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Review

Neuroestradiol and neuronal development: Not an exclusive male tale anymore

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ABSTRACT

The brain synthesizes a variety of neurosteroids, including neuroestradiol. Inhibition of neuroestradiol synthesis results in alterations in basic neurodevelopmental processes, such as neurogenesis, neuroblast migration, neuritogenesis and synaptogenesis. Although the neurodevelopmental actions of neuroestradiol are exerted in both sexes, some of them are sex-specific, such as the well characterized effects of neuroestradiol derived from the metabolism of testicular testosterone during critical periods of male brain development. In addition, recent findings have shown sex-specific actions of neuroestradiol on neuroblast migration, neuritic growth and synaptogenesis in females. Among other factors, the epigenetic regulation exerted by X linked genes, such as *Kdm6a/Utx*, may determine sex-specific actions of neuroestradiol in the female brain. This review evidences the impact of neuroestradiol on brain formation in both sexes and highlights the interaction of neural steriodogenesis, hormones and sex chromosomes in sex-specific brain development.

1. Introduction

In addition to an endocrine ovarian hormone, estradiol acts as an autocrine or paracrine factor synthesized in different body tissues and organs. This includes the nervous system, which expresses all the molecular machinery for steroidogenesis (Shibuya et al., 2003; Giatti et al., 2020), produces estradiol from the metabolism of endogenous cholesterol (Hojo et al., 2004; Fester et al., 2009) and expresses all types of estradiol cellular receptors (ERs) (Barth et al., 2015). The levels of estradiol in the brain depend not only on the amount of the circulating hormone that cross the blood brain barrier but also on the amount of estradiol synthetized locally from local or peripherally-derived precursors.

For practical reasons it is useful to use the term neuroestradiol to refer to estradiol produced within the brain and differentiate local and peripheral sources of estradiol. This distinction is important, not only because local sources contribute to higher levels of estradiol detected in the brain compared to plasma (Hojo et al., 2009), but also because neuroestradiol synthesis is rapidly regulated by neuronal activity in restricted cellular domains, such as the synaptic cleft, and in proximity to ERs (Balthazart and Ball, 1998; Shibuya et al., 2003; Peterson et al., 2005; Remage-Healey et al., 2009; Saldanha, 2023). Therefore, acting as an autocrine or paracrine neuromodulator, neuroestradiol exerts a more precise spaciotemporal regulation of brain function than hormonal estradiol.

The final step in the steroidogenic pathway conducting to estradiol synthesis is catalyzed by the enzyme aromatase or estrogen synthase (EC1.14.14.14), which in mammals is encoded by the *cyp19A1* gene. This enzyme converts testosterone in estradiol and androstenedione in estrone. Aromatase activity in the central nervous system was first detected by Naftolin et al (1971), using samples of the diencephalon of human male fetuses. Subsequently, its expression and activity has been characterized in the nervous system of males and females in all vertebrate groups. Cells expressing aromatase in the brain include specific populations of neurons, glial cells, and brain endothelial cells and its cellular distribution present phylogenetic variations. For instance, glial cells show high expression of the enzyme in teleost fish, while its glial expression is more restricted in mammals. Concerning neuronal types, the enzyme is expressed in excitatory neurons and in specific populations of interneurons, in agreement with the finding that neuroestradiol regulates both inhibition and excitation (Azcoitia et al., 2022; Hernández-Vivanco et al., 2022).

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Although the anatomical distribution of aromatase expressing cells within the brain varies depending on the species, the enzyme is in general detected in regions related with neuroendocrine and metabolic control, reproductive behavior, sensory processing, sensorimotor coordination, cognition, memory, and social behavior, in agreement with the role of neuroestradiol on the regulation of these functions (Azcoitia et al., 2021; Brann et al, 2022).

Aromatase is also expressed in the developing brain of males and females. The enzyme is localized in neural stem cells, migrating neuroblasts and differentiating neurons (see below), suggesting that neuroestradiol participates in early neuronal developmental processes in both sexes. As it will be discussed in the following sections, studies using genetic deletion of aromatase or inhibitors of aromatase activity have confirmed the role of neuroestradiol on different neurodevelopmental events. These developmental actions of neuroestradiol are exerted at different fetal or postnatal ages, depending on the intrinsic developmental program of each brain region. For instance, in rodents neuroestradiol participates in the developmental organization of the cerebral cortex during fetal life (Sellers et al., 2020) and of the cerebellum during early postnatal life (Sakamoto et al., 2003).

Developmental actions of estradiol at early embryonic ages occurs when the peripheral organs that produce estradiol or estradiol precursors, such as testosterone, are still under maturation. This means that the steroid precursors for neuroestradiol synthesis are probably originated within the nervous tissue. Nevertheless, after the maturation of the gonads, neuroestradiol is also generated in the male brain from the metabolism of testosterone produced in the testes. Therefore, depending on the sex and the developmental timing of each brain region, the origin of neuroestradiol involved in the regulation of developmental events may be the endogenous steroidogenesis, testicular testosterone, or both.

Neuroestradiol regulates common developmental events in both male and female brain. However, some of the developmental actions of neuroestradiol result in sex-specific effects. The role of neuroestradiol derived from testicular testosterone as a mediator of androgenic effects in male brain sexual differentiation in rodents was originally proposed by MacLusky and Naftolin (1981). This mechanism has been confirmed in numerous species (Balthazart, 2019), although testosterone may also generate sex differences in brain organization through the activation of androgen receptors. Furthermore, in humans and related primates, male brain sex differentiation depends mainly on the activation of androgen receptors (Bakker, 2022) and neuroestradiol probably has a minor role, if any, on brain masculinization.

In addition to these well characterized sex-specific actions in males, recent studies suggest that neuroestradiol is also involved in the regulation of developmental mechanism in the female brain. Some of these observations have been performed in experimental systems, such as primary neuronal cultures, in which the implications of peripheral steroids can be excluded. The information concerning developmental actions of neuroestradiol in the female brain is at present limited and fragmentary, but merits to be taken in consideration because aromatase expression is detected as early as E9 in the embryonic ventricular zone of the mouse neocortex in both sexes (Martínez-Cerdeño et al., 2006). Moreover, neuroestradiol participates in the regulation of early developmental events also in females at embryonic ages that precede the peak of testicular production of testosterone in males (see below).

Our focus here is to address the role of neuroestradiol on the regulation of neurodevelopmental processes, focusing our emphasis on the cellular mechanisms, rather than on the behavioral consequences. It is also important to clarify that our aim is not to specifically address the mechanisms involved in the generation of sexually dimorphic brain structures, even if some of the developmental actions of neuroestradiol are involved in their genesis. In addition, we have restricted our analysis to only four developmental processes: neurogenesis, neuroblast migration, neuritogenesis and synaptogenesis. Other neurodevelopmental processes regulated by neuroestradiol, including the survival and molecular differentiation of newly generated neurons and the development of glial cells will not be discussed here.

We will start by considering in section 2 evidence for the regulation by neuroestradiol of these neurodevelopmental mechanisms, regardless of sex. Then, in section 3 we will discuss sex-specific developmental actions of neuroestradiol, including new information available for the female brain. Since neuroestradiol is synthetized in the developing brain in both sexes, and seem to regulate in males and females the same neurodevelopmental processes, an important issue is to identify the factors that determine sex-specificity in some of its neurodevelopmental actions. This will be addressed in Section 4, discussing the implication of sex chromosomes, X-liked genes, epigenetic regulation, and temporal factors in the sex-specific actions of neuroestradiol. Finally, section 5 is concerned with the challenge of identifying the potential functional consequences of the developmental actions of neuroestradiol in the female brain.

2. Neurodevelopmental mechanisms regulated by neuroestradiol

2.1. Neurogenesis

Developmental neurogenesis in vertebrates consists in the generation of mature neurons and glial cells by progenitor cells located in the germinal layers during the embryonic and early postnatal periods. The newly generated cells migrate to reach their definitive destination in the developing brain. During this process, they acquire their final molecular, morphological, and functional phenotype and contribute to build mature neural circuits (Fig. 1). Studies in different vertebrate species have shown that the enzyme aromatase is expressed in progenitor cells in the ventricular and the subventricular zone (SVZ), including radial glial cells, which also provide scaffoldings for migrating neurons. Aromatase is also expressed by newborn and migrating neuroblast. Therefore, neuroestradiol is produced in cells involved in the different steps in the neurogenic process.

The rate of neurogenesis is the result of a balance between cell proliferation and death of the newborn cells. Cell death occurs at different steps of the developmental process and may take place in the same proliferative zone, when the newly generated cells are migrating towards their definitive destination or when neurons are differentiating in their target brain structure. Neuroestradiol is neuroprotective (Azcoitia et al., 2001) and regulates the balance between proapoptotic and antiapoptotic proteins preventing developmental neuronal death (Hill et al., 2004; Fester et al., 2006; Hisasue et al., 2010). Although the role of neuroestradiol in the regulation of apoptosis and other forms of programed neuronal death, such as necrosis or autophagic cell death, will not be discussed here, it is important to keep in mind that the regulation of neuronal death is one of the mechanisms that participate in the effects of neuroestradiol derived from testicular testosterone in the control of the final neuronal number in different brain regions, such as the sexually dimorphic nucleus of the preoptic area (Gorski, 1985; Tsukahara and Morishita, 2020).

Neurogenesis declines as neurodevelopment progresses, but persists beyond puberty with variable intensity depending on the species and the brain regions. Adult neurogenesis is possible because pluripotent cells that remain along the lateral ventricles and the hippocampal subgranular zone (SGZ) have the capacity to differentiate in mature neurons and glial cells (Garcia-Verdugo et al., 2002). In some vertebrate groups, such as teleost fish, the brain continues to growth in adult life in parallel with unceasing body growth. Therefore, these animals maintain active neurogenesis during all their life.

Radial glial cells in the ventricular layer of the brain of teleost fish persist throughout adult life as neuronal stem cells and maintain high active neurogenesis during all lifespan, in parallel with continuous brain growth. The discovery of high aromatase expression in these radial glia progenitor cells in the ventricular layer of the brain of male and female teleost fish suggested that neuroestradiol plays a role in the control of

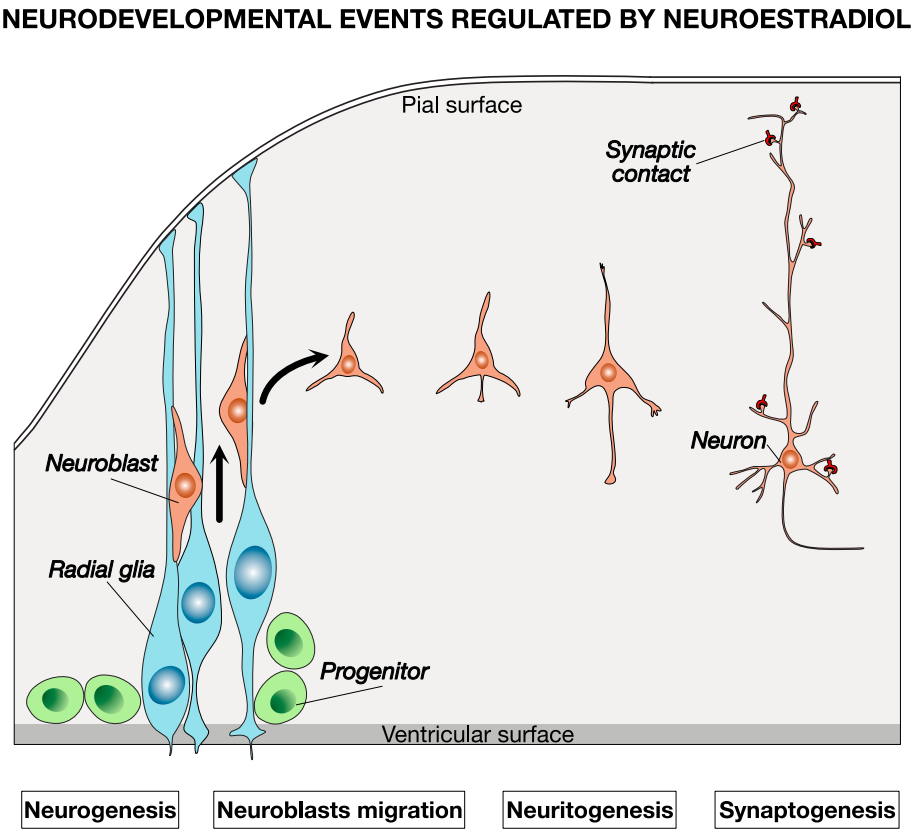

Fig. 1. Neurodevelopmental events that are regulated by neuroestradiol and are discussed in this paper. Aromatase is expressed by progenitor cells, radial glia, migrating neuroblasts and developing neurons. Neuroestradiol regulates neurogenesis, neuroblast migration, neuritogenesis and synaptogenesis in both sexes.

neurogenesis, not only during development, but also in adult life (Forlano et al., 2001; Pellegrini et al., 2007; Mouriec et al., 2008, 2009). Aromatase immunoreactive radial glial cells in the ventricular layer of the brain of young and adult teleost fish actively divide to generate newborn cells, which further divide and migrate away from the ventricles following the path of radial glial processes. Some of these migrating cells are newly generated neurons, identified by the expression of neuronal markers, while others are differentiated in astrocytes, identified with the glial marker S-100 (Pellegrini et al., 2007; Strobl-Mazzulla et al., 2010). Therefore, neuroestradiol generated by aromatase expressing radial glial cells in the neurogenic zones of the teleost fish brain may potentially impact in several steps of the neurogenic process (Diotel et al., 2013; Coumailleau et al., 2015), including the down-regulation of fast cycling and postmitotic cells, as it has been observed in adult female zebrafish (Makantasi and Dermon, 2014).

In birds, as in teleost fish, neurogenesis persists in adulthood. Progenitor cells located in the ventricular zone surrounding the lateral ventricles give rise to neurons that migrate to several regions of the adult forebrain (Paredes et al., 2016). This has been well studied in songbirds, where new cells are added to vocal control centers not only during development, but also during breeding seasons with song learning in both sexes. Proliferation, recruitment, and survival of newborn cells seem to contribute to the increase in the volume of some vocal centers during song development (Kirn and DeVoogd, 1989; Alvarez-Buylla et al.,1992; Zeng et al., 2007; Katz et al., 2008; Diez et al., 2021). In addition, neurogenesis persists in adult life (Diez et al., 2021), participating in song production and perception in males (Chiver et al., 2023). The ventricular region of the songbird brain has all the necessary enzymes for steroidogenesis, including high aromatase expression levels (London and Schlinger, 2007). As in teleost fish, radial glial express aromatase in birds and neuroestradiol is known to promote neurogenesis in the ventricular zone of the injured adult zebra finch brain (Lee et al., 2007; Peterson et al., 2007; Walters et al., 2011). In addition, testosterone and estradiol enhance the incorporation of new neurons to vocal centers (Nottebohm, 1980; Nordeen and Nordeen, 1989; Hidalgo et al., 1995; Yamamura et al., 2011), and neuroestradiol, as a metabolite of testicular testosterone, may participate in this process (Chen et al., 2013).

As in teleost fish, radial glial cells participate in neurogenesis in the mammalian brain. Aromatase expression has been localized in radial glial cells and intermediate progenitor cells in the embryonic ventricular zone and the SVZ of the male and female mouse neocortex from E9 (Martínez-Cerdeño et al., 2006). Furthermore, inhibition of aromatase activity in organotypic cultures at E15 impairs the proliferation of cortical progenitor cells (Martínez-Cerdeño et al., 2006) and reduces proliferation in dissociated cultures of hippocampal granule cells (Fester et al., 2006). Aromatase is also expressed by stem cells in neurospheres derived from the developing rodent hippocampus (Fig. 2) and cell proliferation is reduced in dissociated cultures of hippocampal granule cells after the silencing steroidogenic acute regulatory protein (Fester et al., 2006), which regulates the incorporation of cholesterol to the inner mitochondrial membrane to initiate steroidogenesis. This suggests an effect of endogenous steroidogenesis on neurogenesis.

Although adult neurogenesis in mammals does not reach the high levels of activity observed in teleost fish, neural stem cells remain in different structures of the adult brain, such as the SVZ in the lateral ventricles and the SGZ of the dentate gyrus in the hippocampus. These neural stem cells generate new neurons in the adult mammalian brain. Interestingly, undifferentiated neural stem cells isolated from three-month-old male and female rat brains express aromatase, together with α and β estrogen receptors (ERs) (Waldron et al., 2010a,b). However, the role of neuroestradiol on adult physiological neurogenesis is unclear. From one side, no differences in the number of proliferating cells in the SVZ have been observed between aromatase KO and wild

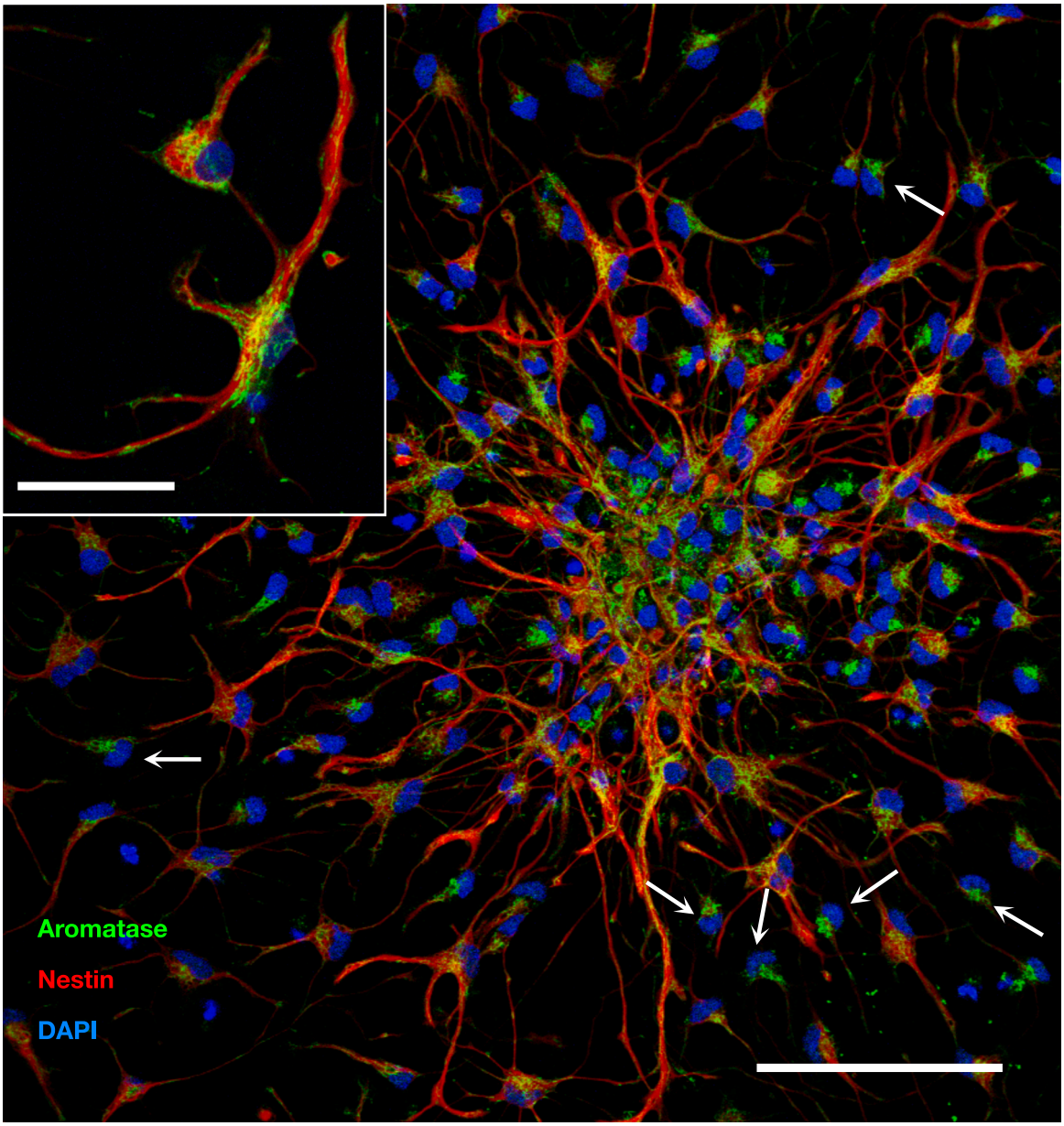

Fig. 2. Aromatase expression in neural stem cells. Aromatase immunoreactivity in a neurosphere derived from P0 mouse hippocampus, plated for 24 h, immunostained for aromatase (green) and the neural stem cell marker nestin (red) and counterstained with the nuclear marker DAPI (blue). Most cells show red and green signals, indicating that neural stem cells express aromatase. The inset show an example of two of these cells that are immunoreactive for aromatase and nestin. Aromatase immunoreactivity shows a reticular aspect corresponding to the localization of the enzyme in the endoplasmic reticulum, while the immunoreactivity for nestin, an intermediate filament, labels the cytoskeleton. Arrows point to aromatase immunoreactive cells that have migrated from the neurosphere and are either nesting negative or show a low level of nestin immunoreactivity, suggesting that they are committed to neuronal differentiation. Scale bar = 50 μm; inset, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

type female mice (Veyrac and Bakker, 2011), suggesting that neuroestradiol does not affect adult physiological neurogenesis in the SVZ, at least in females. On the other hand, the use of aromatase inhibitors or aromatase deletion to assess the effect on adult physiological neurogenesis in the hippocampus has given unconclusive results. Cell proliferation in the SGZ is decreased in young adult aromatase KO female mice compared to wild type animals (Brus et al., 2016), but chronic systemic administration of the aromatase inhibitor letrozole to middle aged female rats increases the production of new neurons in the SGZ (Chaiton et al., 2019). Since in both cases aromatase was targeted in all tissues it is not possible to determine whether the effects of aromatase deletion on the SGZ were due to the deficit in hormonal estradiol or to the deficit in neuroestradiol.

A clearer picture on the role of neuroestradiol has been obtained when neurogenesis is stimulated by brain injury, a phenomenon known as reactive neurogenesis and that probably represents an endogenous repair mechanism. Thus, in the hippocampus of adult ovariectomized female zebra finches receiving a penetrating brain injury, intracerebral aromatase inhibition reduces cell proliferation and estradiol treatment recovers proliferation, (Lee et al., 2007; Peterson et al., 2007; Walters et al., 2011), suggesting that neuroestradiol promotes reactive neurogenesis. Similar results have been obtained in mammals, where exogenous estradiol enhances neurogenesis after brain injury in both the SGZ and the SVZ (Suzuki et al., 2007; Li et al., 2011). Furthermore, injury-induced neurogenesis in the SGZ and the SVZ are significantly reduced in both the ipsilateral and injured hemispheres of aromatase KO (ArKO)

female mice submitted to focal cerebral ischemia caused by middle cerebral artery occlusion (MCAO) (Li et al., 2011). Interestingly, in this model exogenous estradiol enhances neurogenesis in both the SGZ and the SVZ of male and female mice submitted to MCAO. However, the effects of estradiol were not detected in aromatase ArKO females (ArKO males were not studied), suggesting that neuroestradiol is necessary for the effects of exogenous estradiol (Li et al., 2011). This suggests that neuroestradiol is also involved in the promotion of neurogenesis after brain injury in rodents. This is also supported by a study in three-month-old ovariectomized female rats submitted to global cerebral ischemia, where aromatase inhibition with letrozole prevented the promotion of hippocampal neurogenesis by the G protein-coupled ER (GPER1) selective agonist G1 (Wang et al., 2021).

2.2. Neuroblast migration

After neurogenesis the newly generated neurons migrate to their final anatomical destination where they should establish adequate synaptic contacts to be integrated in functional neuronal circuits (Nakajima et al., 2021). Neuroblast migration is regulated by a variety of adhesion molecules and soluble factors (Ferent et al., 2020; Bressan and Saghatelyan, 2021; Geribaldi-Doldán et al., 2023), some of which are regulated by estradiol (Denley et al., 2018). In this process, radial glial cells provide scaffolds for neuronal migration, guide the placement and allocation of migrating neuroblasts and participate in the organization of the developing neuronal circuits (Alvarez-Buylla and Nottebohm, 1988; Casingal et al., 2022) (Fig. 1). As mentioned before, radial glia express high aromatase levels in teleost fish, birds, and mammals, suggesting that neuroestradiol produced by these cells is involved in the regulation of neuroblast migration both under basal conditions and after brain injury, contributing, for instance, to the high regenerative capacity of the adult teleost fish brain (Xing et al., 2014).

In songbirds, neuroestradiol produced by radial glia or other aromatase expressing cells may enhance neuronal migration from the ventricular zone and the incorporation of new neurons to the vocal centers. Indeed, steroid regulation of vocal centers depends more on the incorporation of new neurons than on changes in neuronal proliferation (Mirzatoni et al., 2010; Yamamura et al., 2011; Hall and Macdougall-Shackleton, 2012; Chen et al., 2013; Barker et al., 2014; Louissaint et al., 2002). In this regard it is important to mention that in explant cultures from the adult songbird brain, estradiol is required in newly generated neurons for the coupling of the neuron-glia cell adhesion molecule (NgCAM) to calcium-dependent signaling pathways required for the initiation of neuronal migration from the ventricular zone (Williams et al., 1999). Furthermore, the findings of Louissaint et al. (2002) suggest that in addition to promote the initiation of neuronal migration from the ventricular zone, neuroestradiol activates mechanisms to direct the movement of newly generated neurons towards their target regions. These authors have shown that testosterone, through its metabolite neuroestradiol, enhance the production of vascular endothelial growth factor (VEGF) in the HVC of adult canaries. VEGF induces first angiogenesis in the HVC, and then acts on the newly formed endothelial cells to promote the release of brain-derived neurotrophic factor (BDNF), which in turn promotes neuronal recruitment to the HVC. Therefore, according to this mechanism, neuroestradiol derived from testicular testosterone directs neuronal migration to HVC in the adult canary brain.

Indirect evidence suggesting a role of neuroestradiol in the regulation of the migration of neuroblast in mammals has been obtained in the rostral migratory stream (RMS) of the adult mouse brain. The dorsal migratory stream is formed by neuroblast produced in the SVZ of the lateral ventricles that migrate to the olfactory bulb. Migratory neuroblast in the RMS are targets of estradiol, because they express high levels of G protein-coupled ER (GPER1). Acting on this ER, estradiol promotes the migration of neuroblast from the RMS to the olfactory bulb (Haumann et al., 2020). Interestingly, Haumann et al (2020) have detected that aromatase is expressed in astrocytes surrounding migratory neuroblasts in the RMS. Therefore, neuroestradiol production by aromatase positive astrocytes in the RMS may potentially target GPER1 in RMS neuroblasts, promoting their migration to the olfactory bulb (Haumann et al., 2020). In addition, the survival of newly generated neurons in the olfactory bulb is decreased in aromatase KO young adult female mice compared to wild type animals (Brus et al., 2016), suggesting that aromatase activity may not only regulate migration of neuroblast but also their survival when they have reached their final destination.

In the developing rodent brain, aromatase is expressed in migrating neuroblasts (Sellers et al., 2020). For instance, aromatase immunoreactivity is detected in migrating granule cell neuroblasts during the development of the mouse cerebellar cortex (Fig. 3). Cerebellar neurons, such as Purkinje and granule cells, are generated in a germinal zone situated in the fourth ventricle. Then, granule neuron precursors tangentially migrate beneath the pia as transient amplifying cells and constitute the so-called external granular layer. Purkinje and granule neuroblasts migrate then in opposite directions: Purkinje neuroblast migrate outward, toward the pia, while granule cell neuroblast migrate deeper in the cerebellar cortex, crossing the layer formed by Purkinje cells and generating the inner granular layer. As shown in Fig. 3, granule cells are transiently immunoreactive for aromatase during their migration from the outer to the inner granule layer, suggesting a possible role of neuroestradiol in this process.

Direct evidence for the implication of neuroestradiol in neuroblast migration has been obtained in the developing cerebral cortex. Both neuroblasts and radial glial in the developing male and female cerebral cortex express aromatase and the *in vivo* silencing of the enzyme in migrating neuroblasts results in sex-specific alteration in the migratory pattern (Sellers et al., 2020) (see section 3.2. for detailed discussion). In addition, aromatase expression is downregulated in the cerebral cortex of mouse embryos deficient for steroidogenic factor SF1, in parallel by an arrest in the migration of neuroblast (Komada et al., 2015).

The laminar organization of the cerebral cortex is generated during development by a process of radial migration in which the first migrating cells form the inner layers and successive migrating cells form the outer superficial layers. In a first step, cells from the ventricular and subventricular zones (VZ/SVZ) migrate to an intermediate zone (IZ) in the developing cortex. Form the IZ, cells migrate along radial glial processes until they reach their destination in the cortical plate. In this developmental process of laminar cortical organization an essential molecule is the extracellular matrix glycoprotein reelin (Frotscher et al., 2009; Jossin, 2020), which is expressed and released by Cajal-Retzius cells (Causeret et al., 2021), the first cortical neurons generated.

Reelin is proangiogenic and mediates the communication of endothelial cells and radial glia to control neuroblast migration (Segarra et al, 2018). In the cerebral cortex, reelin is also required for the ordered formation of cortical layers and controls the vertical organization of neuronal dendrites (Frotscher et al., 2009). Interestingly, estradiol upregulates reelin expression. This has been detected in the cerebellum and the hippocampus, two brain regions with laminar structure whose development is also controlled by reelin. The injection of estradiol in the cisterna magna at P5 increases the expression of reelin in the developing cerebellum of reelin haploinsufficient heterozygous *reeler* (rl/+) mouse, preventing Purkinje neuron loss caused by reelin deficiency in males (Biamonte et al., 2009). Similar results were obtained in studies assessing the effect of the inhibition of aromatase activity in organotypic slice cultures of the hippocampus, indicating that neuroestradiol upregulates reelin expression by Cajal-Retzius cells (Bender et al., 2010). Neuroestradiol may therefore affect the developmental organization of brain laminar structures by regulating reelin expression.

2.3. Neuritogenesis and synaptogenesis

Neuritogenesis consists in the growth and the maturation of the dendrites and the axon of the developing neuron. It is initiated with the

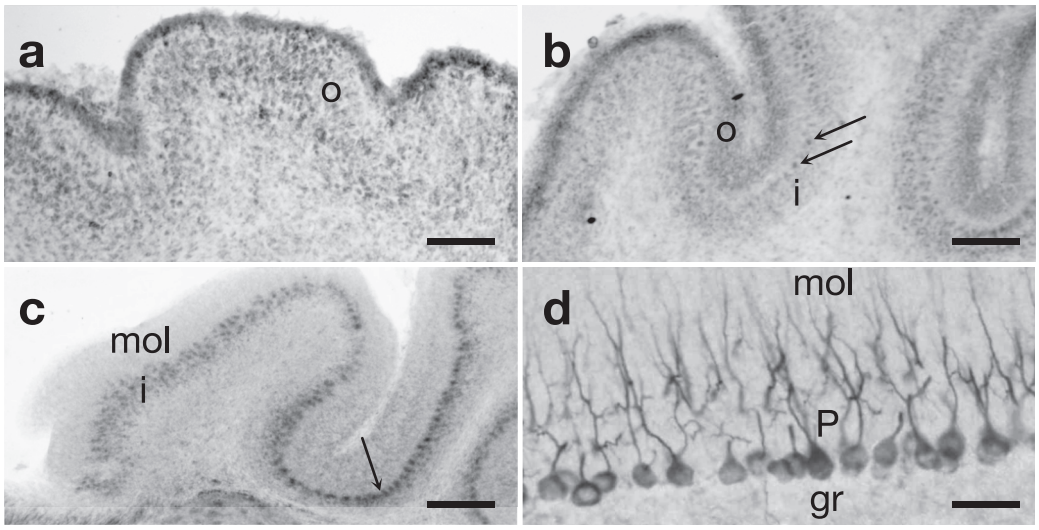

Fig. 3. Transient aromatase immunoreactivity in migrating granule cell neuroblasts in the developing mouse cerebellar cortex. Examples of histological sections of the mouse cerebellar cortex at postnatal developmental stages P2 (a), P4 (b), P7 (c) and P21 (d). The histological sections were immunostained with an antibody raised against mouse aromatase (Garcia-Segura et al., 1999), without counterstaining. a, At postnatal day P2 aromatase immunoreactivity is confined to migrating neuroblast in the outer granular layer (o). b, At P4 migrating neuroblasts have formed an inner granular layer (i), which is separated from the outer (o) granular layer by alienated aromatase-immunoreactive cell bodies of Purkinje cells (arrows). c, At P7 all granule cells have reached the inner granular layer (i) and initiate to turn off aromatase expression, while Purkinje neurons (arrow) increase such expression. A new aromatase-negative molecular layer (mol) has been formed by the growing dendrites of Purkinje cells and the growing axons of granule cells, while the external granular layer has disappeared. d, By the third postnatal week, both Purkinje cell perikarya and dendrites (P) show strong aromatase immunoreactivity, while the granule cell perikarya in the granule cell layer (gr) and the granule cell axons in the molecular layer (mol) are not immunostained. Scale bars = 350 μm (a); 500 μm (b and c); 100 μm (d).

emission of one or several primary growing sprouts by the cell soma. These neuritic sprouts are progressively differentiated in one or several growing dendritic processes and a unique growing axonal process. Finally, these growing processes generate several ramifications and acquire the molecular and morphological characteristics of mature dendrites and axon (Fig. 1).

Torand-Allerand (1976) was the first to demonstrate that, regardless of genetic sex, estradiol induces neuritic growth in organotypic cultures of the newborn mouse hypothalamus/preoptic area. Further studies *in vivo* and in culture models have shown that the administration of exogenous estradiol promotes neuritogenesis (Cambiasso et al., 1995) by acting on nuclear and membrane ERs (ERα, ERβ and GPER1) (Arevalo et al., 2012) and by activating a variety of molecular mechanisms that still are not fully characterized. These include the activation of intracellular kinase signaling pathways, the regulation of intracellular [Ca^{2+}], and the modulation of the action of other neuritogenic factors, such as BDNF, insulin-like growth factor 1 (IGF-1) and neurogenin 3 (Ngn 3) (Carrer et al., 2003; Gorosito and Cambiasso, 2008; Arevalo et al., 2012; Haraguchi et al., 2012; Cabrera Zapata et al., 2019; Ganchala et al., 2023).

The development of the cerebellar cortex occurs during the 3 first postnatal weeks in rodents. As previously mentioned, aromatase immunoreactivity is detected in migrating granule cells and in developing Purkinje neurons (Fig. 3). Studies by the laboratory of Tsutsui, focusing on the development of cerebellar Purkinje cells, have identified neuroestradiol as a source of estradiol involved in the regulation of neuritogenesis. These studies have shown that the Purkinje neurons are provided with the necessary steroidogenic enzymes to convert cholesterol in a variety of neurosteroids, including neuroestradiol (Ukena et al., 1998). They also demonstrated that during the period of neuritogenesis and synaptogenesis Purkinje neurons produce neuroestradiol (Sakamoto et al., 2003) and that this production is necessary for their full dendritic development (Sasahara et al., 2007; Haraguchi et al., 2012; Tsutsui, 2012). Endogenous factors, such as prostaglandin E2, which stimulates aromatase activity and estradiol production in the developing cerebellum (Dean et al., 2012), may regulate this process.

During the final phases of the process of neuritogenesis the axon and dendrites of the differentiating neuron establish functional synaptic contacts with other neurons. As a final step in dendritogenesis, dendrites develop dendritic spines, which are postsynaptic elements for excitatory synaptic inputs. Studies from Tsutsui's laboratory have characterized the promotion of spinogenesis and synaptogenesis in the developing Purkinje cells of the cerebellum by neurostesradiol, which is mediated by the upregulation of BDNF (Sasahara et al., 2007). On the other hand, Rune and collaborators have shown that aromatase inhibition decreases synaptogenesis on dendritic spines and the expression of synaptic proteins, such as synaptophysin and spinophilin in hippocampal cultures of male and female pups (Kretz et al., 2004; Prange-Kiel et al., 2006; Fester et al., 2009). In addition, aromatase inhibition during postnatal development reduces synapses on spines in the molecular layer of the dentate gyrus of female rats (Bender et al., 2010).

The role of neuroestradiol on synaptogenesis has also been demonstrated in male and female mice with selective KO of aromatase in forebrain excitatory neurons. In these animals, there is a significant reduction in the density of dendritic spines in hippocampal CA1 and cerebral cortex pyramidal neurons and in the expression of synaptic markers synaptophysin and PSD95 compared to control mice (Lu et al., 2019).

Neuroestradiol also regulates spinogenesis and synaptic plasticity in the adult brain (Azcoitia et al., 2018). Thus, aromatase inhibition or the silencing of its expression in the adult female mouse hippocampus results in the impairment of long-term potentiation (LTP), the downregulation of CA1 synaptic and spine density, and functional memory deficits (Vierk et al., 2015; Lan et al., 2021). The effect of hippocampal aromatase silencing is more pronounced when combined with ovariectomy, suggesting that both central and peripheral estrogens regulate synaptic plasticity in the adult female brain (Lan et al., 2021), although it should be noted that ovariectomy may also reduce the levels of other neuroactive factors released by the ovaries in addition to estradiol (see, for instance, Hidalgo et al., 1995).

Although all these studies have shown the implication of neuroestradiol on synaptogenesis, it is important to mention that the studies from Kawato's and Rune's laboratories, among others, have in addition demonstrated the importance of endogenous steroidogenesis as the source of neuroestradiol (Kretz et al., 2004; Mukai et al., 2006; Fester et al., 2009). For instance, in the study of Fester et al. (2009), addition of cholesterol to the hippocampal cultures resulted in increased levels of neuroestradiol and in increased synaptogenesis on dendritic spines. In

contrast, inhibition of the cholesterol biosynthetic enzyme 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase in the hippocampal cultures resulted in a reduction in synaptogenesis. Furthermore, the effect of cholesterol on synaptogenesis was blocked by aromatase inhibition, indicating that neuroestradiol mediates the synaptogenic effect of cholesterol.

3. Sex-specific developmental actions of neuroestradiol

The studies reviewed above indicate that neuroestradiol regulates several steps of neuronal development in both sexes. Some of these developmental actions of neuroestradiol have effects that are detectable in only one sex. Although neuroestradiol is a product of endogenous brain steroidogenesis in males and females, its synthesis is boosted in the developing male brain by the high levels of circulating testosterone produced by the fetal testes. Although the role of neuroestradiol derived from testicular testosterone on brain sexual differentiation has been confirmed by numerous experimental studies (Balthazart, 2019), the precise mechanisms involved in these sex-specific developmental effects of neuroestradiol are still under investigation. In the following sections we discuss examples of sex-specific regulation of neurogenesis, neuronal migration, neuritogenesis and synaptogenesis by neuroestradiol in the developing male brain.

On the other hand, while the role of neuroestradiol on male brain development is well documented, much less is known about its potential neurodevelopmental actions in females. However, neuroestradiol may act in the developing female brain as well, because aromatase is expressed in radial glial cells, intermediate neuronal progenitors, migratory neuroblasts and differentiating neurons in both sexes (see section 2; Fig. 1). Indeed, some recent studies suggest that neuroestradiol also has sex-specific neurodevelopmental effects in females. A discussion of these findings is also included in the following sections.

3.1. Neurogenesis

Sex-specific effects of neuroestradiol on male brain neurogenesis have been detected in songbirds and rodents. Chen et al. (2014) injected Bengalese finches with [H^3]-thymidine at post-hatching day 15 to assess cell proliferation in the ventricular zone of the songbird brain, where HVC progenitors are generated. They find that males have higher [H^3]-thymidine incorporation in the ventricular proliferative zone at post-hatching day 15 and higher number of [H^3]-thymidine labeled migrating neuroblasts along vimentin-immunoreactive radial glial fibers at post-hatching days 21 and 26 than females. Furthermore, after detecting similar sex differences in cell proliferation and migration in brain explant cultures, these authors demonstrated that sex differences in cell proliferation and in the incorporation of newly generated neurons into the HVC were due to neuroestradiol and mediated by the release of BDNF. Therefore, these findings in songbirds indicate a sex-specific effect of neuroestradiol on males. In addition to regulate cell proliferation and neuroblast migration, neuroestradiol participates in the addition of new neurons to the HVC in male adult songbirds by promoting the survival of newly generated cells (Hidalgo et al., 1995). This effect of neuroestradiol is mediated by the induction of the release of trophic factors, such as IGF-1, by radial glial cells (Jiang et al., 1998).

In rodents, the expression of aromatase is detected in neural stem cells, suggesting a possible participation of neuroestradiol on developmental neurogenesis and in the generation of sex differences in the different proportion of neuronal and glial markers detected when male and female neurospheres are differentiated with retinoic acid (Waldron et al., 2010b). Indeed, the study by Bowers et al (2010) suggests that the effect of neuroestradiol on developmental hippocampal neurogenesis is sex-specific. They assessed cell proliferation in the developing rat dentate gyrus with bromodeoxyuridine (BrdU) immunostaining, detecting a higher number of newly generated cells in males compared to females. They also find that the treatment of newborn rats with an aromatase inhibitor reduced cell proliferation in developing males but not in developing females, suggesting a male sex-specific effect of neuroestradiol on neurogenesis. Indeed, the exogenous administration of estradiol increased the number of BrdU + cells and the survival time of the newly proliferated cells in females and enhanced the acquisition of a neuronal phenotype by the newly generated cells in both sexes (Bowers et al. 2010). These findings suggest that neuroestradiol generated from testicular testosterone promotes developmental neurogenesis in the male hippocampus.

While these studies indicate male-specific effects of neuroestradiol on developmental neurogenesis, further studies are still necessary to determine whether neuroestradiol also exerts sex-specific effects on adult neurogenesis in rodents. Adult neurogenesis in rodents is regulated by estradiol, presents sex-specific characteristics, and contribute to the generation of sex differences in behavior and cognition during adolescence and adulthood (Ahmed et al., 2008; Juraska et al., 2013; Yagi and Galea, 2019; Trova et al., 2021; Hodges et al., 2022; Mohr et al., 2022). For instance, sex differences in several parameters of adult neurogenesis have been observed in the SGZ of the dentate gyrus (Yagi et al., 2020) and estradiol treatment increases cell proliferation, decreases the survival of new neurons, and decreases cell death in the dentate gyrus of adult female rats, while none of these effects of exogenous estradiol are observed in males (Barker and Galea, 2008). These differences in the results of exogenous estradiol treatments may be due to a sex-specific effect of neuroestradiol on neurogenesis. However, this possibility has not been directly tested. Furthermore, potential sex-specific effects of neuroestradiol regulated neurogenesis under conditions of brain injury in mammals remain to be explored. This is a relevant question to be addressed in future studies, given the reported sex differences after different acute neurological insults, including traumatic brain injury and stroke.

3.2. Neuroblast migration

In the previous section we have seen examples of sex-specific effects of neuroestradiol, promoting developmental neurogenesis in the male brain. In this section we will discuss evidence of a sex-specific effect of neuroestradiol that results in the acceleration of neuroblast migration in females.

The studies that have analyzed possible sex differences in neuroblast migration in the developing rodent brain have provided controversial results. Park et al (1996) injected BrdU on E24 and localized the newly generated cells in the developing preoptic area/anterior hypothalamus (POA/AH) at E30, E34 or E38 in ferrets. Using this approach, they did not detect significant differences in the localization of migrating BrdU positive cells. However, Jacobson and Gorski (1981) reported that neuroblast division and migration to the rat sexual dimorphic nucleus of the POA (SDN-POA) occurs earlier in females than in males, although this difference is restricted to specific moments in brain development and, for instance, is not detected in cells born at day 18 of gestation (Jacobson et al., 1985), which in males corresponds to the peak of testosterone production by the fetal testis (O'Shaughnessy et al., 1998, 2006). These findings suggest that there is an advanced neuroblast migration in female embryos during a time window that precedes the generation of neuroestradiol from testicular testosterone in the male embryos.

Studies on neuroblast migration on brain slices have also revealed subtle but significant sex differences. Thus, Henderson et al. (1999) detected sex differences in the orientation of neuroblast migration into the POA/AH in E15 mouse brain slices, and Knoll et al. (2007) observed that migrating cells in POA/AH slices move nearly three times faster and frequently in females than in males at E14, but not at E13. In addition, treatment of slices with estradiol, increased migration speed and movement in E14 male cells, but did not affect migration speed in females, a difference that could be explained by an endogenous effect of neuroestradiol in females.

Overall, these findings suggest that the timing or speed of cell migration may differ between males and females, with female neuroblasts showing an earlier migration than males at restricted periods during brain development. Faster migration speed of female neuroblasts is also suggested by the more recent study of Sellers et al. (2020) in the developing cerebral cortex. In addition, this study reveals a sex-specific effect of neuroestradiol on neuroblast migration. These authors obtained *in vivo* aromatase knockdown in the cerebral cortex using *in utero* electroporation at E14.5 of shRNA constructs against *Cyp19a1*. Aromatase silencing in the cerebral cortex did not affect the total number of migrating cells, neither in males nor in females. This suggests that neuroestradiol is not involved in the capacity of neuroblasts to migrate. However, aromatase silencing in the brain of male embryos resulted in increased number of migrating cells in the upper most portion of the cortical plate and reduced number of migrating cells in the VZ/SVZ compared to control animals. This effect of aromatase silencing may reflect an inhibitory effect of neuroestradiol on the speed of neuroblast migration in males. However, it is also possible that in males cell migration is affected by testosterone, which is expected to have been accumulated in the developing cortex after aromatase silencing.

However, the most interesting result in the study of Sellers et al. (2020) was obtained in female animals, in which aromatase knockdown decreased the number of neuroblast that reached the cortical plate at P0, producing an accumulation of neuroblasts in the VZ/SVZ. This was associated with an increase in the proportion of neuroblasts with a round morphology and a decrease in the proportion of neuroblasts with a bipolar morphology, characteristic of migrating cells. This suggests that neuroestradiol promotes neuroblast migration in the female cerebral cortex. However, as previously mentioned, a similar number of migrated neuroblast reach the cortical plate at P0 in males and females under basal conditions. Therefore, the effect of neuroestradiol in females is to accelerate neuroblast migration by increasing its speed or by advancing its time of initiation compared to males, rather than to increase the total number of migrating cells. A similar conclusion may be reached from the earlier findings of Jacobson and Gorski (1981) and Knoll et al. (2007), where an accelerated cell migration towards the POA in females do not correlate with a higher number of POA neurons in this sex. Thus, sex-specific effects of neuroestradiol on neuroblast migration in females may have evolved to compensate sex differences in the migration process rather than to generate sex differences in the final number of neurons in the POA and the cerebral cortex (see further discussion on section 5).

3.3. Neuritogenesis

The involvement of neuroestradiol in the generation of sex differences in neuritogenesis was suggested by Holloway and Clayton (2001) in a study on long-term finch brain slice cultures. The authors assessed the growth of neurites from the nucleus HVC to the nucleus robustus (RA), a group of premotor neurons in the song motor pathway that under normal conditions develops only in males. Neuritic outgrowth from the HVC was observed in brain slices of both sexes. In males, the neurites emerged from the HVC reached the RA, as observed in the male brain *in vivo*. However, in females the neurites from the HVC stopped their growth in a semicircle outside the RA.

Holloway and Clayton (2001) first assessed the effect of estradiol on neuritogenesis and they find that the treatment with the hormone induced a male-specific neuritic growth between HVC and RA in female slices. In contrast, estradiol did not affect neuritic growth in male cultures. An interesting observation was that the co-culture of male and female slices induced the development of a male-like neuritic growth in females, with neurites reaching the RA from the HVC. This suggested that a factor released by male cultures was the cause of the masculinization of the HVC-RA neuritic pathway.

To test the hypothesis that neuroestradiol produced by male slices was involved in the generation of the observed sex differences in neuritogenesis, Holloway and Clayton (2001) assessed the effect of inhibiting aromatase activity in the cultures. For this they used the aromatase inhibitor fadrozole. Treatment of male slices with fadrozole resulted in neuritic growth from HVC with similar characteristics to that observed in females. In contrast, aromatase inhibition did not affect the normal pattern of neuritogenesis in female slices, but prevented their masculinization caused by the co-culture with male slices. These findings strongly suggest that neuroestradiol promotes neuritogenesis from HVC to RA in the songbird male brain.

Sex differences in neuritogenesis have been also observed in several rodent brain regions and *in vitro* models (Díaz et al., 1992; Mong et al., 1999; 2001; Arbo et al., 2017; Keil et al., 2017; Marraudino et al., 2019). At least in part, these differences in rodent neuritogenesis are due to an accelerated speed of neuritogenesis in females. Thus, in primary neuronal cultures from the hippocampus and the hypothalamus, the growth and branching of primary neurites and the differentiation of the axon, occurs earlier in female neurons than in male neurons (Scerbo et al., 2014; Ruiz-Palmero et al., 2016).

In contrast with the sex differences of neuritogenesis in the zebra finch brain discussed earlier, which are dependent on testicular testosterone, sex differences in the speed of neuritogenesis in rodents are observed before the perinatal peak of testosterone production by the fetal testes (Cambiasso et al., 1995, 2000; Scerbo et al., 2014; Ruiz-Palmero et al. 2016; Cisternas et al., 2020). An implication of neuroestradiol in the accelerated neuritogenesis of female neurons is indicated by the reduction in the speed of its neuritogenesis to male levels when aromatase is inhibited in primary neuronal hippocampal cultures (Ruiz-Palmero et al., 2016).

One of the factors involved in the effect of neuroestradiol on neuritogenesis in rodent neurons is neurogenin 3 (Ngn3), a basic helix-loop-helix (bHLH) transcription factor regulated by Notch signaling. In the pancreatic islets, Ngn3 plays an essential role in the development of endocrine cells (Rukstalis and Habener, 2009). In the nervous system, Notch activation represses the transcription of *Ngn3*, preventing the differentiation of neurites. When Notch signaling is inhibited, Ngn3 production is activated, promoting axogenesis and dendritogenesis (Simon-Areces et al., 2011, 2013; Arevalo et al., 2012).

Studies in primary hypothalamic neurons (Scerbo et al., 2014) and primary hippocampal neurons (Ruiz-Palmero et al., 2016) have shown that Ngn3 is involved in the generation of sex differences in neuritogenesis. This molecule shows higher expression in female neurons compared to male neurons in primary mouse hypothalamic neuronal cultures. More important, when Ngn3 is silenced in the cultures, sex differences in neuritogenesis are abolished (Scerbo et al., 2014). Similar results have been obtained using primary mouse hippocampal neurons, where it has been confirmed that sex differences in neuronal development are associated with a higher expression of Ngn3 in female neurons at the earlier phases of neuritogenesis and that Ngn3 silencing decreases neuritogenesis in male and female neurons, abolishing sex differences (Ruiz-Palmero et al., 2016). Thus, although Ngn3 is involved in the neuritogenesis of both male and female neurons, neuritogenesis is advanced in females compared to males because there is an earlier peak of Ngn3 in the neurons of this sex. To determine whether the earlier peak of Ngn3 expression in females depend on neuroestradiol, neuronal aromatase activity was inhibited. Under these conditions, the expression of Ngn3 and the neuritogenesis of female neurons was substantially reduced to male levels (Ruiz-Palmero et al. 2016). This finding indicates that neuroestradiol generated by neuronal aromatase promotes an accelerated neuritogenesis in female neurons by upregulating Ngn3 expression.

These studies suggest that a different time of action of neuroestradiol in female and male neurons, probably coupled to a different tissular origin of neuroestradiol precursors (neurons vs testis), advances the expression of Ngn3 and the differentiation of neurites in the female hypothalamus and hippocampus. Thus, neuroestradiol derived from endogenous brain steroiodogenesis will enhance neurite differentiation

in the female hypothalamus and hippocampus at an age when the male embryonic brain has not yet started to metabolize testicular testosterone in neuroestradiol (Scerbo et al., 2014; Ruiz-Palmero et al., 2016).

3.4. Synaptogenesis

At least part of the sex differences in synaptic connectivity that have been detected in the vertebrate brain are likely generated during development. Indeed, early electron microscopic studies of the developing rodent hypothalamus revealed sex differences in synaptogenesis associated with permanent sex differences in synaptic connectivity (Matsumoto and Arai, 1980; Pérez et al., 1990; Pozzo Miller and Aoki, 1991). Studies of dendritic spines with the Golgi staining method also revealed developmental sex differences in the number of dendritic spines in hypothalamic neurons (Mong and McCarthy, 1999; Todd et al., 2007). These studies also indicate that synaptogenesis and dendritic spine formation are regulated by gonadal steroids, suggesting that neuroestradiol derived from testicular androgens regulate synaptic formation in males.

In addition, there is also evidence that neuroestradiol regulates synaptogenesis in females. Studies from Rune's laboratory showed that aromatase inhibition reduces the density of dendritic spines and spine synapses and the expression of synaptic proteins in hippocampal slice cultures and in primary hippocampal neurons derived from female pups, indicating that neuroestradiol is necessary for synapse spine formation in females (Prange-Kiel et al., 2013; Brandt et al., 2020). Furthermore, systemic aromatase inhibition decreases the density of spine synapses in the hippocampal CA1 region in both intact and ovariectomized mice, suggesting that extragonadal estradiol synthesis, probably neuroestradiol, is necessary for synapse formation in females *in vivo* (Zhou et al., 2010).

The studies by Rune and collaborators have also shown that the effect of neuroestradiol on synapse and spine formation in the hippocampus is specific for females (Fester et al., 2012; Prange-Kiel et al., 2013; Brandt et al., 2020). Indeed, in contrast to its effect on females, aromatase inhibition increases the number of mature dendritic spines in male neurons. This is probably due to the elevation in neuronal androgen levels after aromatase inhibition. Indeed, the testosterone metabolite dihydrotestosterone (DHT) promotes an increase in the number of mature dendritic spines in males, but not in females, and the combination of DHT with letrozole further increases the number of mature dendritic spines in males, probably because aromatase inhibition results in more testosterone available for conversion to DHT by the enzyme 5α-reductase. These findings suggest that spine maturation is promoted by androgens in males, while neuroestradiol derived from neuronal steroidogenesis promotes spine maturation in females (Brandt et al., 2020).

Neuroestradiol actions on synaptogenesis may be mediated by actions on glial cells, because astrocytes and microglia express ERs and show sex differences in migration and phagocytosis, two parameters involved in the shaping of neuronal circuits during brain development (Nelson et al., 2017; Yanguas-Casás et al., 2018; Pinto-Benito et al., 2022; Pickett et al., 2023). In agreement with this possibility, Lenz et al. (2013) have shown that estradiol induces the growth of dendritic spines in female rat pup POA primary cultures through the mediation of microglia. Since the number of dendrites spines in the POA is higher in males, this finding suggests that neuroestradiol, acting on microglia, promotes synaptogenesis in males.

Transient sex differences in microglia volume and phagocytic activity in association with transient sex differences in synaptogenesis have been detected in the *stratum radiatum* of the mouse hippocampal CA1 region. Higher microglia volume and phagocytic activity in females compared to males at P8 was followed by higher density of synaptic boutons in females compared to males at P15. However, by P40, females and males have similar microglia volume, similar microglia phagocytic activity and similar density of dendritic spines and synaptic boutons (Weinhard et al., 2018). These findings not only suggest that microglia are involved in the generation of sex differences in synaptogenesis, they also reveal that, as observed for neuritogenesis, female hippocampal neurons have an advanced rate of synaptogenesis compared to males. Although it is unknown if neuroestradiol promotes the advanced female synaptogenesis observed in this study, the findings of Rune's laboratory (Fester et al., 2012; Prange-Kiel et al., 2013; Brandt et al., 2020) suggest that this is the case.

Developmental sex differences in astrocytes occur in parallel to the generation of sex differences in synaptic formation (Garcia-Segura et al., 1995; Amateau and McCarthy, 2002), suggesting that these cells may also play an active role in the generation of sex differences in synaptogenesis. This has been recently demonstrated to be the case by Mazur et al. (2021) in separate male and female rat pup cerebral cortex neuronal cultures. They observed that the co-culture with astrocytes, or the treatment with astrocyte conditioned medium, increases the density of excitatory synapses on neurons of both sexes, being this effect more pronounced in male neurons. Furthermore, they detected that the synaptogenic effect of astrocytes is mediated by thrombospondin-2 (TSP2). Interestingly, aromatase inhibition with letrozole in the cultures decreases the synaptogenic effect of TSP2 on male neurons and enhances the synaptogenic effect of TSP2 in female neurons, abolishing sex differences in synaptogenesis. Therefore, it can be concluded that by facilitating or inhibiting the action of the astrocytic released factor TSP2, neuroestradiol regulates cortical neuron synaptogenesis in both sexes.

4. Do X-linked genes generate an epigenetic landscape permissive for the developmental actions of neuroestradiol in female neurons?

It is now recognized that cell autonomous actions of sex chromosome genes, in addition to gonadal hormones, participate in the generation of phenotypic sex differences in the brain (Carruth et al., 2002; Agate et al., 2003; Abel et al., 2011; Forger et al., 2016). Both sex chromosomes and gonadal hormones influence the expression of epigenetic enzymes and may therefore contribute to brain sexual differentiation through modifications in the structure of chromatin (Nugent et al., 2015; Cortes et al., 2019; Cortes and Forger, 2023).

Some mouse models have facilitated the analysis of the effects of sex chromosome genes in the generation of sex differences in specific traits. One of these models is the four core genotypes (FCG) mouse model, in which the deletion of the testis-determination gene Sry from the Y chromosome (Y^-), in combination with the insertion of Sry in an autosome (+Sry) allows the generation of XX or XY^- mice that are phenotypically female (develop ovaries) and XX + Sry or XY^- +Sry mice that are phenotypically male (develop testes). This mouse model reveals that sex chromosomes and gonadal hormones interact in the control of the expression of aromatase (Cisternas et al., 2015, 2018) and ERβ (Cisternas et al., 2017) in some brain regions of the developing brain, suggesting that sex chromosome complement may influence the synthesis and action of neuroestradiol involved in the generation of developmental sex differences.

A role for sex chromosomes in determining the developmental actions of neuroestradiol may help to explain male/female asynchrony in neurogenesis (Jacobson and Gorski, 1981), neuronal migration (Jacobson and Gorski, 1981; Sellers et al., 2020), neuritogenesis (Scerbo et al., 2014; Ruiz-Palmero et al., 2016) and synaptogenesis (Weinhard et al., 2018) observed in some studies. The asynchrony in neurogenesis, neuronal migration and neuritogenesis is observed before the peak of testosterone production by the fetal testes and therefore is not exclusively attributable to an effect of testicular androgens.

As mentioned before, neuroestradiol in females regulate at least some of these asynchronous developmental events. This is the case for neuritogenesis that, as discussed in section 3.3., is stimulated by neuroestradiol in female neurons through the upregulation of the

neuritogenic factor Ngn3. Interestingly, studies on hypothalamic cultures of FCG mice have determined that is the sex chromosome complement and not gonadal hormones the cause of the advanced expression of the neuritogenic factor Ngn3 and the advanced neuritogenesis of female neurons compared to male neurons (Scerbo et al., 2014; Cisternas et al., 2020). This finding suggests that one or several X linked genes may be involved in the determination of sex differences in Ngn3 and neuritogenesis.

Analysis of X linked genes in hypothalamic neurons from FCG mice have identified three genes that have higher expression levels in XX neurons compared to XY neurons, regardless of gonadal sex: *Kdm6a/Utx*, *Eif2s3x* and *Ddx3x*. Other X linked genes are also expressed by hypothalamic neurons, but at similar levels in XX and XY neurons, such as *Kdm5c*, *Mecp2*, *Usp9x* and *Syp* (Cabrera Zapata et al., 2021). *Kdm6a/Utx* encodes for lysine demethylase 6a, which removes methyl groups in lysine at position 27 of histone H3 (Hong et al., 2007). Trimethylation at this lysine of histone H3 generates a chromatin repressive state that prevents gene expression. Therefore, the demethylation of H3K27 by *Kdm6a/Utx* participates in the epigenetic and transcriptional regulation by promoting chromatin remodeling and accessibility.

In the developing nervous system, *Kdm6a/Utx* regulates neural stem cell proliferation, differentiation, and lineage specification (Lei and Jiao, 2018; Yang et al., 2019; Shan et al., 2020) and loss of this gene in human neural progenitor cells inhibits neuronal differentiation and reduces neuritogenesis in differentiated neurons (Tang et al., 2020). Furthermore, impaired long-term synaptic plasticity in the hippocampus, in parallel with decreased arborization and spine density in dendrites of pyramidal hippocampal neurons, has been detected in *Kdm6a/Utx* deficient mice. These animals also show anxiety-like behaviors and cognitive deficits (Tang et al., 2017).

Given these implications of Kdm6a/Utx in neuritogenesis and dendritic arborization, its role on the generation of sex differences in neuritogenesis was tested on primary hypothalamic neurons (Cabrera Zapata et al., 2021, 2022b). First, *Kdm6a/Utx* expression levels were assessed in the hypothalamus of FCG mice, observing that they are higher in XX males and XX females, compared to XY males and XY females, both at embryonic day 14 and at postnatal days P0 and at p60. In addition, *Kdm6a/Utx* expression levels are also higher in hypothalamic neurons from XX animals compared to XY animals, regardless of the gonadal sex. To confirm the implication of Kdm6a/Utx in neuritogenesis, its methylase activity was pharmacologically inhibited in hypothalamic neurons, observing that neuritogenesis was selectively reduced in female neurons, resulting in the abolishment of sex differences. Identical results are obtained after silencing *Kdm6a/Utx* gene expression (Cabrera Zapata et al., 2021, 2022b). All these findings indicate that X-linked gene Kdm6a/Utx is involved in the generation of sex differences in neuritogenesis in hypothalamic neurons, being essential for enhanced neuritogenesis in females, but not in males.

The effect of X linked genes such as *Kdm6a/Utx* in the generation of sex differences in neuritogenesis may be mediated by the regulation of target genes involved in neuronal development (Tang et al., 2020; Cabrera Zapata et al., 2022a; Koizumi et al., 2022). It has been recently shown that one of the target genes of *Kdm6a/Utx* is *Ngn3*. Thus, *Ngn3* expression is reduced in female neurons after Kdm6 methylase inhibition or *Kdm6a*/Utx silencing (Cabrera Zapata et al., 2021, 2022a,2022b), an effect probably mediated by a direct action of Kdm6a/Utx on the *Ngn3* promoter (Cabrera Zapata et al., 2022a). In contrast, *Kdm6a*/Utx silencing does not affect Ngn3 expression in male neurons (Cabrera Zapata et al., 2022a).

It is important to consider that the sex-specific effect of *Kdm6a*/Utx in female neurons only occurs in presence of neuroestradiol, because aromatase inhibition in female neurons reduces Ngn3 expression to male levels (Ruiz-Palmero et al. 2016). Therefore, the advanced expression on Ngn3 in female neurons requires both the epigenetic modification exerted by Kdm6a/Utx on the Ngn3 promoter and the activation of the neuroestradiol-dependent signaling mechanisms that activate Ngn3 expression, which involve the downregulation of Notch signaling, the upregulation of BDNF and the activation of TrkB receptor signaling (Ruiz-Palmero et al., 2011, 2013; Ganchala et al., 2023). Therefore, neuritogenesis is advanced in females in comparison to males because the X-liked gene *Kdm6a/Utx* exerts an epigenetic modification in Ngn3 in female neurons that allows its subsequent upregulation by neuroestradiol (Fig. 4).

Further studies should determine if X-linked genes also exert a similar epigenetic facilitation of neuroestradiol actions in females in other male/female asynchronous developmental processes. It should be noted that Kdm6a/Utx is expressed in neural stem cells of the embryonic cerebral cortex, with higher expression in females. Moreover, Kdm6a/Utx deficiency has a deeper impact on the cerebral cortex development in females than in males, increasing neural stem cell proliferation, inhibiting cell cycle exit of neural progenitors and decreasing neuronal differentiation and the number of neurons that reach the cortical plate in a sex-specific manner (Lei and Jiao, 2018). Thus, Kdm6a/Utx is a potential candidate gene to participate in sex-specific effects of neuroestradiol on neurogenesis and neuronal migration.

5. Implications of sex-specific developmental actions of neuroestradiol in females

The aromatization hypothesis for the effects of testosterone on rodent male brain sexual differentiation, proposed by MacLusky and Naftolin more than 40 years ago, has received experimental confirmation by numerous studies that have proven that intracerebral conversion of testicular testosterone in neuroestradiol plays a major role in the development of male specific traits. In contrast, the potential effect of maternal estrogens on brain sexual differentiation has been the object of a long debate, but now it is generally accepted that the estrogen-binding protein α-fetoprotein prevents the actions of maternal estrogens on the developing brain (Bakker et al., 2006; González-Martínez et al., 2008). However, there is evidence that developmental actions of estrogens influence adult female behavior (Baum and Tobet, 1986; Ordyan et al., 2007; Pierman et al., 2008; Bakker and Brock, 2010; Royston et al., 2016; Zhou et al., 2020). Indeed, some studies have shown that ovarian estradiol contribute to brain feminization during adolescence and puberty, when brain developmental mechanisms are still operating (Bimonte et al., 2000; Juraska et al., 2013; Bakker, 2022). In addition, the data reviewed here indicate that neurosteroidogenesis is another source of estrogens for the female developing brain, where neuroestradiol exerts sex-specific effects on the timing or speed of neurodevelopmental processes.

There is an apparent asynchrony in some of the sex-specific developmental effects of estradiol in the male and the female brain, because in female rodents some of them are detected at developmental periods (around E14) that in males precede the developmental actions of neuroestradiol derived from testosterone produced by the rodent fetal testis, which peaks around embryonic days E17-E18 (O'Shaughnessy et al., 1998, 2006). Sex-specific effects of neuroestradiol in females may also potentially occur at other developmental periods, depending on the developmental program of each brain region. For instance, they may potentially occur at postnatal ages in the rodent cerebellum, which develops postnatally. However, potential sex-specific effects of neuroestradiol in female cerebellum as in many other brain regions remain to be explored.

The functional consequences of the regulation of female brain development by neuroestradiol also remain to be determined. By accelerating neurogenesis, neuronal migration, neuritogenesis and synaptogenesis compared to males, neuroestradiol may exert a compensatory mechanism to reduce in females the impact of sex differences generated by the neurodevelopmental actions of testicular testosterone in the male brain. However, in the nervous system different types of cells are generated at different times and therefore an accelerated neurogenesis, neuronal migration, neuritogenesis and

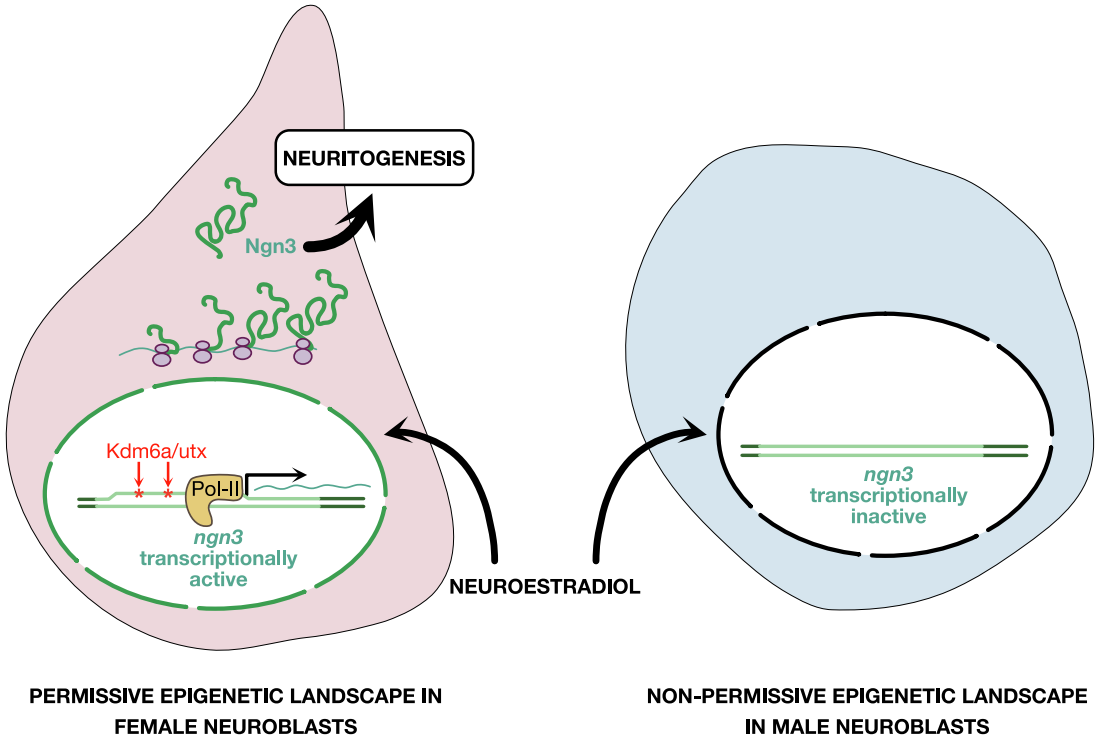

Fig. 4. Role of epigenetic regulation in determining sex-specific actions of neuroestradiol in female neuron neuritogenesis. The X-linked enzyme Kdm6a/Utx, which removes methyl groups in lysine at position 27 of histone H3, exerts an epigenetic regulation of the neuritogenic factor neurogenin 3 (Ngn3) gene, facilitating the upregulation of its transcription by neuroestradiol-activated signaling pathways in developing female neurons.

synaptogenesis may also have, per se, a strong impact on the temporally coordinated development of pre and postsynaptic structures in the female brain, with long term sex-specific effects in the configuration and function of adult neuronal networks. Therefore, further studies are needed to characterize the cellular and molecular mechanism of action of neuroestradiol on female neurodevelopmental program. Understanding the impact of neuroestradiol in specific cellular elements and cell types and identifying the sources and targets of local estradiol in developing neuronal circuits will pave the way to determine their functional consequences for adult behavior and for the vulnerability to affective and neurodegenerative disorders. In addition, neurodevelopmental actions of neuroestradiol in females should be taken in consideration when analyzing the consequences of the exposure to endocrine disrupting chemicals or synthetic estrogens during pregnancy.

6. Concluding remarks

Developmental actions of neuroestradiol have been traditionally analyzed from the point of view of its role in male brain organization. These actions of neuroestradiol as a metabolite of testicular testosterone are exerted at specific critical periods during male brain development. However, the production of neuroestradiol derived from brain cholesterol metabolism is not restricted to these critical periods and occurs in females as well.

We have reviewed here a variety of studies in different species showing that in coordination or by the mediation of other neuronal and glial factors, such as NgCAM, VEGF, BDNF, IGF-1, Reelin, Ngn3 or TSP2, neuroestradiol regulates in both sexes and at different developmental periods basic developmental processes in the nervous system, such as neurogenesis, the migration of neuroblasts, the growth of the dendrites and the axon and the formation of synaptic contacts.

These findings indicate that neuroestradiol controls the same neurodevelopmental processes in both sexes. However, the effects of neuroestradiol are not always identical in males and females and some of its developmental actions are sex-specific. The most obvious examples of such sex-specific actions are those exerted by neuroestradiol derived from testicular testosterone, which result in the generation of sex differences in the organization of different brain structures, being a paradigmatic example the sexual dimorphic nucleus of the preoptic area in rodents. However, not all the sex-specific actions of estradiol result in overt sex differences in brain organization. Examples of these are the effects of neuroestradiol in neuroblast migration, neuritogenesis, and synaptogenesis in females, whose characteristics and possible functional significance have been discussed in the previous sections.

Neuroestradiol synthesis in the brain of both males and females highlights the importance of understanding the mechanisms and processes leading to its sex-specific neurodevelopmental actions. We have discussed here the role of sex chromosome genes and epigenetic factors in determining some of such sex-specific actions in female neurons. However, further research is necessary to clarify the precise mechanisms of other sex-specific neurodevelopmental actions of neuroestradiol in both sexes including, for instance, that puzzling fact that it masculinizes the brain when is derived from testicular testosterone, but not when is derived from brain steroidogenesis.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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