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REVIEW ARTICLE

Interaction of sex chromosome complement, gonadal hormones and neuronal steroid synthesis on the sexual differentiation of mammalian neurons

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ABSTRACT

Female mouse hippocampal and hypothalamic neurons growing *in vitro* show a faster development of neurites than male mouse neurons. This sex difference in neuritogenesis is determined by higher expression levels of the neuritogenic factor neurogenin 3 in female neurons. Experiments with the four core genotype mouse model, in which XX and XY animals with male gonads and XX and XY animals with female gonads are generated, indicate that higher levels of neurogenin 3 in developing neurons are determined by the presence of the XX chromosome complement. Female XX neurons express higher levels of estrogen receptors than male XY neurons. In female XX neurons, neuronal derived estradiol increases neurogenin 3 expression and neuritogenesis. In contrast, neuronal-derived estradiol is not able to upregulate neurogenin 3 in male XY neurons, resulting in decreased neuritogenesis compared to female neurons. However, exogenous testosterone increases neurogenin 3 expression and neuritogenesis in male XY neurons. These findings suggest that sex differences in neuronal development are determined by the interaction of sex chromosomes, neuronal derived estradiol and gonadal hormones.

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Androgen receptor; aromatase; estradiol; estrogen receptors; neurogenin 3; neuritogenesis

Introduction

In 1849, Arnold Adolph Berthold (1803–1861) published the results of a series of experiments on the effect of castration in roosters. He observed that juvenile castrated roosters did not develop as adults the typical aggressive, mating and crowing behaviors of male animals. However, these behaviors were recovered if testes were implanted in the body of castrated animals, even if the testes were not innervated (Berthold, 1849). This was the first demonstration of the role of testicular secretions for the development of adult male behavior. This idea was confirmed by experiments in the laboratory of William Caldwell Young (1899–1965) showing that prenatal administered testosterone masculinizes sexual behavior in female guinea pig (Phoenix, Goy, Gerall, & Young, 1959). Phoenix *et al.* (1959) proposed that 'testosterone or some metabolite acts on those central nervous tissues in which patterns of sexual behavior are organized'. Testosterone is metabolized to estradiol by the enzyme aromatase and to dihydrotestosterone (DHT) by the enzyme 5α-reductase. The activity and expression of these enzymes were later identified in the human and rodent brain (Massa, Stupnicka, Kniewald, & Martini, 1972; Naftolin, Ryan, & Petro, 1971, 1972) and we know today that both metabolites of testosterone participate in the sexual differentiation of the brain. In particular, estradiol plays a major role in defeminizing and masculinizing brain and behavior in rodents (MacLusky & Naftolin, 1981; Bakker *et al.*, 2006). The enzyme aromatase, which metabolizes testosterone to estradiol, is expressed together with estrogen receptors in specific structures of the developing brain and participates in the generation of sex differences in neuronal and glial development.

The action of testosterone and its metabolites to induce sex differences in the brain occurs at specific organizational periods during the development. In rats and mice this mainly happens during the late fetal period, when there is a peak of testosterone production by the fetal testes, at E17–18 in mice and at E18.5–19.5 in rats (Huhtaniemi, 1994; O'Shaughnessy *et al.*, 1998; O'Shaughnessy, Baker, & Johnston, 2006; Scott, Mason, & Sharpe, 2009; Warren, Haltmeyer, & Eik-Nes, 1973). Then, testosterone and its metabolites are thought to activate epigenetic changes that will determine sex differences in sexual behavior when the animals reach puberty (Forger, 2016; Matsuda, Mori, & Kawata, 2012; McCarthy *et al.*, 2009; Nugent, Schwarz, &

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McCarthy, 2011). Around puberty, the brain circuits involved in reproductive behavior are activated by the sex hormones produced by adult gonads.

In addition to gonadal hormones, several studies have shown that sex chromosomes have gonadal-independent effects on brain sex differentiation. The evidence has been mainly obtained using the four core genotype (FCG) mouse model (Arnold & Chen, 2009). This model was created using mice with a deletion of the testis-determining gene Sry in the Y chromosome (Y^-). XY^- mice are female, since they do not develop testes and develop ovaries. The insertion of a Sry transgene into an autosome resulted in the generation of XY^-Sry mice, which are fertile males. By crossing XY^-Sry males with normal XX females the FCG is obtained: XX females, XXSry males (XX males), XY^- females (XY females) and XY^-Sry males (XY males) (Table 1). The FCG model allows to differentiate the gonadal effects from the sex chromosome effects in the generation of sex differences. Here, we will review the role of gonadal hormones and sex chromosomes on neuronal differentiation and neuritic growth.

Sex differences in neuronal development

Although sex differences in glial cells have been reported (Schwarz & Bilbo, 2012), many of the described brain sexual dimorphisms correspond to differences in neuronal number, morphology, connectivity and/or gene expression (Abel, Witt, & Rissman, 2011; Arnold & Gorski, 1984; Balan *et al.*, 2000; Carruth, Reisert, & Arnold, 2002; De Vries & Panzica, 2006; Gu, Cornea, & Simerly, 2003; Isgor & Sengelaub, 1998; Juraska, Fitch, & Washburne, 1989; Luque, de Blas, Segovia, & Guillamon, 1992; Oren-Suissa, Bayer, & Hobert, 2016; Panzica & Melcangi, 2016).

Table 1. The four core genotype mouse model.

Offspring	XX female	XX male	XY female	XY male
Sry transgene on autosome 3	No	Yes	No	Yes
Phenotype	Ovaries	Testes	Ovaries	Testes
Genotype	XX	XXSry	XY^-	XY^-Sry

Primary neuronal cultures have been used as a model to determine sex differences in developing neurons (Figure 1). These cultures have been prepared from different brain regions, including the mesencephalon, the hypothalamus and the hippocampus (Beyer, Green, & Hutchison, 1994; Carruth *et al.*, 2002; Keil, Sethi, Wilson, Chen, & Lein, 2017; Lorenzo, Díaz, Carrer, & Cáceres, 1992; Raab, Pilgrim, & Reisert, 1995; Reisert & Pilgrim, 1991). These studies have shown sex differences in neuronal differentiation (Beyer *et al.*, 1994; Carruth *et al.*, 2002; Keil *et al.*, 2017; Lorenzo *et al.*, 1992; Raab *et al.*, 1995; Reisert & Pilgrim, 1991; Ruiz-Palmero *et al.*, 2016; Scerbo *et al.*, 2014). For instance, in hypothalamic cultures obtained from E14 mouse embryos, before the peak in testosterone production by the fetal testes at E17–18, female neurons developed faster than male neurons (Scerbo *et al.*, 2014). Neurons were classified according to their developmental stage of differentiation *in vitro* (Díaz, Lorenzo, Carrer, & Cáceres, 1992). In stage I, cells extend lamella around the cell body; in stage II, cells display short and thin neurites with symmetric appearance; in stage III, cells show one neurite several times longer than the others that acquires axonal characteristic, whereas the remaining neurites become branching and tapering dendrites in stage IV. In stage V, spines are observed in the dendrites. The analysis of hypothalamic neuronal cultures revealed a progressive increase in the proportion of neurons in more differentiated stages as time in culture increases (Scerbo *et al.*, 2014). Sex differences in the proportion of neurons in the

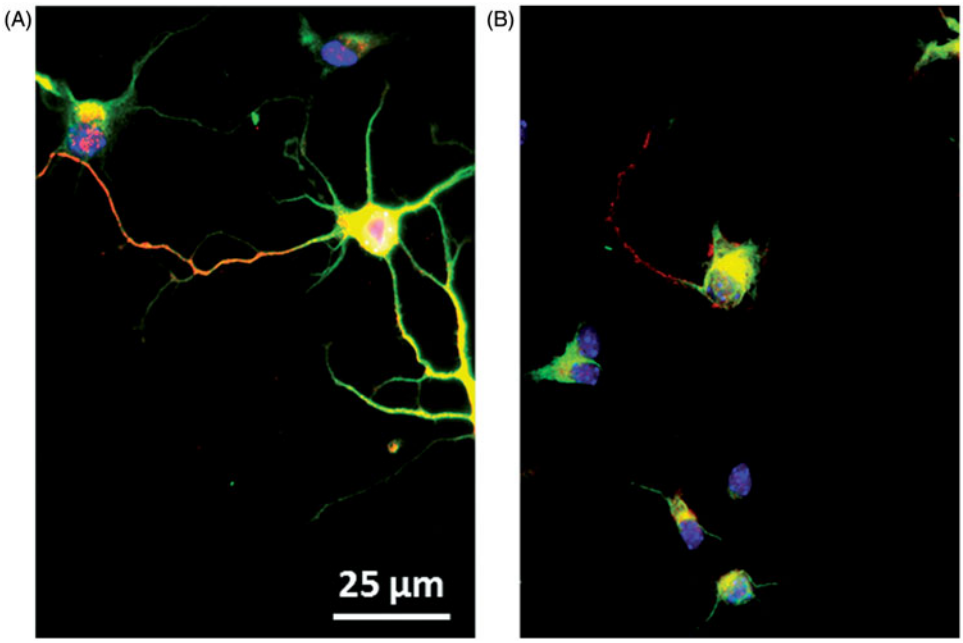

Figure 1. Representative examples of female (A) and male (B) hippocampal neuronal cultures at 2 days *in vitro*. Cultures were prepared from embryonic day 17 mouse embryos. Neurons were immunostained for the dendritic marker microtubule-associated protein 2 and the axonal marker Tau. Cell nuclei were stained with DAPI.

different stages of differentiation were observed from 1 day *in vitro* (DIV) to 6 DIV. Thus, after 1 DIV neurons were in stages I and II, but most neurons in male cultures were in stage I, while in female cultures most cells were in stage II. At 2 DIV neurons in stage III appeared, but only in female cultures, while in male cultures neurons in stage I predominated. At 3 DIV neurons in stages I, II, III and IV were observed. Female cultures showed neurons in stages II, III and IV, being stage III the predominant form. In contrast, in male cultures most neurons were in stage II; neurons in stage I were still present and the proportion of neurons in stages III and IV were significantly lower than in female cultures. At 4 DIV, neurons in stage I disappeared. Most neurons in male cultures were in stage II, while most neurons in female cultures were in stage III. Sex differences in neuronal development were maintained until 7 DIV, where all neurons were in stages III or IV and no sex differences in the proportion of neurons in each developmental stage were detected (Scerbo *et al.*, 2014).

Similar sex differences in neuronal differentiation were observed in hippocampal neurons obtained from E17 mouse embryos (Ruiz-Palmero *et al.*, 2016). The cultures were examined at 2 DIV where 42.00% of neurons in male cultures were in stage I versus the 14.75% in female cultures. In contrast, female cultures showed an increased proportion of neurons in stage II (58.75%) than male cultures (34.00%). A similar proportion of cells in stage III were observed in male and female cultures.

Sex differences in the proportion of neurons in the different developmental stages of differentiation were accompanied by sex differences in neuritogenesis. For instance, the axonal length was significantly higher in female hypothalamic neurons at 2, 3, 4 and 5 DIV. The number of hypothalamic neurons with branched neurites was also higher in female neurons at 1, 2, 3, 4 and 5 DIV. Female hypothalamic neurons also had an increased dendritic length compared to male neurons (Scerbo *et al.*, 2014). Similar results were obtained in hippocampal cultures, where female neurons at 2 DIV showed an increased number of primary neurites, and increased axonal length and an increased complexity of the dendritic arbor, assessed by Sholl analysis (Ruiz-Palmero *et al.*, 2016) (Table 2).

Role of neurogenin 3 in the generation of sex differences in neuritogenesis

Neurogenin 3 (Ngn3) is a Notch regulated gene that is involved in neurite extension and remodeling in developing neurons (Ruiz-Palmero, Simon-Areces, Garcia-Segura, & Arevalo, 2011; Salama-Cohen, Arévalo, Grantyn, & Rodríguez-Tebar, 2006; Simon-Areces, Membrive, Garcia-Fernandez, Garcia-Segura, & Arevalo, 2010). Ngn3 expression in developing neurons is repressed by Hairy and Enhancer of Split (Hes) 1, which in turn is upregulated by Notch. The inhibition of Notch pathway results therefore in the downregulation of Hes 1, in increased expression of Ngn3 and in increased axonal and dendritic outgrowth (Ruiz-Palmero *et al.*, 2011; Salama-Cohen *et al.*, 2006). Overexpression of Ngn3 in E17 primary mouse hippocampal neurons increases neuritogenesis. In contrast, Ngn3 silencing in E17 primary hippocampal cultures results in decreased neuritogenesis in both male and female neurons (Ruiz-Palmero *et al.*, 2011, 2016; Salama-Cohen *et al.*, 2006) and abolishes sex difference in neuritogenesis (see below).

The expression of Ngn3 in primary hippocampal neurons presents sex differences (Ruiz-Palmero *et al.*, 2016). In hippocampal neurons obtained from E17 male embryos there is a peak in Ngn3 protein levels at 2 DIV. In contrast, in female neurons obtained from E17 embryos, Ngn3 protein levels are already elevated at 1 DIV. Thus, at 1 DIV Ngn3 mRNA and protein levels are higher in female than in male neurons (Table 2). This sex difference is also observed in the hippocampus at E17 *in vivo*, where Ngn3 protein levels are significantly higher in females than in males (Ruiz-Palmero *et al.*, 2016). This sex difference is transient, since at P0 and P1 the hippocampus of males and females had similar Ngn3 protein levels (Ruiz-Palmero *et al.*, 2016). Sex differences in the expression of Ngn3 have been also observed in cultures from mouse hypothalamus obtained from E14 embryos. As in the hippocampus, in these cultures female neurons also express higher levels of Ngn3 than male neurons (Scerbo *et al.*, 2014).

The different expression level of Ngn3 between male and female neurons seems to be involved in the generation of sex differences in neuronal development. Thus, the silencing of Ngn3 in male and female hippocampal cultures abolished the sex difference in the proportion of neurons in different stages of development (Ruiz-Palmero *et al.*, 2016). Ngn3 silencing also decreased the number of primary neurites and the complexity of the dendritic arbor in both sexes, abolishing the sex difference in the number of primary neurites (Table 2) and reducing the sex difference in dendritic arborization. Ngn3 silencing in hypothalamic cultures also abolished sex differences in neuronal differentiation and neuritogenesis (Scerbo *et al.*, 2014).

Role of sex chromosomes in the generation of sex differences in neurogenin 3 expression

Sex chromosomes determine some sexual dimorphisms in the brain. For instance, using the FCG model, Carruth et al (2002) observed that in dissociated mesencephalic cultures obtained from E14.5 mouse embryos, sex chromosomes

Table 2. Effects of exogenous hormones and endogenous estradiol synthesis on Ngn3 expression and neuritogenesis in male and female primary hippocampal neurons.

	Ngn3 expression	Neuritogenesis
Basal conditions	**C**F > **C**M	**C**F > **C**M
Ngn3 silencing	↓ in M & F **N**F = **N**M	↓ in M & F **N**F = **N**M
Estradiol	↑ in M; ↓ in F **E**F = **C**M; **C**F = **E**M	↑ in M **C**F = **E**M = **E**F
Letrozole (inhibion of estradiol synthesis)	↑ in M, ↓ in F **C**F = **L**M; **L**F = **C**M	↑ in M, ↓ in F **C**F = **L**M; **L**F = **C**M
DHT	↑ in M, ↓ in F **DHT**F = **C**M	↑ in M, ↓ in F **DHT**F = **C**M; **C**F = **DHT**M

↑, increase; ↓, decrease; M, male neurons; F, female neurons; **C**, Control neurons (basal conditions); **N**, Ngn3 silenced neurons; **E**, Estradiol treated neurons; **L**, Letrozole treated neurons; **DHT**, neurons treated with DHT. Based on Ruiz-Palmero *et al.* (2016).

determine the proportion of neurons expressing tyrosine hydroxylase. In addition, sex differences in calbindin expression in the Purkinje cells of the cerebellum depend on sex chromosomes, while sex differences in calbindin expression in the frontal cortex depend on an interaction between sex chromosomes and estrogen receptor (ER) α (Abel *et al.*, 2011).

The role of sex chromosomes in the generation of sex differences in Ngn3 expression between male and female neurons has been tested in hypothalamic neuronal cultures using the FCG model. The levels of Ngn3 were higher in the cultures obtained from XX embryos than in cultures obtained from XY embryos, with independence of the gonadal sex. Thus, neurons obtained from XX females and XX males showed higher Ngn3 mRNA levels than the neurons obtained from XY females and XY males (Scerbo *et al.*, 2014). This finding indicates that sex chromosomes generate the basal sex differences in Ngn3 expression in hypothalamic neurons.

Sex differences in the regulation of neurogenin 3 and neuronal development by estradiol

Although the basal sex differences in Ngn3 expression in hypothalamic neurons are determined by sex chromosomes, gonadal secretions may also regulate the expression of this neuritogenic factor. According to the classical aromatization hypothesis of brain sexual differentiation (MacLusky & Naftolin, 1981) testosterone produced by the developing testes is converted to estradiol within the brain by the enzyme aromatase. Then, estradiol produced within the brain, is the steroid that causes brain masculinization. Since the classical work of Toran-Allerand (1976), it is known that estradiol promotes neuritogenesis in different neuronal populations (Arevalo *et al.*, 2012; Beyer & Karolczak, 2000; Blanco, Diaz, Carrer, & Beaugé, 1990; Cambiasso, Díaz, Cáceres, & Carrer, 1995; Haraguchi *et al.*, 2012; Lorenzo *et al.*, 1992; Miñano *et al.*, 2008; Nathan, Barsukova, Shen, McAsey, & Struble, 2004). Several mechanisms are involved in the neuritogenic effect of estradiol, including the activation of PKA, MAPK and PI3K signaling (Arevalo *et al.*, 2012; Beyer & Karolczak, 2000; Cambiasso & Carrer, 2001; Gorosito & Cambiasso, 2008; Miñano *et al.*, 2008). In addition, estradiol may interact with other factors, such as brain derived neurotrophic factor and insulin-like growth factor-I to promote neuritogenesis (Arevalo *et al.*, 2012; Duenas, Torres-Aleman, Naftolin, & Garcia-Segura, 1996; Haraguchi *et al.*, 2012; Topalli & Etgen, 2004).

The effect of estradiol on Ngn3 expression and neuronal development was assessed in male and female hypothalamic cultures. Surprisingly, estradiol treatment increased Ngn3 expression and promoted neuritogenesis only in male neurons. In fact, estradiol increased Ngn3 and neuronal differentiation in male cultures to female levels, abolishing sex differences in Ngn3 expression and neuronal development (Scerbo *et al.*, 2014). Similar findings were obtained in hippocampal cultures, where estradiol treatment increased Ngn3 expression and neuritogenesis only in male neurons (Table 2). As observed in hypothalamic cultures, estradiol increased the differentiation of male neurons to the levels of female neurons (Ruiz-Palmero *et al.*, 2016).

Role of neuronal aromatase and steroid receptors in the generation of sex differences in neuritogenesis

Mouse hippocampal neurons *in vitro* express the enzyme aromatase, which converts testosterone into estradiol (von Schassen *et al.*, 2006). The role of aromatase on the generation of sex differences in neuronal development was assessed using letrozole, a selective inhibitor of the enzymatic activity (Ruiz-Palmero *et al.*, 2016). Treatment of male and female hippocampal cultures with letrozole resulted in a significant increase in Ngn3 expression and neuronal development in male cultures and in a significant decrease in Ngn3 expression and neuronal development in female cultures (Ruiz-Palmero *et al.*, 2016). In fact, aromatase inhibition abolished sex differences in neuronal development. Thus, control female neurons had a similar morphology than male neurons treated with letrozole and control male neurons had a similar morphology than female neurons treated with letrozole (Table 2). The effect of aromatase inhibition on female neurons was reverted when the cultures were treated with estradiol (Ruiz-Palmero *et al.*, 2016). These findings suggest that estradiol produced by female neurons is the cause of its increased expression of Ngn3 and its increased development, compared to males. The effect of aromatase inhibition in male neurons, which had the opposite effect than in female neurons, can be due to the accumulation of testosterone, which could be converted to DHT, which increased Ngn3 expression and neuronal development in male neurons (Ruiz-Palmero *et al.*, 2016). Interestingly, DHT decreased Ngn3 expression and neuritogenesis in female neurons to control male levels (Table 2), in agreement with the observation that DHT participates in the masculinization or defeminization of the brain in androgenized females (Foecking, Szabo, Schwartz, & Levine, 2005; Resko & Roselli, 1997; Thornton, Zehr, & Loose, 2009; Wu *et al.*, 2010).

Although estradiol synthesized by the enzyme aromatase in female neurons was the cause of their increased Ngn3 expression and neuritogenesis, the levels of estradiol were similar in male and female cultures. In addition, the expression of aromatase was higher in male neurons than in female neurons (Ruiz-Palmero *et al.*, 2016). This suggests that the difference in Ngn3 expression and neuritogenesis was not due to an increased production of estradiol by female neurons. In contrast, the expression of ERα, ERβ and G protein-coupled ER was higher in female neurons than in male neurons, while the expression of androgen receptor was higher in male neurons (Ruiz-Palmero *et al.*, 2016). This suggests that sex differences in estrogen and androgen signaling are the cause of the sex differences in the expression of Ngn3 and neuritogenesis.

Sex differences in the expression of aromatase and steroid receptors may be the consequence of epigenetic modifications in their promoters (Forger 2016, 2017; McCarthy, Nugent, & Lenz, 2017; Mosley *et al.*, 2017; Nugent *et al.*, 2011, 2015) and microRNA (miRNA) regulation (Morgan &

Bale, 2017). Interestingly, recent findings indicate that miRNA expression profile in the adipose tissue is regulated both by gonadal sex and sex chromosome complement (Link *et al.*, 2017). Further studies are needed to determine the role of epigenetic modifications in the generation of sex differences in Ngn3 expression and neuritogenesis.

Proposed model for the interaction of sex chromosomes, neuronal derived estradiol and sex steroids on the generation of sex differences in neuritogenesis

The studies reviewed here indicate that sex differences in neuritogenesis are the result of an interaction of cell autonomous actions of sex chromosomes with the production of estradiol by female neurons. The studies with the FCG model suggest that either genes located in the X chromosome, which escape X inactivation and are expressed higher in XX, cause an increased expression of Ngn3 in female neurons or genes located in the Y chromosome (absence in XX) induce a downregulation of Ngn3 in male neurons. However, the effect seems to be mediated by a sex difference in endogenous estradiol signaling and not by a direct regulation of Ngn3 by sex chromosome genes (Figure 2). In this regard, it is of interest to note that in the anterior amygdala of the mouse developing brain, sex chromosome complement determines the levels of expression of ERβ. XX animals, either male or female, had lower expression levels of ERβ than XY females and XY males (Cisternas, Cabrera Zapata, Arevalo, Garcia-Segura, & Cambiasso, 2017). Therefore, it is conceivable that sex chromosome genes may also determine the levels of expression of steroid receptors in hippocampal and hypothalamic developing neurons, allowing or preventing the neuritogenic action of neuronal derived estradiol. Thus, in XX neurons, with higher expression of estrogen receptors, the local production of estradiol upregulates Ngn3 and this will induce increased neuritogenesis (Figure 2(A)). In contrast, in XY neurons, with lower expression levels of estrogen receptors, endogenous estradiol is not able to upregulate Ngn3 and their neuritogenesis is therefore decreased compared to female neurons (Figure 2(B)).

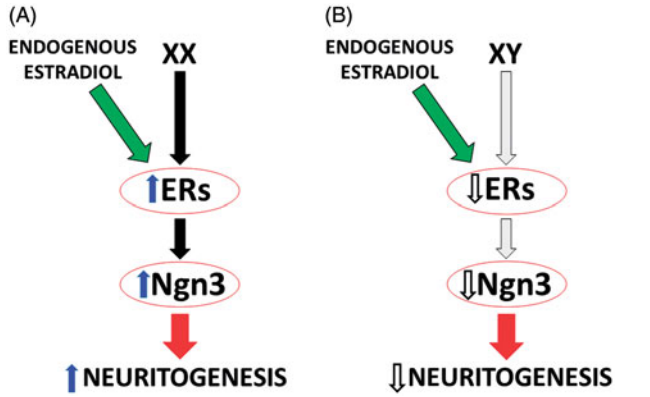

Figure 2. Proposed model for the interaction of sex chromosomes and neuronal derived estradiol on the generation of basal sex differences in neuritogenesis. In XX neurons (A), neuronal derived estradiol (endogenous estradiol) induces the expression of the neuritogenic factor neurogenin 3 (Ngn3), which in turn induces neuritogenesis. In contrast, in XY neurons (B), with lower expression of estrogen receptors, endogenous estradiol is not able to induce the expression of Ngn3. Therefore, XY neurons have a decreased neuritogenic activity compared to XX neurons.

The effect of endogenous estradiol on female neurons, increasing their expression of Ngn3, may be the cause of its lack of response to exogenous estradiol. Thus, endogenous estradiol in female neurons may have a ceiling effect on Ngn3 expression, not allowing further regulation by the exogenous hormone. In contrast, male neurons are able to respond to exogenous testosterone, which after its local conversion in estradiol and DHT increases Ngn3 expression and neuritogenesis (Ruiz-Palmero *et al.*, 2016). Thus, testosterone produced by the fetal testes contributes to the regulation of neuronal development in males, while neuronal synthesized estradiol regulates neuronal development in females.

The transient sex difference in neuritogenesis may cause the generation of permanent sex differences in neuronal connectivity, since a different speed in the development of male and female neurons may generate a different matching between developing presynaptic and postsynaptic inputs (Ruiz-Palmero *et al.*, 2016; Scerbo *et al.*, 2014). Alternatively, the sex difference in basal neuritogenesis may represent a compensatory mechanism, as proposed by De Vries (2004), to prevent the generation of permanent sex differences caused by the fetal peak of testosterone in males. Thus, estradiol synthesized by female neurons would promote their development to match with the development induced in male neurons by gonadal testosterone. Further research is needed to test these alternative hypotheses.

Concluding remarks

Differences between males and females in brain structure and function are due to differences in sex chromosome complement of neurons, as well as differences in exposure to sexual steroids derived from the gonads or from *de novo* synthesis within the brain (as neurosteroids) from cholesterol. Estradiol availability to XX or XY brain cells affects differentially the growth and developmental pattern of neurons arranging the sex specific synaptic connections and their functional profile. This complex process requires the participation of specific receptors for estrogens as well as androgen, which may be also under the regulation of sex chromosome genes. Both, genetic and hormonal factors interact to produce additive, potentiating or interdependently complementary effects on growth and differentiation. The challenge of the coming studies is the identification of specific X and Y genes implicated in the regulation of neuritogenesis.

Disclosure statement

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