well as the number of dendritic spine-like protrusions on mPOA neurons. Oestradiol or PGE_2 treatment will also masculinize POA neurons in a dish, as indicated by an increased density of spine-like processes. However, this response does not occur if the culture is deprived of microglia[43]. *In vivo*, oestradiol-induced release of PGE_2 in the mPOA is blunted by minocycline, an antibiotic known to inhibit microglia activity, confirming the requirement for microglia in the inflammatory signalling that is responsible for male-typical synaptic patterning. Furthermore, masculinization of copulatory behaviour in females by neonatal oestradiol treatment can be prevented by co-treatment with minocycline[43]. Even brief microglial ablation from the neonatal brain of males leads to complete loss of male typical copulatory behaviour in adulthood[52], along with other sex-specific effects[53].

In the nearby anteroventral periventricular nucleus (AVPV) of the POA, a parallel process of sexual differentiation is underway during the sensitive period. In this region, oestradiol increases cell death in males, significantly reducing the size of the AVPV in comparison to that of females[54]. Once again, sex differences in inflammatory signalling are responsible for masculinization. An unbiased screen of AVPV gene expression revealed that several members of the tumour necrosis family of cytokines — tumour necrosis factor (TNF), TNF receptor 2 (TNFR2; also known as TNFRSF1B) and nuclear factor-κB (NF-κB) — are constitutively active in neurons of the female AVPV, but this activity is blocked in males by testosterone-induced expression of E3 ubiquitin-protein ligase TRAIP, which allows apoptosis to proceed[55,56].

Resident immune cells may not be the only immune players in the sexual differentiation process. Indirect evidence supports a role for immune cells derived from the bone marrow and thymus. T cells from the thymus reside in the meninges, and recent studies have found that T cell-deficient mice have impairments in adult behaviours, including spatial learning and social behaviour[57,58]. A separate study examined the role of T cells on brain development[59]. In adulthood, the bed nucleus of the stria terminalis (BNST), a brain region that is contiguous with the POA and is both highly sexually dimorphic and sexually differentiated by steroid hormones during development[60], was masculinized, that is, increased in size, in female mice congenitally lacking T cells. The BNST is necessary for sociosexual behaviours, including territoriality and olfactory investigation, as well as mood-related behaviours. The T cell-deficient females showed a decrease in anxiety behaviour that may or may not be a result of the increased volume of the BNST. Social and sexual behaviours have not been investigated in these animals to date but certainly warrant future

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inquiry. Overall, this study suggests T cells are active participants in brain feminization, but the mechanism by which they influence physiology in the CNS remains unclear.

Mast cells are granulocytes of myeloid lineage derived from the bone marrow and are distributed throughout the body, including the brain[61,62]. Some mast cells synthesize gonadotropin-releasing hormone[63], a peptide crucial for the control of the anterior pituitary and gonadal function. Mast cell numbers increase in specific brain regions in both male and female rodents when exposed to sexually receptive stimuli of the other sex[63,64]. Furthermore, the transcriptome of peripheral mast cells is markedly different in males and females[65]. Mast cells have not yet been implicated directly in brain sexual differentiation, but, given their involvement in reproductive responses, the potential for such a role is high.

Neuroepigenetics and brain sex differences

The term ‘epigenetics’ describes modifications (epigenetic marks) to the DNA or associated histones that do not involve alterations of nucleotide sequences but have an impact on levels of gene expression in the long term. Canonical epigenetic modifications are methylation of the 5′ carbon of cytosines that are proximal to guanines (CpG) and post-translational modifications of histone tails, the most prevalent of which are acetylation and methylation. Epigenetic mechanisms also include additional sites of DNA methylation and other modifications of histones along with the actions of non-coding RNAs and the generation of a constellation of epigenetic marks that inactivate the X chromosome. The observation that epigenetic modifications in the nervous system happen rapidly and are reversible within a lifespan has led to a re-synthesis of the traditional view of cell fate determination and is aptly named neuroepigenetics[66].

A neuroepigenetic contribution to sex differences is at once obvious and mysterious. For example, every cell in the female nervous system has one epigenetically inactivated X chromosome, but how that contributes to functional sex differences remains obscure. As mentioned above, it is obvious that sex chromosome composition is a contributing variable to sex differences in the brain[16,67], but it is not clear whether X-chromosome genes that escape X inactivation in females drive these differences or whether the effect is due to the heterochromatic ‘sink’ that is created when large amounts of epigenetic regulatory proteins are required to maintain silencing of the second X chromosome: that is, the reduction in available epigenetic modifiers in cells with two X chromosomes may dilute the capacity for other forms of epigenetic regulation. It is also important not to forget the potential direct effects of Y chromosome genes (see above).

When contemplating an early life programming event that only manifests in adulthood, such as hormone- mediated sexual differentiation, it seems to be obvious that some form of cellular ‘memory’ must be established. Steroids bind to nuclear transcription factors (which interact directly with DNA), as well as to larger transcriptional complexes that include histone-modifying enzymes[68,69], thereby increasing the likelihood that epigenetics is involved. Indeed the early life programming scenario outlined above predicts that masculinization is driven by direct transcriptional regulation of a set of masculinizing genes that are turned on early (and possibly continuously), thereby differentiating the male brain. There are hints that this is true, to at least some degree, in the developing rat amygdala. Male

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adult rats have more vasopressin mRNA in their amygdala than females, a sex difference that is determined developmentally by higher androgen levels in males[70,71]. It has been shown that an experimentally induced transient reduction in the levels of the DNA methyl-binding protein methyl-CpG-binding protein 2 (MeCP2) during the sensitive period for sexual differentiation in males can permanently reduce amygdala vasopressin expression to the level of females, suggesting that an epigenetic modification (DNA methylation and the subsequent binding of MeCP2) establishes and maintains the sex difference in amygdala vasopressin levels[72] (FIG. 4). The amygdala is a site of multiple neuroanatomical sex differences and is central to the control of multiple behaviours that differ in males and females, including juvenile social play (which is displayed at higher levels by males across a wide range of species)[73].

Epigenetic regulation of vasopressin and MeCP2 expression is presumed to be downstream of steroid hormone action, but what about regulation of the steroid receptors themselves? Direct modulation of these transcription factor genes should provide a wide ranging and enduring regulation of hormonal responsiveness in adulthood. There have been studies of promoter methylation of both isoforms of the oestrogen receptor and the progesterone receptor in rats[74,75]. However, there is little agreement about the existence, direction and magnitude of any sex differences in promoter methylation[76,77]. In the few instances in which more than one time point was measured, sex-specific epigenetic patterns established early in life were replaced by different but still sex-specific patterns later in life[75]. This was most dramatically seen in a genome-wide analysis of genomic areas that are highly methylated. When comparing male mice, female mice and female mice treated with testosterone at birth to induce masculinization, the investigators found relatively few genes for which methylation levels were different in newborn males and females or that were modified by testosterone treatment of females. But, when animals treated the same way were assessed in adulthood, there were hundreds of genes that were differentially methylated by the testosterone treatment[78]. Among the challenges to epigenetic research is the inability to comprehensively assess DNA methylation and histone modifications at the same time on the same gene, and — even more importantly — to do so retrospectively. Thus, when an epigenetic modification is observed, it is impossible to know if it happened long ago and endured until now or was placed there just yesterday. Instead, we are limited to a single snapshot in time of a single end point. Nevertheless, these studies suggest that the epigenetic impacts of early life hormone exposure are enduring, but dynamic, generating an epigenetic ‘echo’ that grows and distorts across the lifespan but retains aspects of its original form.

As noted above, steroid-mediated transcription is an obvious means by which males could be differentiated from females. In addition, a recent study[79] showed that DNA methyltransferase (DNMT) activity in the POA of female rats was higher than that in males and that treating females with a masculinizing dose of steroid reduced enzymatic activity to that of males. This sex difference lasted for only the first few days after birth. DNA isolated from the female mPOA had more global methylation and a higher number of 100% methylated CpG sites than that isolated from both males and masculinized females, consistent with higher DNMT activity. But is this differential methylation important? RNA-sequencing analyses of the transcriptome from neonatal males and females with and without treatment that reduces DNA methylation was employed to explore this question. As

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expected, reducing DNA methylation increased the number of genes upregulated in females more than it did in males. More interestingly, when the transcriptome of males and females was directly compared, a surprisingly small number of genes (70) were differentially expressed. Furthermore, about half were expressed at higher levels in males, whereas half were higher in females. This is surprising because a scenario in which steroid receptor-induced transcription differentiates males from females would predict increased gene expression in males. Moreover, most sex differences in differential gene expression were reversed in animals in which DNA methylation was reduced, confirming that epigenetics was the basis for the sex-specific transcriptomes. Further proof was found in the reversal of sex differences in mPOA neuron synaptic density and adult mating behaviour in females subject to reduced DNA methylation as neonates. The mechanism by which steroids reduce DNMT activity is not known, but the capacity is lost by the end of the first week of life, coincident with the closing of the critical period. However, if females are treated with a demethylating agent at the normal end of the critical period, the critical period remains open and females are masculinized. Thus, DNA methylation seems to be essential to both the initiation and maintenance of sexual differentiation of the mPOA.

In contrast to the effects of DNA methylation (which prevent masculinization), histone deacetylation- mediated repression of gene expression is required for masculinization[80]. This is again surprising, as DNA methylation and histone deacetylation usually work in concert to suppress gene expression. Even more interestingly, there is evidence that these two opposing forces are at work in the same general brain region. As described above, the BNST is larger in males than in females because more cells die in females. This sex difference can be reversed by giving females a masculinizing dose of steroid during the critical period, indicating that steroids support survival[60]. It takes several days for the pro-survival effects of steroids to manifest, suggesting that an epigenetic change is induced by the steroids that then protects the cells from a subsequent apoptotic programme. Treatment of neonatal mouse pups with a histone deacetylase (HDAC) inhibitor increased histone acetylation and prevented the masculinization of the BNST, suggesting that pro-death genes that are normally suppressed in males were activated[81]. A functional impact of neonatal HDAC inhibition is evident in the impaired sexual ability of adult males[80], an effect that is attributed at least in part to dysregulation of the *Cyp19a1* gene, which encodes the enzyme aromatase, responsible for converting testosterone to oestradiol.

There are two approaches to discover sex differences in the epigenome. One is the use of broad based surveys that assay most or all potential targets. The second approach is to focus on specific candidate genes or epigenetic marks. An advantage of broad survey approaches is that intragenic regions, promoters, regulatory elements and reading frames of genes are all included in the analyses. Whole-genome bisulfite sequencing analysis of the neonatal POA demonstrated that most sex differences in DNA methylation levels are in the intergenic region, the functional significance of which remains a mystery[79]. A more focused view was gained by analysing an epigenetic mark known to cluster at transcription start sites, histone H3 lysine 4 trimethylation (H3K4me3), which is generally permissive of gene expression[82]. Chromatin immunoprecipiation followed by sequencing (CHIP–seq) established that most genes of males and females exhibited the same number of H3K4me3 marks but that ~200 genes differed in the number of H3K4me3 marks carried (most of them being higher in

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females)[83]. Thirteen genes were confirmed by quantitative polymerase chain reaction to be differentially expressed at the moment of assay, and two of these were located on the X chromosome, where they escape X inactivation. Together, these findings highlight the importance of considering the 'sexome' — the constellation of gene expression changes related to sex — rather than sex differences in individual genes[84].

Intersection of neuroimmunity and neuroepigenetics

Given the findings outlined above, it is natural to question whether there is crosstalk between neuroimmune and neuroepigenetic mechanisms of sex differentiation. The X chromosome has the highest concentration of immune-related genes of any chromosome[85]. Thus, X-chromosome inactivation in females is crucial to keeping overexpression of these immune-related genes repressed, and dysregulated X-chromosome silencing is proposed as a possible reason that females suffer more auto-inflammatory conditions[85].

Gene ontology analyses of the genes regulated by DNA methylation in the POA showed an almost fourfold enrichment of immune-related genes in female rat pups treated with a DNMT inhibitor (FIG. 3). These included cytokines, chemokines and their receptors, microglia- specific and/or macrophage-specific genes, genes related to phagocytosis, complement-related proteins and antigen presentation and receptor genes[79]. There were also many gene isoforms differentially expressed in males and females. Among these were many immune-related genes, including intriguingly a mast cell protease[79], implicating this non-neuronal cell type in the process of sexual differentiation.

A complementary possibility is that immunocompetent cells possess sexually differentiated epigenetic tags. Exposure to early life stress via neonatal handling of rat pups induces microglial-specific decreases in methylation of the anti-inflammatory immune gene interleukin-10, resulting in long-term increases in cytokine levels and subsequent resilience to morphine-induced conditioned place preference[86]. Although no sex differences were reported in this study, future exploration of immune cell-specific sex differences in the methylome or histone modifications would answer the question of whether the epigenetic regulation of these cells contributes to or maintains sexual differentiation of the brain.

Gender biased human neuropathology

A diagnosis of autism spectrum disorder (ASD) is 4–5 times more common in males than in females[87–89] and is greater in individuals exposed to higher levels of fetal testosterone as a result of maternal conditions such as polycystic ovarian syndrome[90,91]. Higher levels of several steroid hormones present in amniotic fluid during pregnancy, including cortisol, progesterone and testosterone, predict higher rates of autism diagnosis[92]. Low maternal levels of the oestrogen oestriol and both low and high levels of the placental hormone human chorionic gonadotropin during pregnancy have been associated with a higher risk of autism in male, but not female, offspring[93]. Thus, too much exposure to hormones involved in sexual differentiation may lead to ASD and possibly contribute to the male bias in autism risk. However, the role of these alterations in sex hormone signalling in brain processes associated with autism is not known.

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The centrality of gonadal steroids to the developmental process of brain masculinization was the basis for the ‘extreme male brain’ theory of autism[94]; however, in the absence of a thorough understanding of the cellular mechanisms by which steroids masculinize the brain, it is difficult to evaluate what is ‘extreme’. As reviewed above, neuroinflammatory mediators direct many sex differences in the brains of rodents. New analysis of existing gene expression profiles from post-mortem human cerebral cortex[95,96] reveals that many markers associated with activated astrocytes and microglia are higher in developing males than in females, suggesting that these cells have a role in normal human brain masculinization[97]. More interestingly, when the gene expression profile of post-mortem tissue of adult males with ASD is compared with that of neurotypical males, neuroimmune genes are expressed at even higher levels in those with ASD[97]. The paucity of females with a diagnosis of ASD precludes analyses of sex differences among affected individuals at this time but is an important future goal. Nonetheless, these data make a persuasive case that the normal biology of male-typical brain development seen in rodents also applies to humans and interacts with autism risk genes or environmental factors to make males more vulnerable.

Schizophrenia too is diagnosed more often in males than in females (in the order of 1.5 male diagnoses:1 female diagnosis)[98], with an earlier onset and greater severity in males that include more positive and negative symptoms[99]. By contrast, females tend towards more affective symptoms and a spike in psychosis around menopause, which has been hypothesized to depend on loss of the antipsychotic and antidepressant properties of oestrogens[100]. Neuroimaging indicates that regions of the cerebral cortex that exhibit sex differences in neurotypical individuals also show the largest abnormalities in individuals with schizophrenia[101], suggesting that sexual differentiation of the brain is disrupted in individuals that go on to develop schizophrenia.

Behavioural disorders that begin in childhood, including attention deficit hyperactivity disorder (ADHD), oppositional defiant disorder, conduct disorder[102], stuttering[103], dyslexia[104,105] and Tourette syndrome[106], are also more common in males. Of these disorders, ADHD has been the most extensively studied in terms of sex differences. As with schizophrenia, males and females show phenotypic differences in presentation, with girls showing less impulsivity, hyperactivity and externalizing relative to boys, as well as more mood disturbances and inattention[107,108]. Neuroimaging reveals sex differences, with ADHD diagnosed females having a smaller prefrontal cortex and ADHD males having a smaller premotor cortex[109], as well as significant sex differences in white matter tracts[110]. Certain ADHD risk alleles, such as catechol *O*-methyltransferase (COMT), an enzyme that helps to degrade neurotransmitters such as dopamine and noradrenaline, seem to play a stronger part in ADHD incidence in males than in females (others alleles, including monoamine oxidase type A (MAOA) and sodium-dependent serotonin transporter (SERT; also known as SLC6A4) seem to have a greater role in female ADHD)[111]. As of yet, the role of these genes in normal sex-specific brain development or risk of ADHD has not been well studied in animal models.

The only neurodevelopmental disorder diagnosed more often in females than in males is Rett syndrome, an X-linked genetic disorder that is embryonically lethal in males[112] (thus explaining its sex bias). This highlights a very important caveat to any discussion of sex

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differences in neurodevelopmental disorders: it is important to note that the measured incidence of a particular disorder does not account for cases in which the disrupted development underlying the disorder is embryonically fatal. Thus, it must be considered that any disorder that seems to be more biased towards males may be the result of more males exposed to a particular genetic or environmental risk surviving through fetal development than females.

Conclusions

The impact of sex and gender on health and disease is complex, multifactorial and pervasive. Discriminating biological origins of sex differences in disease susceptibility is one tool among many for improving the diagnosis and treatment of conditions that affect both men and women. Dissociating the many variables contributing to sex differences in brain and behaviour is particularly challenging, but animal models are uniquely powerful in this regard. Recent discoveries hint at novel biological sources of sex differences in the brain that involve use of inflammatory mediators as natural agents for differentiation between males and females. Divergent neuroepigenetic profiles in males and females seem to partner with differences in neuroinflammation, and, although the rules of engagement remain elusive, they surely warrant further study.

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Glossary

Functional MRI
The detection of changes in regional brain activity through their effects on blood flow and blood oxygenation that in turn affect the brightness of magnetic resonance images.

Diffusion tensor imaging
An MRI technique that provides a three-dimensional image of water diffusion in the brain. As water diffuses more readily along the axis of myelinated nerve fibre tracts, this method can be used to obtain a non-invasive estimate of anatomical connectivity between brain areas.

Connectome
A comprehensive map of neural connections within the nervous system of an organism.

Steroid hormone
A signalling molecule that is synthesized from cholesterol and is released into the circulation from endocrine glands including the gonads and adrenals. It binds to nuclear transcription factor receptors and can directly and indirectly modify gene expression.

Early life programming

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The phenomenon whereby events during development, such as stress, altered nutrition or endogenous hormone exposure, exert enduring influences on the nervous system in anticipation of the adult environment and experiences.

Critical period
A developmental window during which specific cellular events must occur or will be forever precluded. Sexual differentiation of the brain mediated by steroid hormones occurs during a perinatal critical period in the rodent and prenatally in humans.

Hormone response elements
Sequences of DNA in promoter regions that are recognized and bound to by steroid hormone receptors after binding of hormone, thereby promoting transcription.

Humoral signalling
Signalling molecules released into the blood stream that then act at a distance, such as steroid hormones.

Cytokine
Originally defined as an immune system protein that modifies biological responses; cytokines are now known to be released by most cells and are important in regulating intercellular communication, cell function and cell survival.

Immune surveillance
The hypothesized process by which the immune system constantly monitors the body for both invading pathogens and aberrant cell pathology, such as that seen in cancer.

Neuromodulators
Endogenous chemical substances that change the intrinsic properties of a neuron and the dynamics and strength of neurotransmission. Neuromodulators can modify neuronal responses to synaptic inputs on potentially long timescales.

Neurohormones
Steroid hormones that are further modified in the brain or synthesized *de novo* from cholesterol in the brain and are thus distinguished from those synthesized in the endocrine glands. Other neurohormones are peptides synthesized in the brain and released into the periphery such as oxytocin and vasopressin.

T cells
Lymphocytes produced by the thymus gland that actively participate in the immune response.

Mast cells
Multigranular cells that function as stores for several key inflammatory and/or pain mediators (including nerve growth factor, tumour necrosis factor, chemokines and histamine) and that originate in bone marrow but have a resident population in the brain.

Epigenetic marks

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Modifications to the genome that do not change the nucleotide sequence but have an impact on gene regulation. Methylation groups added to cytosine nucleotides or histones on the chromatin, along with other chemical groups, are examples of epigenetic marks.

Chemokines

A subfamily of inflammatory molecules that were initially described as regulators of the chemotaxis of inflammatory cells but that also have important roles in other processes, such as cell growth and differentiation.

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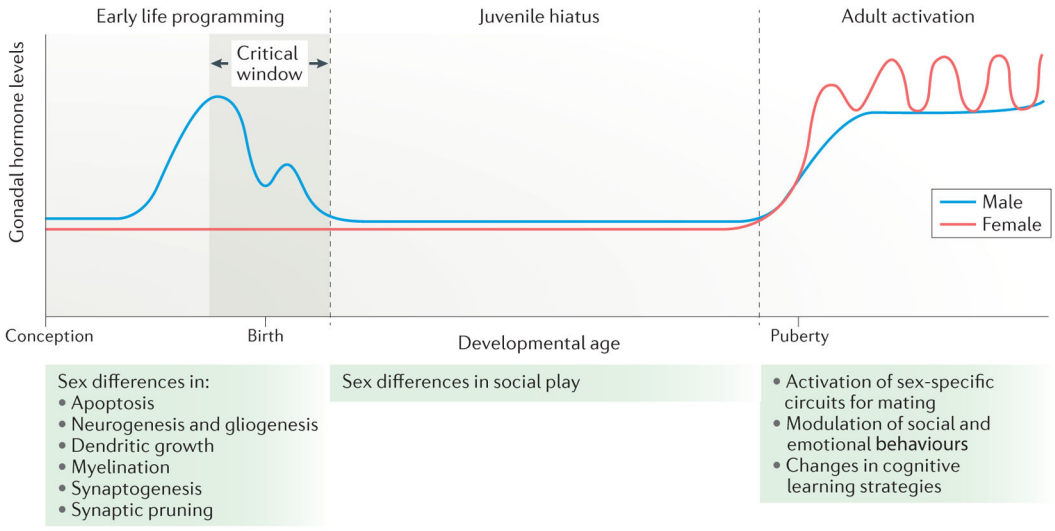

Figure 1. Early life programming of adult sex differences

The role of hormones in the establishment of sex differences in brain and behaviour can be broadly divided into three stages. In the rodent, early life programming occurs during a perinatal sensitive period (the critical window) that is marked by the onset of gonadal steroid production in males. This occurs prenatally and around birth, a period during which there is no corresponding steroidogenesis in females. During this sensitive period, numerous neuroanatomical end points are differentiated in males versus females in a region-specific manner. In some areas, there are sex differences in apoptosis, in others, there are differences in synaptogenesis or neurogenesis. The juvenile hiatus begins shortly after birth and extends until puberty. It is characterized by low to non-existent steroid levels; however, during this time, social play behaviour is more frequent among males than among females, a behavioural sex difference that is organized during early life programming. The adult period is characterized by sex-specific hormonal milieus with males having high and steady levels of testosterone (with some daily and annual variations), whereas females undergo cyclic changes in hormone levels that are associated with ovulation. The steroid milieu of each sex activates the neural circuits of mating that were organized during the perinatal period. In this way, the gonadal phenotype and neural phenotype controlling mating behaviour are synchronized. Non-reproductive behaviours such as stress and anxiety, spatial learning, locomotion and social affiliation are also modulated by steroids in adulthood, and this modulation may or may not depend on earlier programming effects during the sensitive period.

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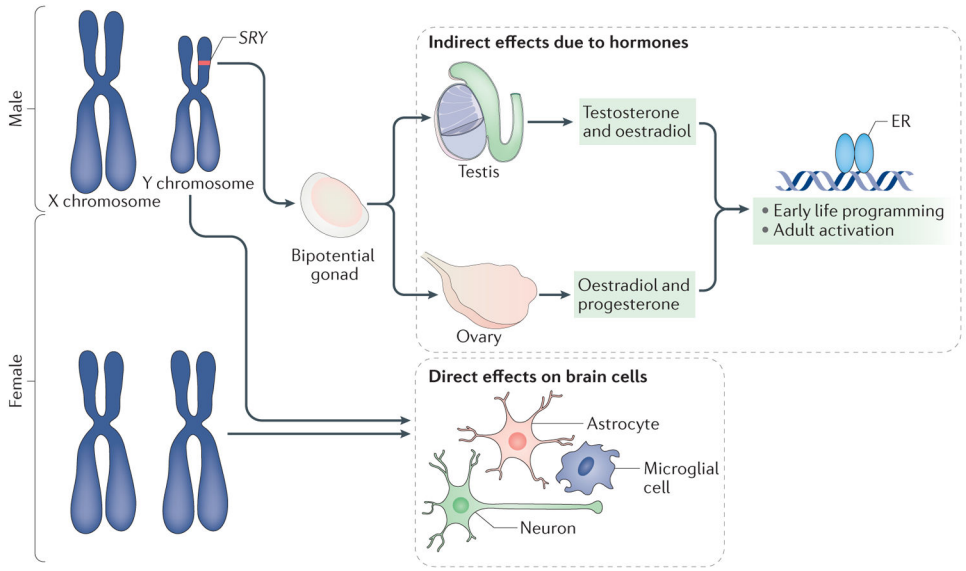

Figure 2. Sex chromosomes affect brain development directly and indirectly

The differentiation of the bipotential gonad into a testis versus an ovary depends on the *SRY* gene, which is located on the Y chromosome. If present, the gonad will become a testis, and, if not, it will become an ovary[18]. Testosterone and its aromatized end product oestradiol produced by the embryonic testis bind to their cognate receptors, which regulate gene transcription and exert an organizing influence on cells in the developing brain. Ovarian and testicular steroids also activate sex-specific physiology and behaviour in adulthood. Moreover, every cell in the brain is XY in male mammals and XX in female mammals. Genes on the X chromosome may be expressed at different levels in males and females (in cases in which they escape from X inactivation in females)[113], whereas genes on the Y chromosome (such as *SRY*) are expressed only in the brain of males[20]. In females, the presence of a second X chromosome could create a heterochromatic sink by monopolizing the cellular machinery used for epigenetic regulation and thus alter the expression of other genes in a manner that is distinct from males. Thus, the sex chromosomes can exert both specific and broad influences on the developing brain. ER, oestrogen receptor.

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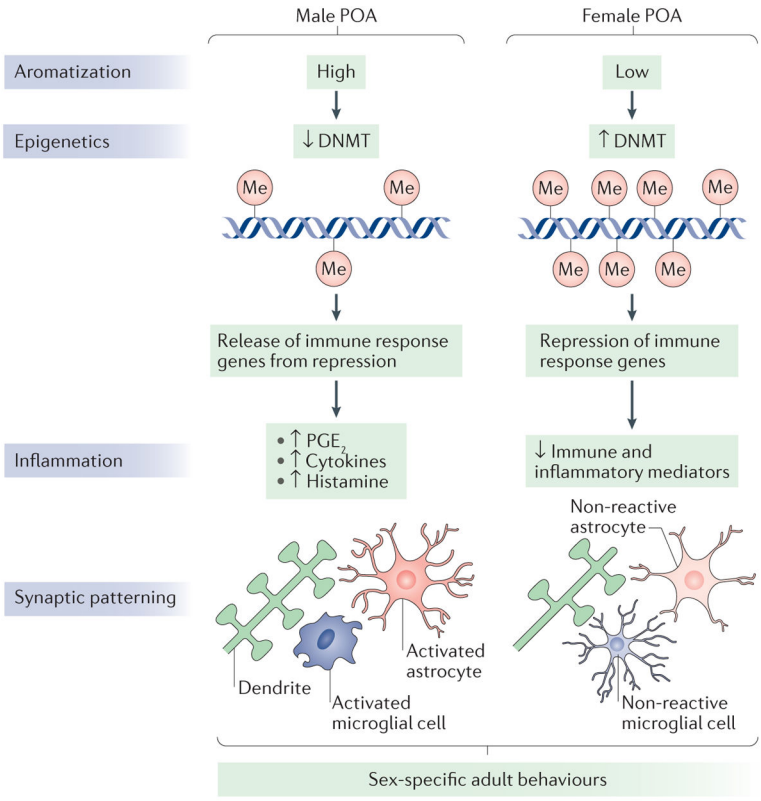

Figure 3. Neuroepigenetic and neuroinflammatory contributions to sex differences in the preoptic area

The rodent preoptic area (POA) is robustly sexually dimorphic and is therefore an ideal model system to understand the role of neuroepigenetics and neuroinflammation in sexual differentiation. The activity of DNA methyltransferase (DNMT) enzymes is higher in the developing POA of female rats than in that of males. This is because it is reduced in males, through unknown mechanisms, by the elevated oestradiol levels that are present owing to the aromatization of testosterone made in the testis. As a result, males have less DNA methylation (in the figure, indicated by 'Me') in POA cells[79]. Indirect evidence suggests that the lower DNA methylation in males releases immune response genes from epigenetic repression, resulting in elevation of inflammatory signalling molecules such as prostaglandin E_2 (PGE_2), histamine and cytokines[79]. PGE_2 in particular is known to activate a signal transduction cascade that results in the formation of dendritic spine synapses on the dendrites of POA neurons. This process requires the participation of both astrocytes, as a putative source of glutamate, and neighbouring microglia, which provide much of the prostaglandin. In females, immune response genes are epigenetically silenced by higher levels of DNA methylation. PGE_2 levels are therefore low, and both microglia and astrocytes are in a non-reactive state. As a result, females have half the density of dendritic spine synapses in the POA compared with males. These neuroanatomical sex differences undergird sex-specific reproductive behaviours, including male copulatory behaviour (both motivation to copulate and actual sexual performance), social scent marking, female oestrous cycling and maternal behaviour (for reviews, see REFS 44,114).

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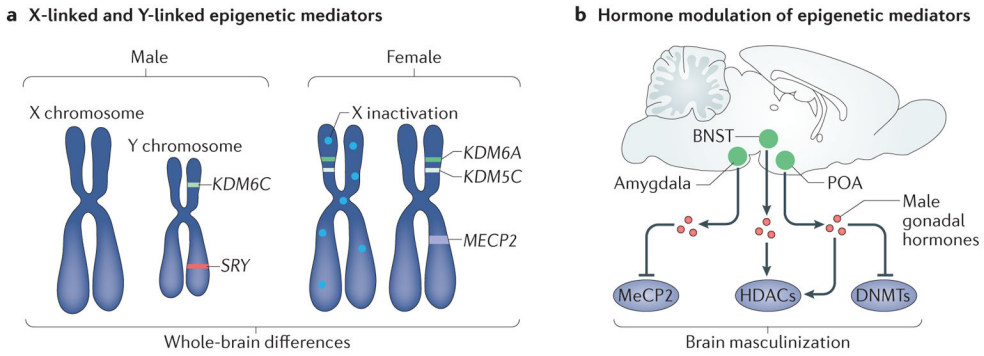

Figure 4. Epigenetic sources of brain sex differences

a | Several crucial epigenetic mediators are X chromosome and Y chromosome linked. These include the histone lysine demethylases KDM6A (also known as UTX) and KDM5C, which escape X inactivation and are thus expressed at higher levels in the female brain[115,116]. The Y-linked homologue of KDM6A, KDM6C (also known as UTY), is expressed at higher levels in the male brain[116]. Sex-specific expression of epigenetic modifiers such as these has the potential to establish widespread sex differences in the chromatin landscape and gene expression and thus to drive structural and functional sex differences in the brain. X-linked chromatin-binding proteins, such as methyl CpG binding protein 2 (MeCP2), have also been shown to be important for establishment of brain sex differences. **b** | Several epigenetic mediators are regulated by sex specific gonadal hormones. For example, male gonadal hormones reduce the expression of MeCP2 in the amygdala[72], reduce DNA methyltransferase (DNMT) activity and methylation genome-wide in the preoptic area (POA)[79], and alter methylation on specific promoters related to brain masculinization such as the oestrogen and progesterone receptors[74,75]. Hormonal modulation at the level of histone methylation and acetylation has also been demonstrated in the POA and bed nucleus of the stria terminalis (BNST), potentially mediating both active and repressive chromatin states[80,83]. HDACs, histone deacetylases.

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Table 1

Examples of neuroanatomical, neurochemical and physiological sex differences in the brains of rodents

End point measured	Measurements displaying male bias	Measurements displaying female bias	Measurements displaying context-, age- or region-specific biases
Volume	• mPOA[117] • VMN[118] • BNST[60] • Binocular area of the visual cortex[119] • Amygdala[120]	• Dorsal agranular insular subarea (orbital prefrontal cortex)[121] • AVPV of the hypothalamus[122] • LC[123]	Differences in medial amygdala volume are dependent on circulating testosterone in adult males[124]
Fibre density	• Lateral septum (vasopressin neurons)[125] • Medial preoptic nucleus (serotonergic fibres)[126] • BNST (vasopressin neurons)[127]	AVPV (kisspeptin fibres)[128]	None known
Synapses	• Excitatory synapses in the mPOA[47,48,129,130] • Hippocampal CA3 primary dendrites and spines[131] • Axosomatic synapses in the arcuate nucleus[132] • Axodendritic synapses in the VMN[133,134]	Axodendritic synapses in the arcuate nucleus[132]	• Hippocampus dendritic spine density (female > male, but this reverses following stress)[135] • CA3 dendritic atrophy (male > female, but this reverses following stress)[136]
Branching	• Length of glial processes in the arcuate nucleus[134] • Dendritic branching in prefrontal cortex cingulate areas[137] • Dendritic branching in the VMN[138]	Dendritic branching in the agranular insular cortex[137]	• Differences in dendritic branching in dentate granule cells following environmental enrichment[139] • Difference in dendritic branching and soma size of spinal nucleus bulbocavernosus is maintained by testosterone in males[140]
Neurochemical phenotype	• Vasopressin cells in the BNST[141] • Androgen receptor-expressing cells in the mPOA and BNST[142]	• Oestrogen receptor-α immunoreactive cells in the median preoptic nucleus and VMN[143] • Kisspeptin-expressing cells in the AVPV[144]	Differences in aromatase activity and progesterone receptor expression are region specific[146–148]

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End point measured	Measurements displaying male bias	Measurements displaying female bias	Measurements displaying context-, age- or region-specific biases
		• Dopamine neurons in the AVPV[54] • Whole-brain levels of serotonin and its metabolites[145]	
Cell number	• Activated microglia in neonatal mPOA[43] • Glia in visual cortex[119] • Glia in the globus pallidus and CA1 (REF. 149) • Astrocytes in the posterodorsal medial amygdala[150]	• Radial glia in the mPOA and hypothalamus[151] • Oligodendrocyte precursors[152]	• Biases in brain-wide microglia counts in neonates and adult depend on age[42] • Sex difference in astrocyte number in the medial amygdala only occurs in the left hemisphere[153]
Activational state	Duration of developmental excitatory actions of GABA[154]	• Hippocampal long-term potentiation, excitatory postsynaptic potential slope, burst depolarization[2] • Hypothalamic proopiomelanocortin-expressing neurons and cannabinoid receptor type 1 mediated miniature inhibitory postsynaptic current frequency[155]	Hormonal control of nigrostriatal dopamine system[156]
Cell genesis	Neurogenesis in CA1 and dentate gyrus[157,158]	• Cell genesis in the medial amygdala[159] • Neurogenesis in the LC[160]	Addition of new cells to sexually dimorphic AVPV and medial amygdala at puberty depends on gonadal steroids[161]

The categorization of sex differences in the rodent brain for over 40 years makes a comprehensive list unwieldy. Here, we note some examples of the range of types of sex differences that are found throughout the brain. AVPV, anteroventral periventricular nucleus; BNST, bed nucleus of the stria terminalis; LC, locus coeruleus; mPOA, medial preoptic area; VMN, ventromedial nucleus of the hypothalamus.

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Table 2

Sex differences in neuropsychiatric, neurological and neurodegenerative conditions

Condition	Sex differences in prevalence	Sex differences in onset	Sex differences in phenotype	Refs
Neuropsychiatric conditions with origins in development				
Autism spectrum disorder	Four to five times higher in males than in females	None	More social impairment in males; more affective symptoms in females	87,88, 162,163
Conduct disorder oppositional defiance disorder	Three times higher in males than in females	Earlier onset in males	More externalizing symptoms in males; more affective symptoms in females	164,165
ADHD	Two to three times higher in males than in females	None	More hyperactivity, externalizing and impulsivity in males; more internalizing, inattention and intellectual impairment in females	166,167
Schizophrenia	1.42 times higher in males than in females	Earlier onset in males than in females (early onset is more likely in males; late onset is more likely in females)	More language disruption, positive symptoms and severe course of illness in males; more affective symptoms in females	98,99, 168–170
Neurological developmental conditions				
Dyslexia and/or reading impairment	Two to three times higher in males than in females	None	None known	104,105
Stuttering	2.3 times higher in males than in females	Adolescent onset four times higher in males than females	None known	103
Tourette syndrome	Three to four times higher in males than in females	Earlier onset in males	Greater tic severity in adulthood in females	106
Adult onset neuropsychiatric conditions				
Major depression	None before puberty; two times higher in females than in males post-puberty	None	None known	171–175
Bipolar disorder	None for bipolar I; bipolar II higher in females than in males	Earlier onset in males	Sex by genotype interaction	176–179
Generalized anxiety	Two times higher in females than in males	None	Higher chronicity and comorbidity with major depression in females	174, 180–182
Panic disorder	2.5 times higher in females than in males	None	None known	180
OCD	1.5 times higher in females than in males	None	None known	183
PTSD	Two times higher in females than in males	None	More likely in females than in males following childhood trauma	184,185
Anorexia nervosa	Three times higher in females than in males	Unknown	None known	186–189
Bulimia	Three to four times higher in females than in males	Unknown	None known	186–189

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Condition	Sex differences in prevalence	Sex differences in onset	Sex differences in phenotype	Refs
Alcoholism or substance abuse	Higher in males than in females	Earlier in females than males	Females progress to addiction more quickly than males	190
Adult neurological conditions				
Migraine	None pre-puberty, but three times higher in females than males post-puberty	None	None known	191,192
Stroke	Higher in males than females before age 85, but higher in females than males after age 85	Males 4 years before females	None known	193,194
Neurodegenerative disease				
MS (with exception of primary progressive MS)	Two times higher in females than males	Earlier onset in females	More severe in males	195–197
Alzheimer disease	1.5–2 times higher in females than males, especially in those over 80 years	Earlier onset in females	More tangles and global pathology in females; pathology more highly correlated with clinical score in females	198–201
Parkinson disease	1.5 times higher in males than females	Males 2 years before females	None known	202
ALS	Three times higher in males than females	Earlier onset in males	None known	197,203
Myasthenia gravis	Four times higher in females than males	Earlier onset in females	None known	196

ADHD, attention deficit hyperactivity disorder; ALS, amyotrophic lateral sclerosis; Bipolar I, bipolar spectrum disorder characterized by at least one manic or mixed episode; Bipolar II, bipolar spectrum disorder characterized by at least one episode of major depression lasting two or more weeks and at least one hypomanic episode; MS, multiple sclerosis; OCD, obsessive compulsive disorder; PTSD, post-traumatic stress disorder.