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REVIEW ARTICLE

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Hormonal and genetic factors interact to control aromatase expression in the developing brain

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Brain expression of the enzyme P450-aromatase has been studied extensively. Subsequent to the aromatisation hypothesis having established brain aromatase as a key factor to convert gonadal testosterone to oestradiol, several studies have investigated the regulation of aromatase during the critical period of brain sexual differentiation. We review previous and recent findings concerning regulation of aromatase. The role of gonadal hormones, sex chromosome genes and neurosteroids is analysed in terms of their contribution to aromatase expression, as well as implications for the organisational effect of steroids during development.

KEYWORDS
amygdala, aromatase, gonadal hormones, sex chromosome complement, sex differences

1 | INTRODUCTION

During the last 60 years, testosterone and its aromatised metabolite 17β-oestradiol (E_2) have been recognised as the main factors that masculinise and defeminise the brain and behaviour in rats. A main contribution that set the basis for the subsequent studies in the field of sexual differentiation was the pioneering work of Phoenix et al[1] It was demonstrated that testosterone treatment during a sensitive period of development was able to masculinise and defeminise the sex behaviour of the female guinea pig. Two remarkable findings need to be highlighted from their work: (i) the effects of testosterone on the female brain and (ii) a developmental sensitive period for these effects. During the following years, numerous laboratories have identified the organisational action of testosterone during critical periods of development as the main cause of the generation of sex differences in the brain.[2] The central

dogma, commonly known as the classical hypothesis, establishes a sequential model to explain the sexual dimorphism in the structure and function of the mammalian brain. As a result of the sex-determining region of the Y chromosome (*Sry*), chromosomal sex (XY/XX) determines gonadal sex (testes/ovaries), which in turn determines brain sex. The presence of *Sry* gene in the Y chromosome initiates testes development[3] which, shortly after differentiation (around embryonic day [E]13), begin to synthesise testosterone in mice.[4,5] Then, a surge in testosterone production by foetal testes occurs at E17-18 in mice and at E18.5-19.5 in rats.[6,7] The prenatal surge in testosterone production signals the beginning of a critical period for hormonal effects, which extends until postnatal day (PN)10, when the female brain becomes insensitive to exogenous testosterone. This organisational action of testosterone in rodents occurs mainly after its intracerebral metabolism to E_2, which masculinises (enhances behaviours and functions typical of males) and defeminises (suppresses behaviours and functions typical of females) the brain.[2] During this critical period for hormonal effects, gonadal testosterone shapes the brain circuitry that controls male sex behaviour and reproductive physiological processes. On the other hand, XX embryos develop ovaries that begin secreting significant amounts of steroids after the first postnatal week.[8] Thus, feminisation of the brain is a process that occurs in the absence of high levels of gonadal steroids during the perinatal sensitive period. Remarkably, the organisational effect of testosterone was proposed to be present only for the male brain because the female ovaries are quiescent during most of the critical period. In adulthood, the organised neural substrate is activated by gonadal steroids in the circulation and required for sex-typical behaviours to be expressed. It was previously considered that, once the perinatal window closes, the brain is permanently wired as either male or female. Recently, the remarkable work of Nugent et al[9] redefined the concept of the critical period at the level of molecular genetics. Pharmacological inhibition of DNA methylation outside of the critical period at PN10 resulted in masculinised neuronal markers and male sexual behaviour in female rats.

For a long time, the organisational-activational dichotomy was applied to the understanding of many sex differences, with hormones being the only factors discussed as proximate signals causing sex differences. The classical hypothesis has been tested extensively and confirmed with regard to numerous reproductive phenotypes.[10] However, this dogma is now considered incomplete because it does not include other sex-specific factors that could act before or in parallel to gonadal hormones to induce sex differences in the brain.

2 | HORMONAL AND GENETIC FACTORS

Although the role of gonadal steroids in the generation of sexual dimorphism is undeniable, a growing body of evidence indicates that some sexually dimorphic traits cannot be entirely explained solely as a result of gonadal steroid action, but also may be ascribed to differences in sex chromosome complement. Males and females carry a different complement of sex chromosomes and are influenced throughout life by different genomes. XY cells, in addition to having the *Sry* gene on the Y chromosome, are equipped with a different set of genes only present on the male-specific region of the Y chromosome. Recent progress in molecular genetics has given us a better understanding of the genes encoded on the sex chromosomes and the different ways by which they regulate autosomal gene expression. It is known that some X and Y genes regulate autosomal gene expression by encoding for transcriptional regulator proteins, which in turn mediate chromatin changes.[11] An inherent inequality in X or Y genes causes a differential regulation in gene expression, which is then translated in a sex-specific manner. The Y-linked *Sry* gene has received special attention because of its role in setting up the early hormonal environment in utero for the males. However, other sex chromosome effects are also possible. The genetic and/or hormone pathways could thus interact or act independently (antagonistically/synergistically) in regulating sexual dimorphic development.[12]

3 | FOUR CORE GENOTYPES MOUSE MODEL

For many years, sex differences arising from the different complements of sex-linked genes in males and females have received little research attention because sex chromosomes are intrinsically associated with gonadal development. The development of the transgenic mouse models has opened new perspectives and allowed us the possibility of a direct in vivo evaluation of sex chromosome effects without altering hormonal levels. The mouse model most often used to study sex chromosome effects is the "four core genotypes" (FCG) model, in which gonadal sex and sex chromosome complement are uncoupled.[10,13,14] This animal model owes its name to the four different types of genotypes that it comprises. As a result of a spontaneous deletion of the *Sry* gene from the Y chromosome, the Y^- chromosome no longer determines the development of testes. One of the most interesting aspects of the FCG model was possible with the subsequent insertion of a *Sry* transgene on chromosome 3.[15-17] The Y^- chromosome and chromosome 3 segregate independently; thus, the four types of genotypes correspond to XX, XY^- (without *Sry* on the Y chromosome), XX*Sry* and XY^-*Sry* (both with *Sry* in autosome 3). All individuals possessing the *Sry* transgene develop testes and have a male external phenotype, regardless of their sex chromosome complement (XXSry and XY^-Sry, are referred as XX male and XY male throughout the text), whereas individuals lacking the transgene have ovaries and external female secondary sex characteristics (XX and XY^-, referred as XX female and XY female) (Table 1). The XX and XY males are masculinised equivalently by testosterone secretions during development, and thus differ phenotypically from both female groups.[13,14,17,18] For the analysis of the origin of a specific sex dimorphism, the FCG model allows discrimination between the effect of the sex chromosome complement and the effect of the gonadal phenotype and also allows the study of their interaction.[14]

TABLE 1 Genotype and gonadal phenotype in the four core genotypes model

Sex chromosome complement	Sry transgene in chromosome 3	Gonadal phenotype	Genotype	Referred to in text as
XY	With	Testes	$XY^{-}Sry$	XY male
XY	Without	Ovaries	XY^{-}	XY female
XX	With	Testes	XXSry	XX male
XX	Without	Ovaries	XX	XX female

4 | AROMATASE

Because the field of sexual differentiation has been largely influenced by the effects of gonadal hormones on the brain, the role of brain aromatase, the enzyme that converts testosterone in E_2, has received wide attention. One of the most intriguing aspects that makes aromatase a functional component for sexual differentiation of the brain[19] is its expression being restricted to discrete regions of the central nervous system.[20] Even more importantly, brain expression corresponds highly to well-known sexually differentiated brain regions such as the hypothalamus and preoptic regions. Based on immunohistochemical evaluation during brain development, the expression of aromatase has been classified into three different groups: (i) foetal, (ii) foetal/neonatal and (iii) young/adult.[21] Aromatase-positive neurones were observed along the medial side of the stria terminalis, forming a striking neuronal group considered to comprise a continuum and also known as the medial preopticoamygdaloid neuronal arc. These neuronal groups positive for aromatase were previously described by Shinoda et al[21] in the embryonic rat brain. In the E16 mouse brain, we found aromatase-positive cells identified as neurones after double immunostaining with a neuronal marker.[22] The highest expression was localised in the stria terminalis (ST) (Figure 1A) and the anterior amygdala area (AAA) (Figure 1B). These neuronal groups positive for aromatase were observed to continue caudally with specific staining in the medial and central amygdala.[21,22] In the adult brain, the pattern of aromatase distribution is restricted to an arc of interconnected nuclei that includes the nucleus of the posteromedial amygdala, the encapsulated region of the bed nucleus of the ST, the ventrolateral portion of the ventromedial hypothalamic nucleus, and the central component of the medial preoptic nucleus.[20] In addition to its hypothalamic and limbic expression, aromatase is also expressed in the cerebral cortex, hippocampus, cerebellum, midbrain and spinal cord,[20] suggesting that aromatase may play a role in the modulation of affective behaviours, mood and/or memory and learning. It has also been recognised to play an important function in neuroprotection.[23]

Coincident with a role in the organisational effect of gonadal testosterone in the male brain, aromatase mRNA expression in the hypothalamus increases gradually to reach peaks shortly before and after birth in rats[25,26] and mice.[27] In addition, several studies have shown that, during the perinatal critical period of hormonal sexual differentiation, there are sex differences in aromatase expression that are region- and time-specific.[28] For example, mRNA expression in the bed nucleus of the ST and the sexually dimorphic nucleus of the preoptic area is higher in male rats at PN2. Later in development, at PN6, the sex differences only remained in the bed nucleus of the ST.[28]

A growing body of evidence collected during the last 15 years indicates that, in addition to gonadal hormones, the sex chromosome complement is also involved in the generation of specific traits in the brain.[14,29] Most of these findings were obtained in the FCG model.[18,30,31] Concerning sex differences during development, the expression and activity of aromatase in the hypothalamus of E16 mice and rats was higher in males than in females.[25,32-34] These sex differences

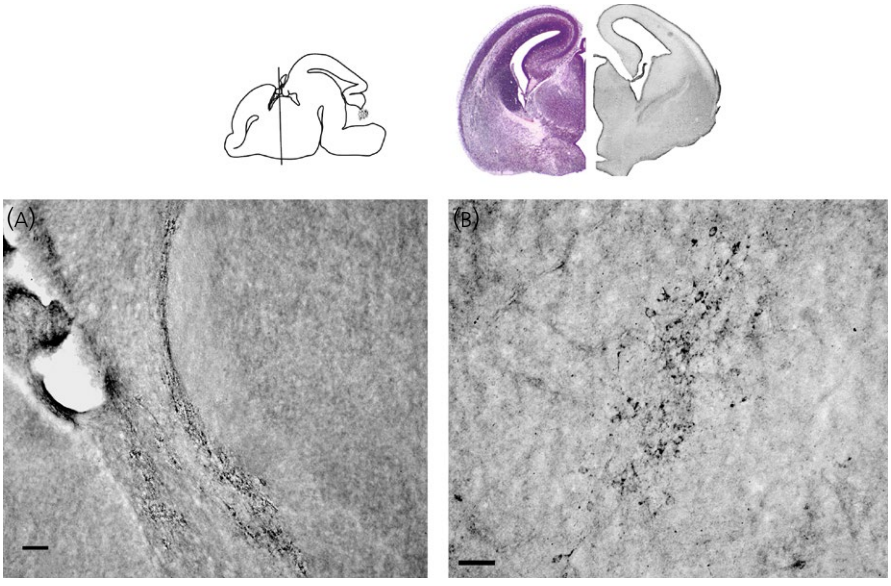

FIGURE 1 Photomicrographs depicting the pattern of aromatase immunoreactivity in the stria terminalis (A) and in the anterior amygdaloid area (B) of XY male brain at embryonic day 16. The schematic drawing of the embryonic brain shows the level at which coronal sections were selected with a colour image of a coronal section stained with haematoxylin based upon the atlas of Schambra.[24] Scale bar=50 μm. Adapted with permission[22]

were found before the in utero exposure to significant increased levels of gonadal testosterone. Recently, we have explored the sex difference in aromatase expression before the critical period of sexual differentiation. Working with FCG mice, our laboratory has found a sex chromosome effect in the sexually dimorphic expression of aromatase in the ST (Figure 2A) and in the AAA (Figure 2B) of the mouse brain at E16.[22] In these particular regions, the XY brain has higher aromatase levels (protein and mRNA) than the XX brain, irrespective of gonadal status (ovaries vs testes), indicating that the presence of a Y chromosome independent of any testicular factors or hormones enhances aromatase expression in these brain areas (Figure 2). The biological significance of this effect is undisclosed; nevertheless, these differences in vivo could result in differential local synthesis of E2, by aromatisation of testosterone, to organise sex specific synaptic connections in XX and XY brains. Lorenzo et al[35] reported that E_2 increases the length of dendrites in primary amygdala neurones in vitro. No sex differences were found in morphological parameters, growth characteristics or

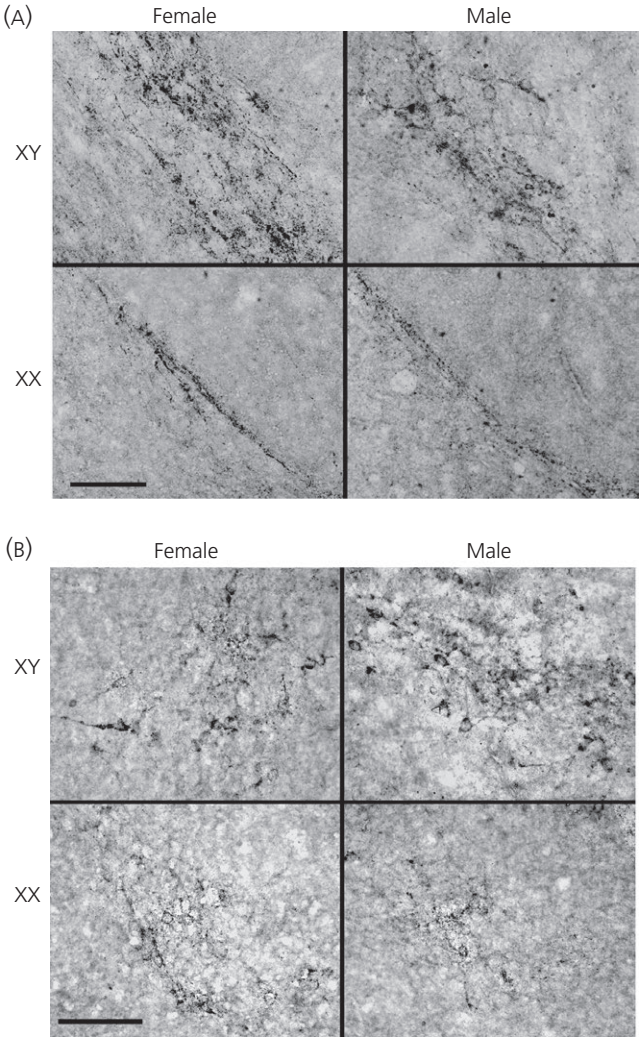

FIGURE 2 Prominent aromatase immunoreactivity in the stria terminalis (A) and anterior amygdaloid area (B) of individuals carrying the XY chromosome respect to those carrying XX sex chromosomes, irrespective of their being male or female

effect of E_2. These results led to the hypothesis proposing that local de novo production of E_2 in the female amygdala compensates for the in utero exposure to gonadal hormones in the male brain (for a more complete discussion, see later in this review).

The modulation of aromatase in the brain is intricate and not completely understood. Because the expression levels of aromatase mRNA are highly correlated with protein levels, it was proposed that the control of this enzyme is mediated by both transcriptional and post-transcriptional mechanisms.[36,37] The regulatory effect of steroid hormones in the expression of brain aromatase has been studied extensively; however, most of the studies had been performed in adult brains[37,38] or in neuronal cell lines.[39] Tabatadze et al[37] reported that, in adult rats, the hormonal up-regulation of aromatase mRNA expression in the amygdala occurs in both sexes. The aromatase gene contains androgen and oestrogen response elements,[40-42] suggesting that aromatase gene regulation could be exerted through both oestrogen and androgen receptor-dependent mechanisms. Given that male and female brains differ in basal levels of aromatase, it may be possible that hormonal regulation is actually starting at a different level to ensure the adequate level of oestrogens required for sexual differentiation. To test this hypothesis, we evaluated the mRNA expression of aromatase in neuronal cultures of amygdala derived from FCG embryos. Interestingly the treatment with either E_2 or dihydrotestosterone (DHT) was able to increase aromatase expression only in neuronal cultures of XX embryos (XX males and XX females) without any effect in XY cultures (Figure 3). The final outcome was the abolition of basal sex differences induced by the sex chromosome complement.[22] Given that neurones were taken from E15 embryos, before the critical period of hormonal differentiation, these findings imply that genetic and gonadal factors interact in the generation of sex differences in some structures of the developing rodent brain. The overall effect of this interaction in the regulation of aromatase expression might be seen as a link between the role of sex chromosome genes and sex hormones in the generation of a specific trait that plays a key role in sexual differentiation of the brain.

It is known that two different enzymatic pathways metabolise testosterone in the brain. Both mechanisms should be seen as parallel because they are both present in the brain throughout life. In addition to being converted to E_2, testosterone can also be metabolised to DHT by the 5α-reductases (5α-R) enzymes. DHT synthesised in the brain also exerts organisational actions on selected nuclei and it is involved in sexual differentiation of specific brain regions and behaviours.[43] In this context, brain masculinisation can be exerted either via ostrogen receptors (ERs) in some brain regions or via the conversion to DHT and the activation of androgen receptors (AR) in others. The role of AR on aromatase expression was assessed using the nonsteroidal pure antiandrogen flutamide that acts by inhibiting the uptake and/or binding of DHT to the target cell receptor, thus interfering with the action of androgen action.[44] Flutamide did not prevent the hormonal effect of DHT, raising the possibility of an indirect signalling through the conversion of DHT to an oestrogenic metabolite such as 3β-diol. As extensively reported in previous studies, DHT is also converted to products with oestrogen-like activity by other enzymes besides aromatase.[45,46]

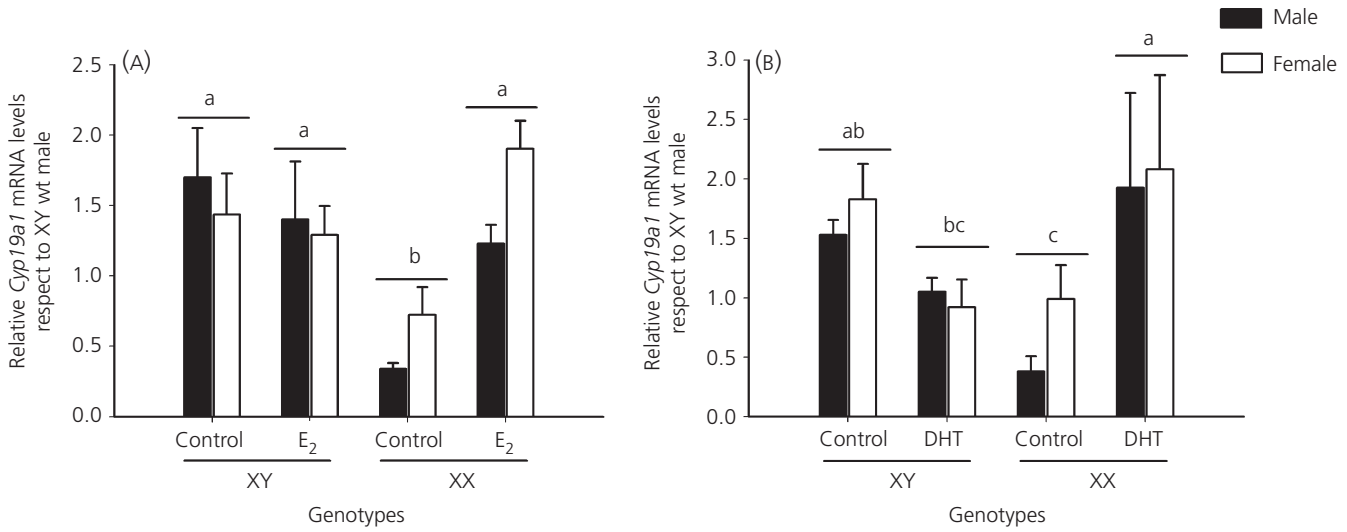

FIGURE 3 17β-oestradiol (E_2) (A) and dihydrotestosterone (DHT) (B) increase aromatase mRNA expression in XX (but not in XY) amygdala neuronal cultures. Different letters indicate significant differences with $P < 0.05$ (LSD Fisher). Data are mean ± SEM. wt, wild-type. Reprinted with permission[22]

One of these products is 3β-diol, which preferentially binds to ERβ and not AR.[47] Surprisingly, 3β-diol treatment increased aromatase expression in neuronal cultures derived from the amygdala of female FCG mice.[48] These findings could shed light on our previous results of hormonal regulation in neuronal cultures. Because aromatase mRNA expression was regulated by both E_2 and DHT and the magnitude of this effect was almost equivalent,[22] we can hypothesise that E_2 and 3β-diol bind to ERβ to regulate aromatase expression in amygdala neurones of the developing mouse brain. This hypothesis was evaluated recently using different agonists and antagonists of ERs. It was found that selective agonists for ERα or for the membrane ER GPR30 are not able to reproduce the effect of E_2 on aromatase mRNA. However, a selective ERβ agonist fully reproduced the effect of E_2 and DHT on aromatase expression.[48] In concordance with a possible ERβ involvement in aromatase regulation by E_2 or DHT, a selective antagonist for ERβ in combination with E_2 or DHT prevented the hormonal effects.[48]

5 | OTHER COMPONENTS OF THE NEUROSTEROIDOGENIC PATHWAY

The expression of the steroidogenic acute regulatory protein (StAR) and the cholesterol side chain cleavage enzyme (P450scc) in the brain are the initial and key steps for neurosteroid synthesis. StAR mRNA is only present in steroidogenic tissues[49] and it is required for the transfer of cholesterol from the external to the internal mitochondrial membrane, where P450scc is situated.[50] The first, rate-limiting and hormonally regulated step in the synthesis of all steroid hormones is the conversion of cholesterol to pregnenolone, catalysed by P450scc. P450scc is expressed in the nervous system of the developing rodent embryo and in cell lineages derived from the neural crest. In addition, P450scc immunoreactive protein is continuously expressed, beginning at E9.5, in the rat central and peripheral nervous systems.[51] Both StAR and P450scc are expressed during early brain development.[51-53] Moreover, StAR transcripts are co-expressed with 3β-hydroxysteroid dehydrogenase and P450scc in different neuronal populations in the brain.[50,54] Two different 5α-R (types I and II) catalyse the conversion of testosterone into the more potent androgen DHT.[55,56] The 5α-R type I mRNA is widely expressed in various tissues including the brain and, during ontogeny, is always detectable in the whole brain from E14 to adult, whereas 5α-R type II mRNA expression increases after E18 showing a peak at PN2 and then decreases gradually to low levels in adults.[57] To determine whether the enzymatic machinery necessary for neurosteroidogenesis is present in aromatase positive regions before the critical period of brain development, we evaluated the expression of StAR, P450scc and 5α-R genes in FCG. Neither StAR, nor P450scc and 5α-R types I and II are regulated by the sex chromosome complement or gonadal sex.[22] We found that, in aromatase-positive brain regions, such as ST and AAA, the enzymatic machinery necessary to initiate neurosteroid synthesis is present at E16. Although not yet explored, the female brain is also equipped with key factors to produce neurosteroids de novo. As noted above, the critical period for hormonal effects in sexual differentiation was largely influenced by gonadal production of testosterone by foetal testes. However, the role of local steroid production during the critical period is not clearly understood, mainly because of technical issues in steroid quantification in specific brain regions of the embryonic/neonatal brain.

6 | IMPLICATIONS IN THE FIELD OF SEXUAL DIFFERENTIATION

Differences between sexes in brain aromatase are a result of differences in the chromosome complement of neurones, as well as differences in exposure to sexual steroids during development. This complex interaction (genetic and hormonal factors) requires the

participation of X/Y linked genes, which could mediate differential autosomal gene regulation, such as specific oestrogen receptor expression at a particular time and brain region. Early in development, before the organisational effect of gonadal steroids, individuals carrying the XY chromosome complement show higher expression levels of aromatase (mRNA and protein) than the XX complement, irrespective of the gonadal status (testes vs ovary). We recently proposed a model in which differences in aromatase expression between XX and XY may produce differences in E_2 availability to amygdala neurones depending on genetic factors. In XY individuals, high levels of aromatase convert testosterone (as released by the perinatal testes or synthesised de novo from cholesterol) to E_2 favouring the action of E_2 (E_2 > testosterone) through ERα/β. On the other hand, in XX individuals, low levels of aromatase may promote testosterone signalling (testosterone > E_2) which can be converted by 5α-reductase to DHT. DHT is further metabolised to the ERβ agonist 3β-diol. Because, during prenatal development, ovaries are quiescent and start to produce oestrogens during postnatal life, the female brain may be exposed to a locally produced testosterone or E_2 from cholesterol. Direct evaluation of brain steroid content in male and female rat brain indicates that E_2 and testosterone levels are much higher in the embryonic vs postnatal brain without any correlation with circulating steroids.[58] Importantly, during a time period when only males are exposed to gonadal testosterone, some regions of the female brain show detectable levels of steroids. In concordance with this evidence, it was shown that neonatal hippocampal neurones are capable of synthesising E_2 because letrozole treatment resulted in a decrease of E_2 in the culture medium.[59] Moreover, Ruiz-Palmero et al[60] have shown that E_2 synthesised by female neurones generates sex differences in neuritogenesis in hippocampal cultures, which were reverted after the inhibition of E_2 synthesis with an aromatase inhibitor. Even though our findings of aromatase regulation were demonstrated in amygdala neurones, they could shed light on a possible mechanism present in XX amygdala that ensures adequate levels of E_2 during female development. The functional relevance of a possible role of prenatal oestrogen derived from locally-synthesised testosterone through brain aromatase needs to be demonstrated in vivo.

7 | CONCLUSIONS

Oestrogen availability to neurones depends on the local aromatisation of testosterone from peripheral gonads or from de novo synthesis (as neurosteroid) from cholesterol in the brain. Here, we have summarised evidence showing that the sex chromosome complement determines the expression and regulation of aromatase in the brain before critical periods of sex differentiation. This complex interaction contributes to regulate the expression of a key enzyme involved in brain masculinisation. The X-Y gene that is involved in the genetic mechanism leading to sex chromosome effects in aromatase needs to be specified in future studies. Current evidence indicates that genetically controlled mechanisms may precede gonadal influences during the genesis of differences between the sexes in rodent brain.

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