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Sex chromosome complement determines sex differences in aromatase expression and regulation in the stria terminalis and anterior amygdala of the developing mouse brain

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ABSTRACT

Aromatase, which converts testosterone in estradiol, is involved in the generation of brain sex dimorphisms. Here we used the "four core genotypes" mouse model, in which the effect of gonadal sex and sex chromosome complement is dissociated, to determine if sex chromosomes influence the expression of brain aromatase. The brain of 16 days old XY mouse embryos showed higher aromatase expression in the stria terminalis and the anterior amygdaloid area than the brain of XX embryos, independent of gonadal sex. Furthermore, estradiol or dihydrotestosterone increased aromatase expression in cultures of anterior amygdala neurons derived from XX embryos, but not in those derived from XY embryos. This effect was also independent of gonadal sex. The expression of other steroidogenic molecules, estrogen receptor-α and androgen receptor was not influenced by sex chromosomes. In conclusion, sex chromosomes determine sex dimorphisms in aromatase expression and regulation in the developing mouse brain.

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1. Introduction

The classical hypothesis for the sexual differentiation of the rodent brain postulates that the fetal testis produces testosterone at critical moments of development and then, this hormone can act as an organizing agent after its conversion to 17β-estradiol (E2) by the enzyme cytochrome P450 aromatase (MacLusky and Naftolin, 1981). The expression pattern of aromatase is restricted to discrete regions of the central nervous system, according to in situ hybridization and immunohistochemical studies (Lauber et al., 1997; Shinoda et al., 1994; Tsuruo et al., 1994). Several studies demonstrated that during the critical period of sexual differentiation there are sex differences in aromatase expression that are time- and regionally specific (Lauber et al., 1997). Most of the studies focused on the expression in sexually dimorphic brain areas, such as the hypothalamus and the preoptic area. During brain differentiation aromatase mRNA in the hypothalamus increases gradually to reach peaks shortly before and after birth in rats (Colciago et al., 2005; Lephart et al., 1992) and mice (Harada and Yamada, 1992). In the bed nucleus of the stria terminalis and the sexually dimorphic nucleus of the preoptic area some sex differences were found with a higher expression of aromatase mRNA in male rats at P2; and later in development, at P6, the sex differences only remained in the bed nucleus of the stria terminalis (Lauber et al., 1997). Some of the sex differences in aromatase expression could not be explained by organizational actions of gonadal hormones. For instance, a higher expression and activity of aromatase was observed in neurons and hypothalamic regions in male mouse and rat brain at E16 (Beyer et al., 1994b, 1993; Colciago et al., 2005; Negri-Cesi et al., 2001). These differences can not be attributed to

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Abbreviations

AR	androgen receptor
Cyp11a1	cytochrome P450, family 11, subfamily a, polypeptide 1; also known as P450scc
Cyp19a1	cytochrome P450, family 19, subfamily a, polypeptide 1; also known as aromatase
DHT	dihydrotestosterone
E2	17β-estradiol
ER	estrogen receptor
ER-α	ER alpha
Esr1	estrogen receptor 1; also known as ER-α
FCG	four core genotypes
Gapdh	glyceraldehyde-3-phosphate dehydrogenase
P450scc	cholesterol side-chain cleavage enzyme
Srd5a1	steroid 5 alpha-reductase 1
Srd5a2	steroid 5 alpha-reductase 2
StAR	steroidogenic acute regulatory protein

the peak of testosterone production by the fetal testis, which in mice is at E17-18 (O'Shaughnessy et al., 2006, 1998) and in rats at E18.5–19.5 (Huhtaniemi, 1994; Scott et al., 2009). However, it is not possible to complete exclude any effect of hormones derived from the gonads at or before the embryonic age used.

Testosterone can also be metabolized to the most potent androgen dihydrotestosterone (DHT) by the enzyme 5α-reductase. Two different 5α-reductase isoenzymes, type I and II, catalyze the conversion of testosterone into DHT (Celotti et al., 1997, 1992). The mRNA for the 5α-reductase type I isoenzyme is constitutively expressed in the rat brain at all stages of development, whereas the mRNA for the 5α-reductase type II isoenzyme is detected at E18, peaks at P2 and then decreases gradually to low levels in adults (Poletti et al., 1998). DHT synthesized in the brain also exerts organizational actions on selected nuclei and it is involved in sexual differentiation of specific brain regions and behaviors (Bodo and Rissman, 2008). In this context, brain masculinization can be exerted via estrogen receptors (ERs) in some brain regions and via the formation of DHT and the activation of androgen receptor (AR) in others.

ERs and AR are critical for the organizational actions of steroids during brain development and their expression overlaps with the expression of aromatase (Simerly et al., 1990). AR mRNA is detected in the hypothalamus and preoptic area of mouse brain from E14 and its expression is sexually dimorphic between E15–E16 (Young and Chang, 1998). ER-α mRNA is detected from E18 in the striohypothalamic nucleus, the caudal portion of the ventromedial hypothalamic nucleus, the bed nucleus of the stria terminalis, the caudal arcuate nucleus and the medial and cortical nuclei of the amygdala in male and female rats (DonCarlos, 1996). In addition, the expression of ER-α and ER-β in subregions of the amygdala, including the posterodorsal part of the medial amygdaloid nucleus, the posterior cortical nucleus, and the amygdalohippocampal area, has been reported in early postnatal rats (Cao and Patisaul, 2013).

The vast bibliography regarding sex dimorphic expression and regulation of aromatase in the brain is based on studies in the hypothalamus and the preoptic area (Beyer et al., 1994b; Hutchinson et al., 1997, 1999; Negri-Cesi et al., 2001) and very little is known about factors that control aromatase expression during development in other brain regions with high aromatase levels, such as stria terminalis and amygdala regions. The regions of interest for the present study were the medial preopticoamygdaloid neuronal arc that involves areas known to be sexually dimorphic and sensitive to organizational actions of steroid hormones (Forger et al., 2004; Morris et al., 2008). Taking into account that recent findings suggest that in addition of gonadal hormones, the sex chromosome complement is also involved in the generation of specific traits in the brain (Arnold, 2009; Scerbo et al., 2014) in this study we have tested the hypothesis that sex chromosome complement influences the expression of aromatase in the stria terminalis and amygdala regions. With this aim, the study was carried out in E15-16 mouse embryos (i.e., before the testosterone surge) of the "four core genotypes" (FCG) model, which combines a deletion of the testis-determining gene Sry from the Y chromosome (Y^-) with the subsequent insertion of a Sry transgene onto an autosome (Lovell-Badge and Robertson, 1990; Mahadevaiah et al., 1998). The Sry gene deletion in XY mice (XY^-) yields in a female phenotype (ovaries). When the Sry transgene is inserted into an autosome of these mice they have testes and are fully fertile (XY^-*Sry*). The Y^- chromosome and the Sry transgene segregates independently, thus, four types of offspring are produced by breeding XY^-*Sry* males to XX females: XX and XY^- females (without Sry on the Y chromosome) and XX*Sry* and XY^-*Sry* male mice (both with *Sry* in an autosome). By comparing these genotypes, it is feasible to segregate the role of a) sex chromosome complement (comparing mice with the same gonadal type but with different sex chromosomes: XX *vs.* XY) b) gonadal phenotype (males *vs.* females regardless of the sex chromosome complement) and c) their interaction (Arnold and Chen, 2009). Throughout the text, we will refer to XX and XY^- as XX and XY females (XXF and XYF), and to XXSry and XY^-Sry as XX and XY male (XXM and XYM), respectively. The study was complemented with the analysis of other steroidogenic molecules and steroid receptors in the same brain regions. Thus, we also assessed the expression of ER-α, AR, 5α-reductase, steroidogenic acute regulatory protein (StAR) and cholesterol side-chain cleavage enzyme (P450scc or *Cyp11a1*). To determine the impact of sex chromosome complement on the regulation of aromatase by E2 and DHT we have used primary cultures of amygdala neurons obtained from embryos of the FCG mouse model.

2. Materials and methods

2.1. Animals

The embryos used for this study were obtained from MF1 "four core genotypes" mice born and reared in the Instituto Ferreyra (Córdoba, Argentina). The day of vaginal plug was defined as E1. All experimental protocols were approved by the appropriate animal care and use committees at our institute and followed the National Institutes of Health guidelines for the care and use of laboratory animals.

2.2. Genotyping

Genotyping of FCG was performed on genomic DNA samples of E15-16 mouse embryos separated by sex. Male fetuses were identified under a dissecting microscope by the presence of the spermatic artery on the developing gonad. PCR was performed for the *Sry* transgene [primers SryF (forward): CTA CAC AGA GAG AAA TAC CCA AAC; SryR (reverse): GTC TTG CCT GTA TGT GAT GG] (Gubbay et al., 1990) and the Y long-arm gene family Ssty [primers SstyF (forward): CTG GAG CTC TAC AGT GAT GA; SstyR (reverse): CAG TTA CCA ATC AAC ACA TCA C] (Turner et al., 2000). The autosomal gene Myogenin [primers MYOF (forward): TTA CGT CCA TCG TGG ACA GCA T; MYOR (reverse): TGG GCT GGG TGT TAG TCT TAT] served as an amplification control (Palaszynski et al., 2005) yielding a 245-bp product in all genotypes. Amplification of DNA yielded the following products according to the genotypes: for XY males (XYM)

the 159-bp Sry and the 302-bp Ssty; for XY females (XYF) the 302-bp Ssty; for XX males (XXM) the 159-bp Sry; meanwhile in XX females (XXF) only the myogenin control product was amplified.

2.3. Immunohistochemistry of brain sections

Brains were obtained from E16 mouse embryos. Dissected brains were fixed by immersion for 48 h in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) and cryoprotected with 30% sucrose in PB at 4 °C.

2.3.1. Antibody specificity testing

In this study we used a rabbit polyclonal anti-aromatase antibody generated against a 15 amino acid peptide corresponding to residues 488–502 of mouse aromatase, a region homologous to human and monkey aromatase (Beyer et al., 1994a). Specificity of this antibody has been previously described and cross reacts with rat, human and monkey cytochrome P450 aromatase (Beyer et al., 1994a; Garcia-Segura et al., 1999; Yague et al., 2010, 2008). In order to corroborate the specificity of this antibody we performed one series of immunohistochemical experiment with another anti-aromatase rabbit polyclonal antibody, (Santa Cruz Biotechnology Inc., sc-30086) which recognizes an epitope that corresponds to aminoacids 209–503 mapping at the C-terminus of CYP19 of human origin. The two anti-aromatase antibodies gave the same staining pattern in wild-type E16 brain. Figures show the results obtained with the first aromatase antibody. Immunostaining was absent when the aromatase antibody was omitted.

2.3.2. Single-labeling procedure

Peroxidase immunohistochemistry was performed on cryostat coronal sections (40 μm thick). Sections were treated for 45 min with a solution of ethanol (50%) and hydrogen peroxide (5%) in PB to quench endogenous peroxidase activity. For single immunohistochemistry, sections were then incubated with a rabbit polyclonal anti-aromatase antibody (1/1000 dilution) for 48 h at 4 °C.

The primary antibody was diluted 1:1.000 in PB with 0.3% Triton X-100 (PBT, 0.3% bovine serum albumin and 5% normal horse serum). After incubation with the primary antibody, sections were washed in PB and incubated, for 1 h at room temperature, in biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch; diluted 1:200 in normal horse serum 1%). Sections were then processed using the Vectastain ABC immunoperoxidase kit (Vector, Burlingame, CA) and the antibody distribution was detected histochemically with 0.05% 3,3′-diaminobenzidine tetrahydrochloride as a chromogen (DAB; Sigma, St. Louis, MO) and 0.01% hydrogen peroxide. The sections were cleared with xylene and coverslipped.

2.3.3. Double-labeling procedures

Double immunohistochemical staining was performed to colocalize aromatase with a neuronal marker. Coronal tissue sections (20 μm thick) were incubated for 48 h at 4 °C with the primary antibodies in PBT containing 5% normal horse serum. The aromatase antibody was combined with a mouse monoclonal antibody against the neuronal marker NeuN (MAB 377, Chemicon, Temecula, CA, and diluted 1:1000). Tissue sections were subsequently washed in PB and incubated for 1 h at room temperature with donkey anti-rabbit IgG conjugated with green Alexa (488 nm) and donkey anti-mouse IgG conjugated with red Alexa (564 nm) (Invitrogen; diluted 1:450 in PBT).

2.4. Image analysis

The regional distribution of aromatase immunoreactivity was evaluated in E16 brain sections of the FCG mice. Representative sections were selected for quantifications according to previous results (Shinoda et al., 1994; Zhao et al., 2007). The brain nuclei exhibiting aromatase immunoreactivity were identified and delimited according to the prenatal mouse brain atlas of U.B. Schambra (Schambra, 2008). The relative amount of aromatase immunoreactivity was measured in four areas: the stria terminalis, the anterior amygdaloid area (GD 16 Coronal 15 of the prenatal mouse brain atlas) and the medial and central nuclei of the amygdala (GD 16 Coronal 18 of the prenatal mouse brain atlas). Coronal slices containing the stria terminalis and the anterior amygdaloid area were identified according to the Coronal Plane 15 GD16 using the lateral ventricles and the internal capsule as landmarks. Caudal coronal slices containing the medial and central nuclei of the amygdala were identified according to the Coronal Plane 18 GD16 (Schambra, 2008) using the optic tract as landmark. Aromatase immunoreactivity was quantified by an experimenter who was blind to the sex/genotype of the mice using computer-assisted image analysis. Sections were digitized through a computerized system that included a Zeiss microscope equipped with a DC 200 Leica digital camera attached to a contrast enhancement device. Images were digitalized and analyzed using ImageJ PC software (version 1.42q). Aromatase immunoreactivity was assessed with an X40 objective in one entire field (area: 0.035 mm^2) placed in a standardized manner, based on predefined anatomical landmarks, within the neuroanatomical region of interest. The amount of aromatase immunoreactivity was determined by measuring the percentage of the area covered by thresholded pixels [those pixels with a gray level higher than a defined threshold density (specific immunoreactive staining)]. For each section threshold was defined as the mean optical density five times the standard deviation higher than the mean background density (Brock et al., 2010; Wagner et al., 2004). The mean background density was measured in a region devoid of specific staining, immediately lateral to the analyzed region containing aromatase immunoreactivity. We measured the relative amount of aromatase immunoreactivity in three consecutive coronal sections for each animal.

2.5. Micropunch technique

Dissected brains of E16 mice embryos were immediately frozen with isopentane at −20 °C and were kept at −80 °C until determination of genotype by PCR. Tissues were micropunched from slices of 280 μm in thickness prepared at −25 °C using a cryostat (Leica). Punches of 0.838 mm of inner diameter were performed with an 18G needle and were obtained from the stria terminalis, the anterior amygdaloid area and the amygdala (a single punch containing the medial and central nuclei of the amygdala). Coronal slices containing the stria terminalis and the anterior amygdaloid area (GD16 Coronal 15; Schambra, 2008) were identified using the lateral ventricles and the internal capsule as landmarks. Caudal coronal slices containing the medial and central nuclei of the amygdala (GD16 Coronal 18; Schambra, 2008) were identified using the optic tract as landmark. For the stria terminalis and the anterior amygdaloid area, unilateral punches were alternately collected from the right or left hemispheres of the same coronal section. Caudal coronal slices containing the medial and central nuclei of the amygdala were punched bilaterally. Samples were punched carefully by holding the punch firmly; the needle syringe was positioned at a right angle above the slice and pushed downward into the brain section, until the needle made contact with the slide. Punches were lifted away slowly from the section along with the brain samples retrieved in the cutting needle. The complete process was carried out inside the cryostat. Temperature was maintained at −25 °C to minimize RNA degradation.

2.6. Primary amygdala neuronal cultures and treatments

Amygdala neurons were obtained from E15 mouse embryos. Cells were cultured separately according to the sex and genotype of fetal donors. The brain was dissected out and the meninges were removed. The anterior amygdala region was transversally sliced between the optic chiasma and the mammilary bodies. The dissected block of tissue mainly contained the anterior amygdaloid area and neighboring piriform cortex from each hemisphere. The tissues were dissociated to single cells after digestion for 15 min at 37 °C with 0.5% trypsin (Invitrogen) and washed in Ca^{2+}/Mg^{2+}-free Hank's Buffered Salt Solution. Neurons were cultured in phenol red-free Neurobasal supplemented with B-27, N-2 and GlutaMAX I (Invitrogen). Cells were plated on 35 × 10 mm cell culture dishes, coated with poly-L-lysine (1 mg/ml, Sigma–Aldrich), at a density of 800 neurons/mm^2. After 3 days *in vitro* (DIV) the medium was replaced for 2 h by fresh medium devoid of supplements. Then, some cultures were incubated for 2 h with E2 (10^{-10} M), DHT (10^{-10} M) or vehicle.

2.7. RNA purification and qRT-PCR

Total RNA was extracted from cell cultures and from micropunches with TRIZOL reagent (Invitrogen) at 4 °C. Total RNA was purified according to manufacturer protocol and quantified by spectrophotometry (NanoDrop; Thermo Scientific) at 260 nm. RNA purity was evaluated with A260/280. The integrity of the isolated RNA was verified by running 7 µl of total RNA on a 2% agarose gel. Total RNA were treated with DNAseI (Fermentas) and 1.5 µg of RNA were reverse-transcribed in a 20 µl reaction using SuperScript II reverse transcriptase (Invitrogen) and random hexameres (Biodynamics). For quantitative real-time PCR (qPCR) primer sets were designed for the specific amplification of murine *Cyp19a1*, *Ar*, *Esr1*, *Srd5a1*, *Srd5a2*, *Cyp11a1*, *Star* and *Gapdh* as a reference gene (Table 1).

Each sample was assayed in duplicate using 200–800 nM of each primer, 2X POWER SYBR Green Master Mix (Applied Biosystems) and 100 ng of cDNA in a total volume of 20 µl. Amplification was carried out in a Step OneTM Real Time PCR System (Applied Biosystems). The PCR amplification efficiency was established by means of 5-point 1:3 calibration curves. The selected dilution for the samples was 1:3 from the initial RT PCR concentration. The Ct values of the samples (tissue and cell cultures) fell into the linear dynamic range of the calibration curve; for example in the case of *Cyp19a1* mRNA expression the range of Ct amplification was 31.5–26 (highest to lowest dilution of the calibration curve) with an average Ct = 29. All primer-pairs were verified to be 90–110% efficient by means of the calibration curve with efficiencies $E = 2 \pm 0.1$ ($E = 10^{[-1/slope]}$) and amplified a single product determined by melting curve analysis. Relative quantifications of mRNA levels were measured by the ΔΔCt method with StepOne Software v2.2 except data for *Cyp19a1* and *Cyp11a1* (E = 2.15 and 2.16 respectively) that were analyzed according to Pfaffl (2001). An expression ratio was determined for each sample by calculating a ratio $= (E^{target})^{\Delta Ct(target)}/(E^{Gapdh})^{\Delta Ct(Gapdh)}$, where E is the efficiency of the primer set and $\Delta Ct = Ct_{(control)} - Ct_{(experimental)}$. Relative quantifications of the target gene were normalized to wild-type MF1 male mice. The $2^{-\Delta CT}$ method was used to determine the expression of *Gapdh* between genotypes (Livak and Schmittgen, 2001). The relative amounts of *Gapdh* were calculated using $2^{-\Delta CT}$ equation where ΔCT = CT treated − CT control (Livak and Schmittgen, 2001). No significant relationships were found between the chromosome complement and the expression of *Gapdh* ($p > 0.05$).

2.8. Statistical analysis

The percentages of covered area by aromatase immunoreactivity were transformed to log10 before the statistical analysis. Statistical significance was assessed by two-way (sex chromosome complement × gonadal sex) or three-way (sex chromosome complement × gonadal sex × hormonal treatment) ANOVA followed by comparison of means by Fisher's least significance difference (LSD) post-hoc. A level of $p < 0.05$ was considered as statistically significant. The n for statistical analysis was the number of embryos or the number of independent cultures.

3. Results

3.1. Aromatase immunoreactivity in the brain of mouse embryos at E16

Aromatase immunoreactivity was evaluated in the brain of male mouse embryos at E16. Aromatase-positive cells were identified as neurons, since aromatase-immunoreactivity colocalized with the neuronal marker NeuN. Immunoreactivity was restricted to neuronal perikarya and fibers. Neuronal nuclei were never immunostained (Fig. 1).

Aromatase immunoreactivity was localized in discrete brain regions, including the paraventricular hypothalamic nucleus, the

Table 1
Primer sequences and conditions used for gene expression assays.

Gene	Genbank	5′–3′ sequence	T Annealing (°C)	Primer concentration (nM)	Primer efficiency	Product (bp)	Cycle numbers (standard curve)
Cyp19a1	NM_007810.3	F: CGGGCTACGTGGATGTGTT R: GAGCTTGCCAGGCGTTAAAG	60	800	115	135	26–31.5
Esr1	NM_007956.4	F: ATGAAAGGCGGCATACGGAAAG R: CACCCATTTCATTTCGGCCTTC	60	200	97	94	30–34
Ar	NM_013476.3	F: GGCGGTCCTTCACTAATGTCAACT R: GAGACTTGTGCATGCGGTACTCAT	59	800	102	84	29–33
Cyp11a1	NM_019779.3	F: GCTGGGCACTTTGGAGTCA R: TGAGAAGAGTATCGACGCATCCT	57	200	116	61	30–35
Star	NM_011485.4	F: GCTGGAAGTCCCTCCAAGACT R: GCCACCCCTTCAGGTCAATA	57	800	101	61	29–35.5
Srd5a1	NM_175283.3	F: GAGGCAGCATCATCAGTGGTAC R: CACCGGGAAGACAGACACTCA	58	800	107	134	22–28
Srd5a2	NM_053188.2	F: ACAGACATGCGGTTTAGCGT R: AACAAGCCACCTTGTGGGAT	60	300	101	134	29–34.5
Gapdh	NM_008084	F: AGTGCCAGCCTCGTCCCGTAG R: GTGCCGTTGAATTTGCCGTGAGTG	62	300	100	196	25–31

stria terminalis and the anterior amygdala area (Fig. 2). These aromatase immunoreactive neuronal groups were observed to continue caudally with other neuronal populations in the medial and central amygdala. Such aromatase immunoreactive neurons in the medial amygdala formed a densely packed cluster of cells and correspond to the posterodorsal part of the medial amygdala observed previously to be aromatase immunoreactive (Shinoda et al., 1994). Collectively, aromatase immunoreactive cells form the preoptico-amygdaloid neuronal group along the medial side of the medial postcommissural arc of the stria terminalis that was described by Shinoda et al. (1994) in the embryonic rat brain.

There was no significant difference in the area covered by aromatase immunoreactivity between wild-type MF1 males and XY^{-}*Sry* (XYM) mice (data not shown).

3.2. Role of sex chromosome factors in the expression of aromatase in the brain of mouse embryos at E16

The existence of a sex dimorphism in aromatase immunoreactivity in the brain of mouse embryos at E16, before the peak of testosterone production by the fetal testis, at E17-18 (O'Shaughnessy et al., 2006, 1998), suggest a possible effect of sex chromosomes in the regulation of aromatase expression. To assess this possibility we analyzed the mRNA levels of *Cyp19a1* and aromatase immunoreactivity in the brain of FCG mice at E16. Given that high levels of aromatase immunoreactivity were detected in the stria terminalis and in amygdaloid regions, these brain areas were selected to determine the role of sex chromosome complement.

Two-way ANOVA of *Cyp19a1* mRNA expression revealed no effect of gonadal sex but a significant main effect of sex chromosome complement in the stria terminalis ($F_{1,8} = 6.40$; $p = 0.035$) and in the anterior amygdaloid area ($F_{1,13} = 7.30$; $p = 0.019$). Individuals carrying the XY chromosome complement showed higher expression levels of *Cyp19a1* in the stria terminalis and the anterior amygdaloid area than individuals carrying XX chromosomes ($p < 0.05$ in both areas), independently of gonadal status (testes *vs*. ovary) (Fig. 3A). There was no interaction of gonadal sex and chromosome complement.

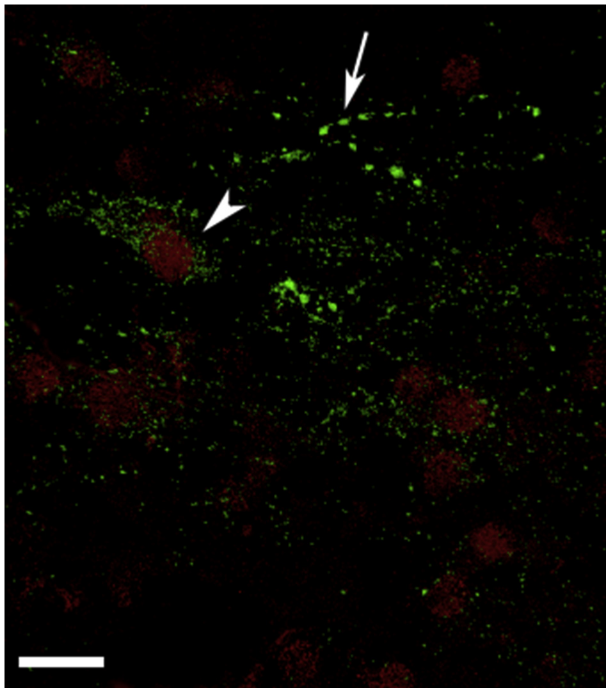

Fig. 1. Confocal laser scanning microscope image demonstrating co-localization of aromatase (green) and NeuN (red) in developing brain of XY male mouse. The image shows aromatase immunostaining both in the perikaryon (arrowhead) as well as in neuronal fibers (arrow). Scale bar: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In order to compare aromatase mRNA expression and protein levels, we quantified aromatase immunoreactivity in the same brain regions in which *Cyp19a1* mRNA was analyzed. The statistical analysis of aromatase immunoreactivity showed a significant effect due to sex chromosome complement in the stria terminalis ($F_{1,11} = 10.50$, $p = 0.008$) and the anterior amygdaloid area ($F_{1,11} = 12.52$, $p = 0.005$). As it was observed for *Cyp19a1* mRNA levels, XY individuals showed higher levels of aromatase immunoreactivity than XX individuals in these brain regions ($p < 0.05$). There was no effect of gonadal sex or interaction of gonadal sex and sex chromosome complement (Fig. 3B).

Neither chromosome complement nor gonadal sex affected *Cyp19a1* mRNA and aromatase protein levels in the medial and central nuclei of the amygdala (Fig. 3).

3.3. Estradiol and dihydrotestosterone compensate sex chromosome-induced sex differences in Cyp19a1 expression

Our previous findings *in vivo* indicate that aromatase expression in the anterior amygdaloid area at E16 was higher in XY embryos than in XX embryos, with independence of the gonadal sex. *Cyp19a1* expression in amygdala neuronal cultures derived from E15 XY embryos showed also higher *Cyp19a1* expression than the cultures derived from XX animals (Fig. 4A–B). Therefore, we used these cultures to evaluate whether the differential *Cyp19a1* expression between genotypes could be regulated by gonadal hormones. With this aim, we treated primary amygdala neuronal cultures with 10^{-10} M E2 or 10^{-10} M DHT. Three-way ANOVA in E2 treated cultures revealed a significant effect of treatment ($F_{1,32} = 4.92$; $p = 0.03$) and due to the interaction of hormone treatment and genotype (E2 × genotype: $F_{1,32} = 7.30$; $p = 0.011$). Three-way ANOVA in DHT treated cultures revealed a significant effect of the interaction of hormone treatment and genotype (DHT × genotype: $F_{1,25} = 10.18$; $p = 0.003$). E2 treatment resulted in a significant increment in *Cyp19a1* expression in XX cultures independently of gonadal sex (XXM and XXF; $p < 0.01$; Fig. 4A). On the contrary, E2 treatment did not significantly affect aromatase mRNA levels in XY cultures (XYM and XYF; Fig. 4A). In a similar way, DHT treatment increased aromatase mRNA levels in XX cultures ($p < 0.01$) but not in XY cultures ($p < 0.05$; Fig. 4B). Thus, the effect of E2 or DHT in XX amygdala neuronal cultures abolished the sex chromosome effect in *Cyp19a1* expression: XX cultures treated with E2 or DHT showed similar aromatase mRNA levels than XY cultures under basal conditions.

3.4. Expression of key steroidogenic enzymes in aromatase positive regions in the brain of E16 mouse embryos

3.4.1. Expression of Star and Cyp11a1 mRNA in aromatase positive regions

Having determined the influence of sex chromosome factors on the expression of aromatase in the brain of E16 mouse embryos, we decided to examine whether these factors also affect the expression of other steroidogenic molecules located upstream of aromatase in the steroidogenic pathway. Therefore we evaluated the hypothesis that aromatase positive regions have the molecular machinery to synthesize neurosteroids from cholesterol and that the expression of this machinery was regulated by sex chromosome factors. *Star* and *Cyp11a1* levels were detectable by qPCR in all analyzed brain

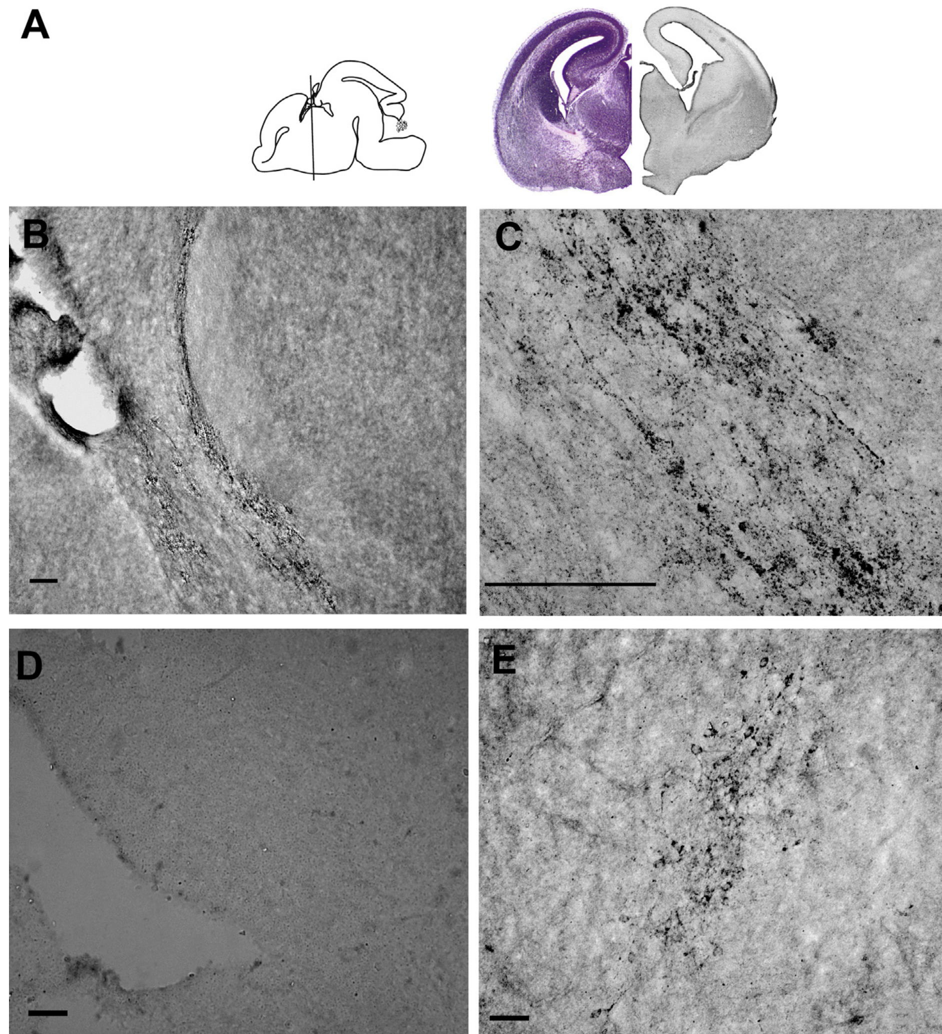

Fig. 2. Aromatase immunoreactivity pattern in the medial postcommissural arc of the stria terminalis at E16. A) Schematic drawing of embryonic brain showing the level at which coronal sections were selected with a color image of a coronal section stained with hematoxylin based upon the atlas of Schambra (2008) and a coronal section stained with antiaromatase antibody showing the region where quantification was carried out. B) Immunoreactive neurons present in the stria terminalis of XY male brain. C) A higher magnification of the immunoreactivity showed in B. D) The same immunohistochemical procedure followed in B omitting the primary antibody. E) Immunoreactive neurons present in the anterior amygdaloid area of XY male brain. Scale bar: 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

regions. The statistical analysis of gene expression did not show a significant effect of neither gonadal sex nor sex chromosome complement factors (Fig. 5).

3.4.2. 5α-Reductase mRNA expression in aromatase positive regions

Since the 5 alpha-reduction of testosterone to DHT is involved in brain masculinization we also analyzed whether the expression of 5α-R Type I (*Srd5a1*) and Type II (*Srd5a2*) was influenced by sex chromosome factors as it was the case for aromatase.

The statistical analysis did not reveal a significant effect of neither gonadal sex nor sex chromosome complement factors in the mRNA levels of 5α-R Type I (*Srd5a1*) and Type II (*Srd5a2*) in the stria terminalis, the anterior amygdaloid area and the amygdala, demonstrating that in these brain areas neither gonadal status nor sex chromosome factors modulated mRNA levels (Fig. 6A–B).

3.5. Expression of estrogen- and androgen-receptors in aromatase positive regions in the brain of E16 mouse embryos

It is known that ER and AR expressing cells are located in aromatase positive brain regions (Simerly et al., 1990). Given that sex chromosome factors regulate the expression of aromatase in some brain regions we decided to determine if this was also the case for ER-α and AR.

3.5.1. ER-α expression

Two-way ANOVA revealed a significant main effect of gonadal status on *Esr1* mRNA expression in the stria terminalis ($F_{1,9} = 5.06$; $p = 0.05$) and in the anterior amygdaloid area ($F_{1,8} = 7.09$; $p = 0.029$), while no effect of genotype ($p = 0.56$ and $p = 0.51$, respectively) nor interaction between genotype and sex ($p = 0.28$ and $p = 0.84$, respectively) was observed. The presence of *Sry* gene in XYM and XXM mice resulted in a lower expression of *Esr1* mRNA levels than in XYF and XXF mice in the stria terminalis and the anterior amygdaloid area ($p < 0.05$, in both areas). ER-α mRNA expression did not show significant differences between genotypes in the amygdala (Fig. 7A).

3.5.2. AR expression

The analysis of *Ar* mRNA expression in the anterior amygdaloid

Fig. 3. *Cyp19a1* mRNA levels (A) and aromatase immunoreactivity (B) in the stria terminalis, the anterior amygdaloid area and the amygdala (containing medial and central nuclei) at E16. XY individuals had higher expression of mRNA and protein levels than XX individuals in the stria terminalis and in the anterior amygdaloid area independently of gonadal sex (LSD Fisher: $p < 0.05$). Data are mean ± SEM. $n = 4–6$ independent embryos for each genotype.

area showed a significant main effect of gonadal sex ($F_{1,9} = 7.86$ $p = 0.021$). Mice carrying Sry gene (XYM and XXM) showed an increased expression of *Ar* mRNA compared with gonadal females (XYF and XXF; $p = 0.05$). On the contrary, in the other areas analyzed the levels of AR mRNA showed no significant differences among genotypes (Fig. 7B).

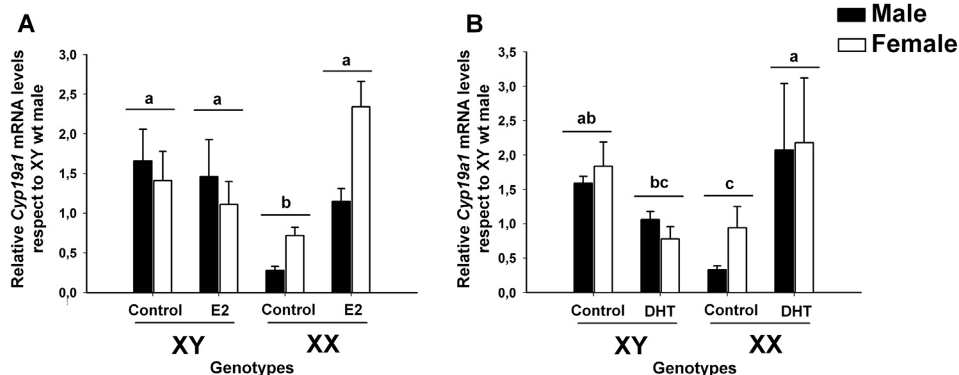

Fig. 4. Effect of 17β-estradiol (E2) or dihydrotestosterone (DHT) on the mRNA levels of *Cyp19a1* (A–B) in primary amygdala cell cultures. Hormonal treatments increased aromatase mRNA levels in XX cultures but not in XY cultures. Different letters indicate significant differences with $p < 0.05$ (LSD Fisher). Data are mean ± SEM. $n = 4–6$ independent cultures for each genotype and treatment.

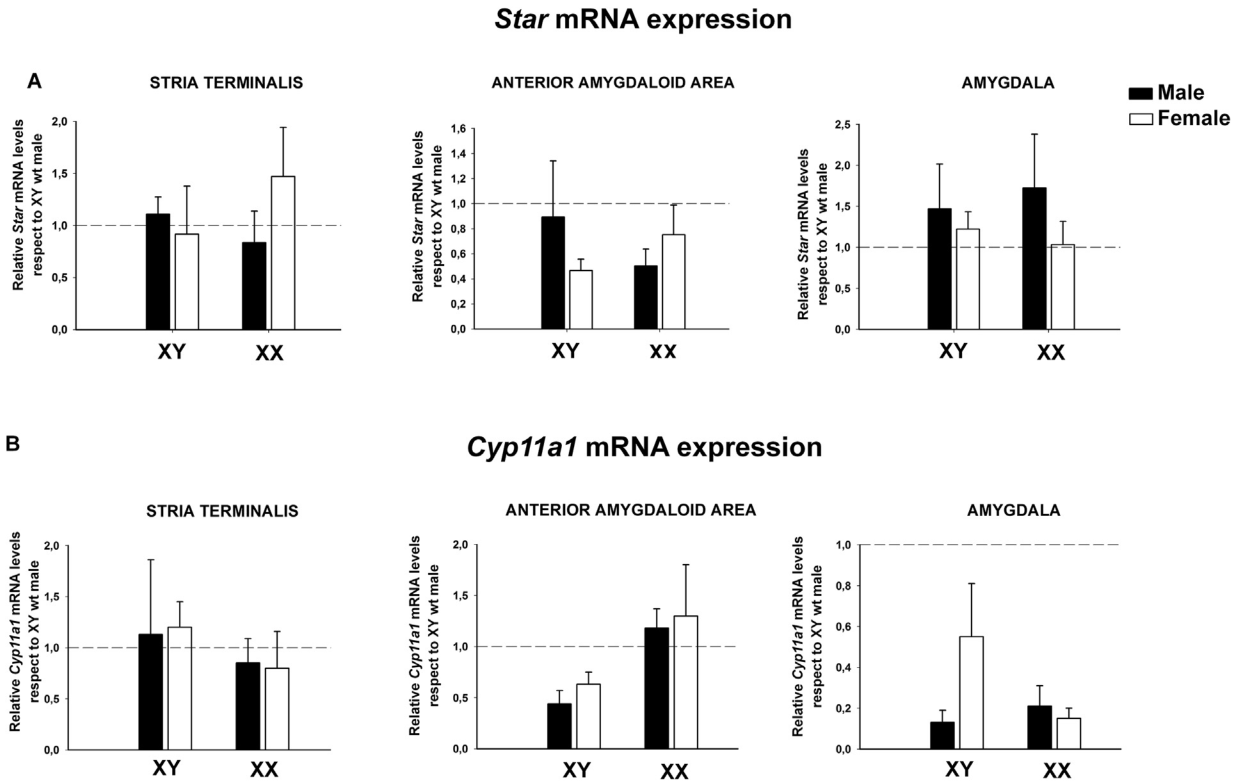

Fig. 5. Expression of *Star* (A) and *Cyp11a1* (B) mRNA in the stria terminalis, the anterior amygdaloid area and the amygdala (containing medial and central nuclei) at E16. There were no significant differences between genotypes. Data are mean ± SEM. $n = 4$ embryos for each genotype.

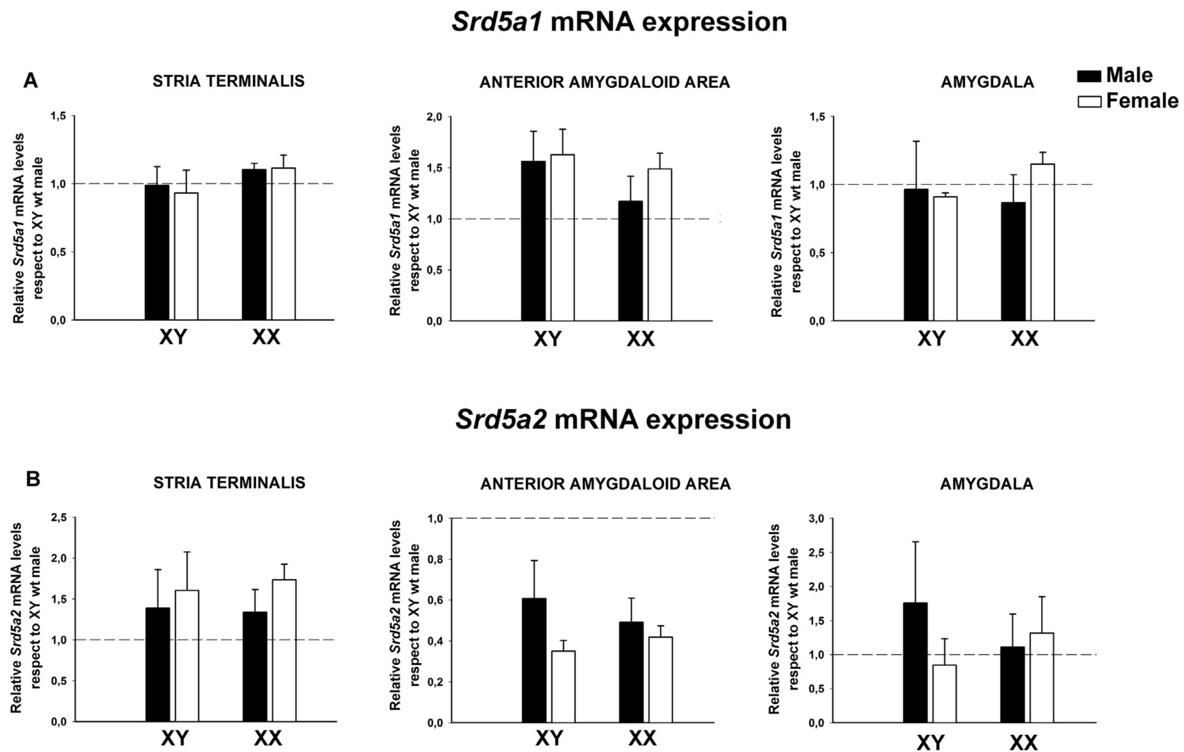

Fig. 6. Expression of *Srd5a1* (A) and *Srd5a2* (B) mRNA in the stria terminalis, the anterior amygdaloid area and the amygdala (containing medial and central nuclei) at E16. There were no significant differences between genotypes. Data are mean ± SEM. $n = 4$ embryos for each genotype.

4. Discussion

Sex differences in aromatase expression have been reported in various brain regions of rats and mice (Beyer et al., 1994b; Colciago et al., 2005; Ivanova and Beyer, 2000; Negri-Cesi et al., 2001). In this study, using the FCG model, we have found significant differences in aromatase immunoreactivity between XY and XX mice brain in the stria terminalis and the anterior amygdaloid area. These differences were detected at E16, before the peak of testosterone production by the fetal testis at E17-18 (O'Shaughnessy et al., 2006, 1998). This suggests a possible effect of sex chromosomes in the regulation of aromatase expression in these brain regions. However, although the peak of testosterone is at E17-18, the production of testosterone by the fetal testis starts earlier and therefore, a gonadal effect can not be excluded. To explore the role of sex chromosomes versus gonadal hormones on the regulation of aromatase expression we used the FCG mouse model. Our findings indicate a sex chromosome effect in the sexually dimorphic expression of aromatase in the stria terminalis and anterior amygdaloid area of the mouse brain at E16, irrespectively of the gonadal status (testes *vs*. ovary). The biological meaning of this effect is unknown; however, such differences *in vivo* could reflect differences in the local production of E2 by aromatization of testosterone in these specific brain areas. According to our findings, XY amygdala neurons would be exposed to the neuritogenic effect of E2 increasing the size of dendritic tree (Lorenzo et al., 1992), establishing a different pattern of synaptic connectivity than XX neurons.

Sex differences in the expression of the autosomal aromatase gene should be the consequence of differences in the expression of X or Y chromosome genes that result from the inherent sex difference in the number (two copies of X) and/or type (presence or absence of Y) of sex chromosomes. Additional studies are necessary to find the primary sex chromosome sex-determining gene(s) responsible to activate downstream pathways that differently regulate aromatase expression in males and females. Several X/Y-linked genes encoding transcriptional regulator proteins have the potential to mediate chromatin changes and regulate autosomal gene expression (Wijchers and Festenstein, 2011). Sry gene in the Y chromosome, which induces testicular differentiation, can be excluded as a potential candidate for generating this sex difference since XX versus XY difference in aromatase expression was found irrespective of the presence or absence of this gene.

Although the expression of aromatase shows sex differences in specific brain regions at E16, an important question is whether testosterone produced by the fetal testis during the critical period for sex differentiation at E17-18 may further affect the expression of this molecule and whether this effect is under the control of sex chromosomes. To answer this question we followed an *in vitro* approach using neuronal cultures from the amygdala obtained from FCG mouse embryos at E15. Importantly, the sex chromosome effects in the expression of aromatase observed in the anterior amygdaloid area *in vivo* were also detected in the cultures. Furthermore, our results showed that basal sex differences in *Cyp19a1* expression, determined by sex chromosomes, were lost after E2 or DHT exposure by a mechanism that was also dependent on sex chromosome factors. Thus, hormonal treatments resulted in the abolishment of sex differences in aromatase expression. In both cases the outcome of the treatments depended on the sex chromosome complement, probably because sex chromosomes had

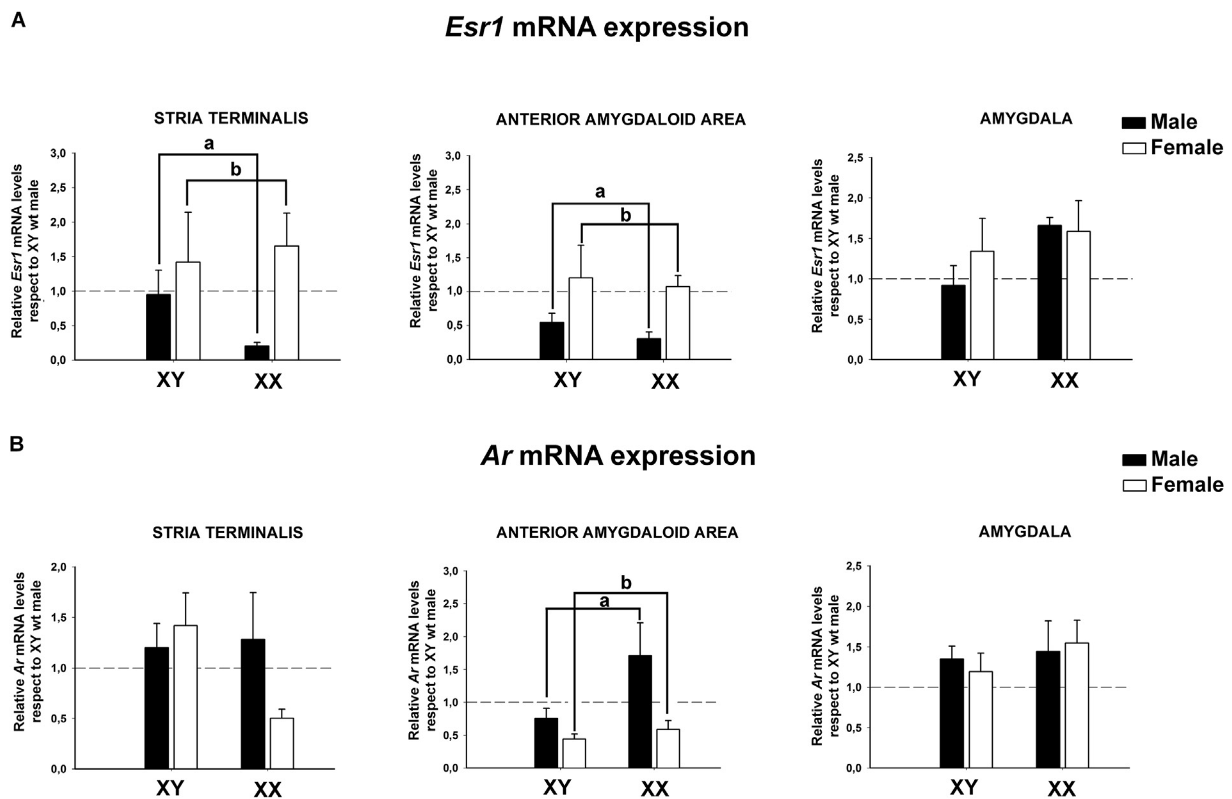

Fig. 7. Expression of mRNA levels for *Esr1* (A) and *Ar* (B) in the stria terminalis, the anterior amygdaloid area and the amygdala (containing medial and central nuclei) at E16. Gonadal males had lower expression of *Esr1* in the stria terminalis and the anterior amygdaloid area and a higher expression of *Ar* mRNA levels than gonadal females in the anterior amygdaloid area independently of sex chromosome complement. Different letters indicate significant differences with $p < 0.05$ (LSD Fisher). Data are mean ± SEM. $n = 4–6$ embryos for each genotype.

previously determined a different basal level of expression of aromatase. Thus, neurons with the XY complement showed a higher basal level of expression of aromatase than neurons with an XX complement. This different basal level of expression may have prevented a further increased in aromatase by hormonal treatments in XY neurons, but not in XX neurons. This interpretation follows the proposed hypothesis of the compensatory effect of the sex-biasing factors (De Vries, 2004), which has received increasing attention in the last years. Recently, Arnold (Arnold, 2014) incorporated the effect of sex chromosome complement in the developing brain as a mechanism to prevent or compensate for the potentially sex differentiating effects of gonadal hormone levels later in life. Thus, sex differences in brain aromatase expression in mouse embryos at E16, caused by sex chromosome factors, may prevent sex-differentiating effects of estradiol and DHT during the testosterone surge by the fetal testis at E17-18.

The above mentioned findings indicate that sex chromosome factors influence the expression of aromatase in specific brain regions of the mouse embryo before the critical period of sex differentiation. An interesting question was to determine whether sex chromosomes also influence the expression of other steroidogenic molecules that may participate in the sexual differentiation of the brain. According to the classical hypothesis for the sexual differentiation of the brain (MacLusky and Naftolin, 1981), the fetal testis produce testosterone at critical moments of development and then, this hormone is transformed locally in the brain to E2 by the enzyme aromatase. However, the rodent brain expresses all the steroidogenic molecules and enzymes to locally produce testosterone from cholesterol. It is unknown whether local synthesis of testosterone may influence the brain sex differentiation process. The first step of steroidogenesis is the transport of cholesterol from the outer to the inner mitochondrial membrane where the first steroidogenic enzyme, P450ssc (product of *Cyp11a1* expression), is located. P450scc converts cholesterol in pregnenolone, the precursor of the other steroids, including testosterone (Baulieu and Robel, 1998). Both StAR and P450scc are expressed during early brain development (Compagnone et al., 1995; Pezzi et al., 2003; Sierra et al., 2003). Moreover, StAR transcripts are co-expressed with P450scc and 3β-HSD in the hippocampus, dentate gyrus, cerebral granular layer and Purkinje cells (Furukawa et al., 1998; King et al., 2002). Our present findings indicate that Star and *Cyp11a1* are expressed in aromatase positive regions of the E16 mouse brain, such as the stria terminalis, the anterior amygdaloid area and the amygdala. This opens the possibility for a role of local steroidogenesis in the generation of substrates for aromatase. It is necessary to demonstrate the presence of the other enzymes of the steroidogenic pathway at E16 in these brain regions. However, our findings with the FCG mouse model indicate that the expression of StAR and P450scc in the stria terminalis, the anterior amygdaloid area and the amygdala is not sexually dimorphic and is not regulated by gonadal or sex chromosome factors.

The generation of some brain sex differences depends on the conversion of testosterone in DHT by the enzyme 5α-reductase (Goto et al., 2005; Ribeiro and Pereira, 2005). The predominant form of 5α-reductase in the brain is type I but type II was detected by RT-PCR followed by Southern analysis in late embryonic rat brain (Poletti et al., 1998). Our present findings indicate that the mRNA levels of 5α-reductases type I and type II do not show sex differences and are not influenced by sex chromosome or gonadal factors in the stria terminalis, the anterior amygdaloid area and the amygdala in the mouse embryo at E16. This finding is in agreement with previous studies that did not detect sex differences in 5α-reductases type I expression in the developing hypothalamus (Karolczak et al., 1998). Our finding are also in agreement with a previous study that assessed the expression of 5α-reductase type I in the brain during ontogeny in SF-1 KO mice, a mouse model for studying sex differences in the brain in the absence of endogenous sex steroids hormones (Spanic et al., 2015). 5α-reductase type I mRNA was detected in the brain of mouse embryos from E12 and the transcript levels were significantly increased at E18 (Spanic et al., 2015). However, the expression of 5α-reductase type I did not show sex differences and was not influenced by the absence of gonadal steroids in SF-1 KO mice.

The process of brain sexual differentiation requires the adequate expression of ERs and AR in specific brain structures to allow the activation of the organizational actions of E2 and DHT. E2 acts on nuclear ERs to regulate transcriptional activity of genes that have estrogen response elements in their promoters (Mangelsdorf et al., 1995). In addition, E2 may bind to ERs associated to the plasma membrane, causing the activation of different signaling pathways that will finally regulate gene transcription through other transcription factor (Arevalo et al., 2012). DHT regulates transcription through AR (Claessens et al., 2008). Previous studies in prenatal rat brain have detected that from E18 there is a robust hybridization signal for ER-α mRNA in the striohypothalamic nucleus, the caudal portion of the ventromedial hypothalamic nucleus, the bed nucleus of the stria terminalis (coextensive with the striohypothalamic nucleus), caudal arcuate nucleus and medial and cortical nuclei of the amygdala (DonCarlos, 1996). Sex differences in the expression of ER-α have been detected in the rat preoptic area at E20, having females higher levels than males (DonCarlos, 1996). Concerning AR, previous studies have shown that in the E16 mouse hypothalamus males have higher expression levels than females (Young and Chang, 1998). Our present results indicate that gonadal sex, rather than the sex chromosome complement influence the expression of ER-α and AR in some regions of the mouse brain at E16. Thus, AR mRNA expression in the anterior amygdaloid area is higher in gonadal males (XYM and XXM) than in gonadal females (XYF and XXF). In contrast, the expression of ER-α mRNA in the stria terminalis and the anterior amygdaloid area was higher in gonadal females than in gonadal males.

5. Conclusions

Taken together our findings indicate that sex chromosome factors determine sex differences in aromatase expression in the stria terminalis and in the anterior amygdaloid area of E16 mouse embryos. Further studies should determine if this is also the situation in some of the other regions of the brain where aromatase is expressed. These sex differences may prevent further sex differentiating effects of gonadal hormones on aromatase expression, since at least in amygdala neurons in culture, only neurons with the XX complement responded to E2 or DHT treatments with an increased expression of aromatase. This differential response of XX and XY neurons to E2 and DHT resulted in the abolishment of sex differences in aromatase expression. The effect of sex chromosome factors was not observed for other steroidogenic molecules, such as StAR, P450scc or 5α-reductases type I and type II. Sex chromosome factors were neither involved in the generation of sex differences in the expression of ER-α and AR in aromatase-positive brain regions at E16. In this case was gonadal sex, rather than sex chromosome factors, the cause of the sex differences. In summary, both sex chromosome factors and gonadal factors interact in the regulation of the molecular machinery involved in the generation of sex differences in some structures of the developing rodent brain.

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