Neuroendocrinology Letters Volume 31 No. 5 2010

ORIGINAL ARTICLE

Expression and distribution of P450-aromatase in the ovine hypothalamus at different stages of fetal development

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Submitted: 2010-09-13 Accepted: 2010-09-21 Published online: 2010-12-05

Key words: **aromatase; brain sexual differentiation; hypothalamus; fetal sheep**

Neuroendocrinol Lett 2010; **31**(5):690–699 **PMID:** 21173749 NEL310510A04

Abstract

OBJECTIVES: An important step of sexual differentiation is the conversion of testosterone to estrogen by aromatase leading to masculinization and defeminization of the fetal brain areas crucial for normal sexual behavior and reproduction. Brain sexual differentiation occurs throughout a critical period starting from different prenatal stages depending on the species. Such period goes on from gestation day (GD) 30 to 100GD in the sheep. The fetal sheep brain is reported to aromatize androgens to estrogens at 64GD. The main goal of this work was to evaluate aromatase expression in sheep hypothalami during the whole period of sexual differentiation (35GD, 55GD, 80GD, 115GD) and whether differences may be observed depending on gestational stage and sex.

METHODS: Sections at the hypothalamic level underwent immunoperoxidase technique employing anti-aromatase and anti-androgen receptor antibodies. Samples from 35GD and 55GD were also processed with in situ hybridization using aromatase cDNA probe. Blot analyses were performed to quantify possible aromatase immunoexpression differences between sexes. For sexing, samples at 35GD and 55GD underwent DNA extraction and SRY amplification.

RESULTS: Our results revealed aromatase and androgen receptor immunoreactivity along the whole period of sexual differentiation. Both molecules were detected in many brain regions and markedly in the periventricular area. The highest aromatase and androgen receptor amounts were observed at 35GD and 55GD, when aromatase was more abundant in females than in males.

CONCLUSIONS: In conclusion, the sheep can be included among the species where aromatase is highly expressed in the hypothalamus during the whole period of sexual differentiation.

INTRODUCTION

The brain is an important site of estrogen synthesis (Roselli, 2007), as demonstrated in mammals by Naftolin *et al.* (1971), who ascertained that the central neuroendocrine tissues are capable of converting androgens into estrogens by means of cytochrome P450-aromatase. In the last years, it has been shown that many of the androgen effects on neural functions are mediated by aromatase through a multistep enzymatic pathway (Roselli and Resko, 2001; Simpson *et al.* 1994). Two pro-

teins are required for aromatase activity: cytochrome P450, that is the CYP19 gene product, and NADP-cytochrome P450-reductase, a ubiquitous flavoprotein (Garcia-Segura, 2008; Negri-Cesi *et al.* 1992). The enzyme complex is located in the smooth endoplasmic reticulum of estrogen producing cells. Aromatase is expressed in many peripheral tissues and organs, such as placenta, fetal tissues, adipose tissue, skin, bones, cartilage, muscle, ovary, testis, etc. (Negri-Cesi *et al.* 1992; Roselli & Resko 2001).

A large number of studies have demonstrated that brain aromatase is predominantly distributed in areas involved in the control of reproductive functions such as the hypothalamus, limbic system and preoptic area (Negri-Cesi *et al.* 2008). These are indeed essential regions for sex-specific control of the reproductive hormone secretion and sex behavior. More precisely, the hypothalamic neurons of the periventricular region and the arcuate nucleus produce reproductive hormones, whereas the medial preoptic, ventromedial, and ventral premammillary nuclei influence sex behavior. Aromatase is also expressed in non-reproductive brain areas such as cerebral cortex, midbrain and spinal cord (Simerly 2002). This enzyme plays an essential role in several neural processes. For instance, aromatase is involved in synaptic plasticity of brain regions related to cognition such as the hippocampus (Von Schassen *et al.* 2006) and it may also be responsible for the early stages of neural cell differentiation (Martínez-Cerdeño *et al.* 2006).

Aromatase is expressed by neurons in baseline condition (Balthazart & Ball 1998; Lephart 1996; Naftolin *et al.* 1971; Negri-Cesi *et al.* 1992), whereas several studies have suggested that it is expressed by astrocytes only after brain injury (Garcia-Segura *et al.* 1999). This indicates that astrocytes have the potential to produce aromatase and therefore to express neuroprotective estrogens (Garcia-Segura 2008).

In addition, aromatase is essential for sexual differentiation of the brain in many mammals and avian species (Herbosa & Foster 1996) leading to the production of estrogens that act permanently in the developing brain. Indeed, during prenatal development, "sex brain" is feminine by default, unless some specific stimuli drive it to a male phenotype (Negri-Cesi *et al.* 2008). Therefore, to achieve male-specific brain it is necessary to activate two independent processes: masculinization and defeminization. The former establishes male-typical copulatory behavior and partner preference, while the latter entails the estrogen-mediated "elimination" of GnRH surge center (Senger 2003). In contrast, female brain is not exposed to testosterone and develops the hypothalamic GnRH surge center. Such processes occur in the brain during prenatal and perinatal development, dependently on the length of gestation. Indeed, in long-gestation species like monkeys masculinization and defeminization mainly take place prenatally, whereas in short-gestation species like rats, they occur postnatally (Herbosa and Foster,1996; Roselli *et al.* 2003).

Recent studies have suggested that these developmental phenomena may also be applied to the sheep, which is a long-gestation species, where masculinization occurs prenatally from day 30 to 100 (Short 1974). Lines of evidence have shown that the fetal sheep brain is able to aromatize androgens to estrogens, but this process has been documented in the literature at day 64 of gestation only (Roselli *et al.* 2003), when no significant sex differences in aromatase activity were found, similarly to what demonstrated for other long-gestation species like monkeys (George & Ojeda 1982; Roselli & Resko 1986; Roselli *et al.* 2003). In contrast, sex differences in hypothalamic aromatase activity were detected in fetuses from short-gestation species like ferrets and rats (Negri-Cesi *et al.* 2008; Tobet *et al.* 1985).

Thus, aim of the present investigation was to detect aromatase hypothalamic immunoexpression throughout the period of sexual differentiation in the fetal sheep brain. Moreover, aromatase expression in male and female fetuses was estimated at early stages of sexual differentiation in order to assess whether sex differences in aromatase activity exist during that crucial period of brain development.

MATERIAL AND METHODS

Sheep fetuses from different gestation days (GD) i.e. 35±2GD (n=13 for immunocytochemistry and *in situ* hybridization, n=6 for Western blot), 55±2GD (n=14 for immunocytochemistry and *in situ* hybridization, n=6 for Western blot), 80±2GD (n=6 for immunocytochemistry), 115±2GD (n=6 for immunocytochemistry) were obtained at local abattoirs, when pregnant sheep were slaughtered. The period of gestation was established measuring fetal crown-rump length, following the tables reported by McGeady *et al.* (2006). Fetal heads from 35GD and 55GD were completely processed, whereas fetal heads from 80GD and 115GD were removed and brains dissected using the ventral surface as landmark. Hypothalamic tissue blocks extending caudally from the anterior border of the optic chiasm to the posterior border of the mammillary body and dorsally to the roof of the third ventricle were obtained. Blocks were fixed with ice-cold 4% paraformaldehyde for 24 hrs, then postfixed with 30% sucrose/PBS as cryoprotectant. Then, they were stored at –80 °C until cutting. Sections, 10 μm thick, were mounted onto microscope slides and underwent immunoperoxidase or *in situ* hybridization techniques. In addition, blocks were homogenised and frozen at –80 °C to be processed by Western blot analysis.

Immunoperoxidase technique (ABC method, Vector, Burlingame, CA, USA) was performed employing the following antibodies: polyclonal anti-P450-aromatase antibody (Acris, Hiddenhausen, Germany) diluted

1:200, monoclonal anti-androgen receptor antibody (Sigma, St. Louis, MO, USA) diluted 1:100 and monoclonal anti-tubulin βIII (Sigma) antibody, a neuronal cell marker (Moody *et al.* 1996). In details, protease digestion (0.1% trypsin, 0.1% calcium chloride, TRIS/HCl 0.005 M, pH 7.6 at 37 °C for 20 min) was made to counteract the possible antigen masking effects of paraformaldehyde fixation. Then, coronal sections were blocked for 20 min with normal goat serum and incubated overnight at 4 °C with the primary antibodies, then for 1 hr in biotinylated goat anti-rabbit or anti-mouse antibodies diluted 1:250. Sections were incubated with avidin-biotin complex for 30 min, then with diaminobenzidine for 1–3 min and finally dehydrated and coverslipped. The anti-P450-aromatase antibody was previously tested by Western blot analysis in order to establish its cross-reactivity to the sheep. Other two anti-P450-aromatase antibodies were used from Sigma and Novus Biological (Littleton, CO, USA) to further confirm the effectiveness of the antibody used.

In order to evaluate aromatase gene expression, *in situ* hybridization technique was performed. A melting curve ranging 37 °C to 42 °C was calculated in a preliminary study carried out at our labs, indicating 38 °C as the optimal temperature (Lepore *et al.* 2009). A biotinylated cDNA probe with 48-nucleotide sequence from Sigma (Roselli *et al.* 2000) was used on samples from 35GD and 55GD, i.e. the earliest stages examined. Then, sections were incubated with streptavidin peroxidase complex and diaminobenzidine as chromogen. A sense probe was used to verify biotinylated cDNA probe and displayed no signal.

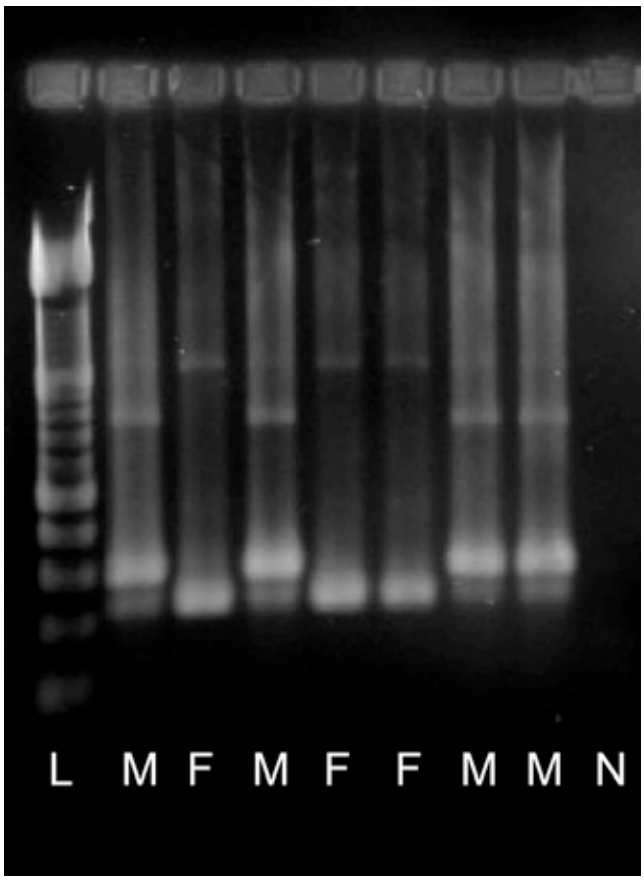

Fig. 1. Sex determination. PCR amplification of SAT (216 bp) and SRY (301 bp) chromosomal regions. L= ladder 100 bp; M= male; F= female; N= negative control.

In addition, possible differences in aromatase expression between sexes were monitored at 35GD and 55GD by immunoperoxidase and Western blot. For sexing, fetal sample tissues underwent DNA extraction and PCR amplification of the sex determining region (SRY) of the Y chromosome following the protocol of Mara *et al.* (2004) specific for the sheep (Figure 1).

As to Western blot, samples were homogenized in lysis buffer with protease inhibitor (Roche Complete Mini, Basel, Switzerland) according to Tabori *et al.* (2005). In order to rule out possible differences in protein estimation and loading, protein concentrations were determined by a colorimetric assay (DC Protein Assay, Bio-Rad, Hercules, CA, USA) setting the spectrophotometer to 750 nm. The lysate was centrifuged at 15,000 rpm for 15 min. Aliquots (40 μg of protein) were prepared in sample buffer (62.5 mM Tris-HCl pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.5% bromophenol blue). Proteins were separated on SDS-PAGE in 10% gel at 200 V for 1hr and then transferred onto nitrocellulose membranes (Trans-Blot transfer medium, 0.45 μm, Bio-Rad) using a semidry transfer unit at a constant current of 20 volts for 20 min. The nitrocellulose membranes were blocked for 1 hr in 5% skim milk PBS and incubated overnight with the same anti-P450-aromatase antibody employed in the immunocytochemical study, diluted 1:500. The membranes were then incubated with a 1:30,000 IgG anti-rabbit alkaline phosphatase-conjugated antibody (Sigma) for 1 hr and the reaction developed using nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate, toluidine salt (NBT/BCIP, Roche) as chromogen. Densitometric values of protein bands were quantified using the computer software Scion Image and statistically processed by analysis of variance (ANOVA).

Quantitative analysis of immunocytochemical results was performed by means of Image J 1.42q software (NIH, Bethesda MD, USA), which detected aromatase and androgen receptor immunopositive cells in all gestation stages examined.

RESULTS

Aromatase immunoexpression in fetal sheep hypothalamus was detected along the whole period of sexual differentiation. Aromatase was widely distributed in different regions, especially in the diencephalic periventricular area, where a high number of immunoreactive cells was detected in dorso- and ventro medial regions. In the early stages (35GD and 55GD), aromatase immunopositivity was more appreciable

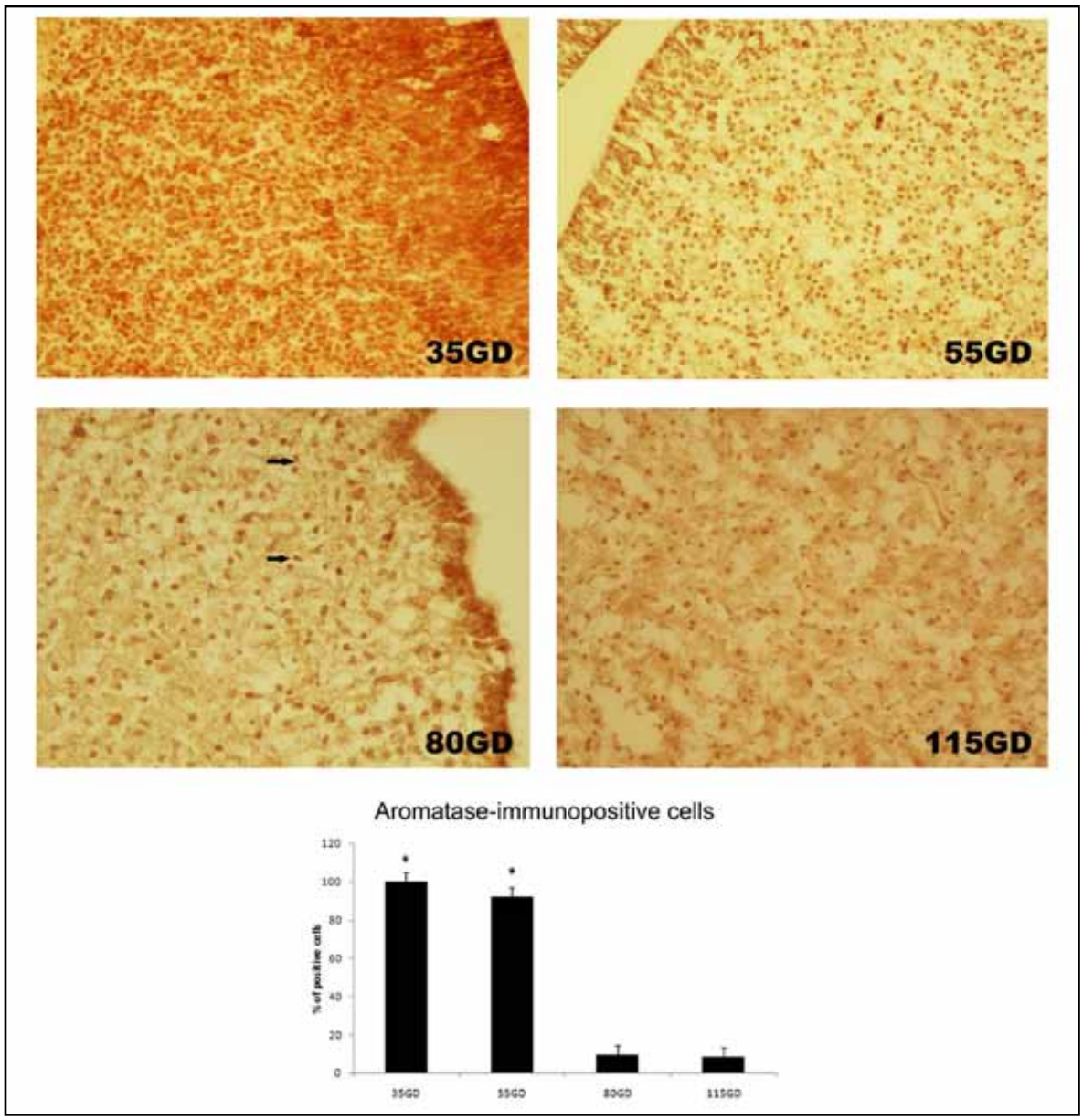

Fig. 2. Aromatase immunoreactivity. Fetal sheep brain at different developmental stages. Arrows indicate neuroblasts with a growth cone. 20×. The diagram below indicates percentage of immunopositive cells. Asterisks indicate statistically significant values ($p<0.05$) from 80GD and 115GD samples.

than in the late stages (80GD and 115GD), as shown in Figure 2. These remarkable differences in aromatase immunoexpression between early and late stages of brain sexual differentiation were highlighted by quantitative analyses (Figure 2). The neuronal specific marker tubulin βIII let identify the immunopositive cells as apolar and unipolar neuroblasts (Figure 3). Indeed, at those early stages of sexual differentiation they had no cytoplasmatic processes and showed globular shape with a large cell body. In contrast, at the later stages neuroblasts were mostly piriform- or oval-shaped and showed one or two processes respectively.

In situ hybridization technique performed at the earliest stages examined (35GD and 55GD), i.e. those showing the strongest aromatase immunoexpression, pointed out that aromatase gene expression occurred at the hypothalamic level ever since the beginning of the crucial period of brain sexual differentiation (Figure 4).

Moreover, immuperoxidase with anti-aromatase antibody at 35GD and 55GD showed more noticeable cell immunoexpression in females than in males, this datum being illustrated in the diagram of Figures 5 and 6.

Western blot analysis revealed a band at 55 kD corresponding to the molecular weight of brain aromatase. In fact, blot analysis confirmed the results of the immunocytochemical studies. Indeed, the densitometric analysis of the bands revealed a small but significant increase in aromatase amount in female than in male sheep fetuses (Figure 7).

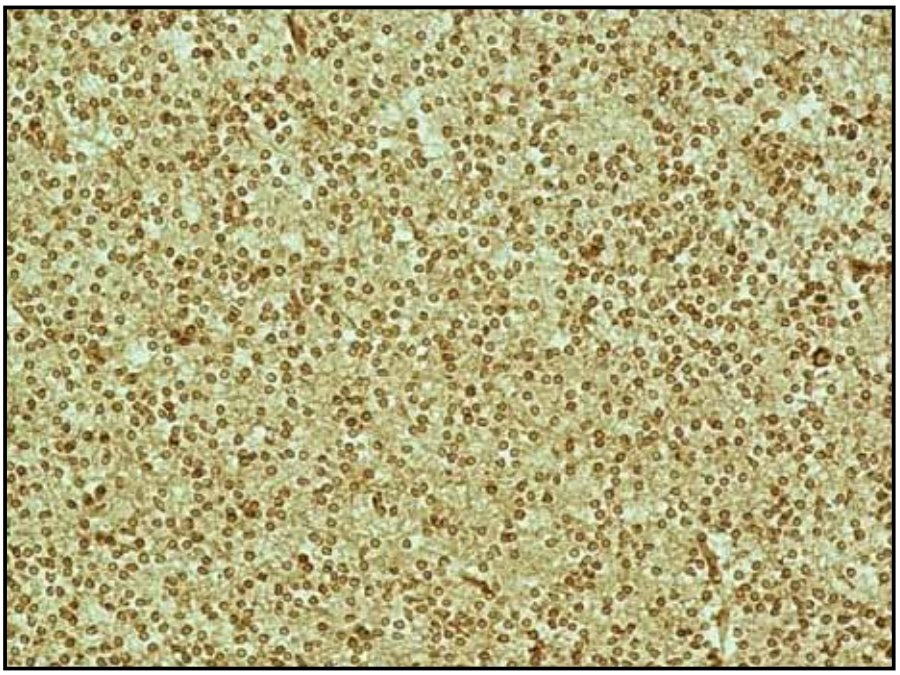

Fig. 3. Tubulin βIII immunoreactivity. The neuronal cell marker stains neuroblasts in the periventricular area at 35GD. 20×.

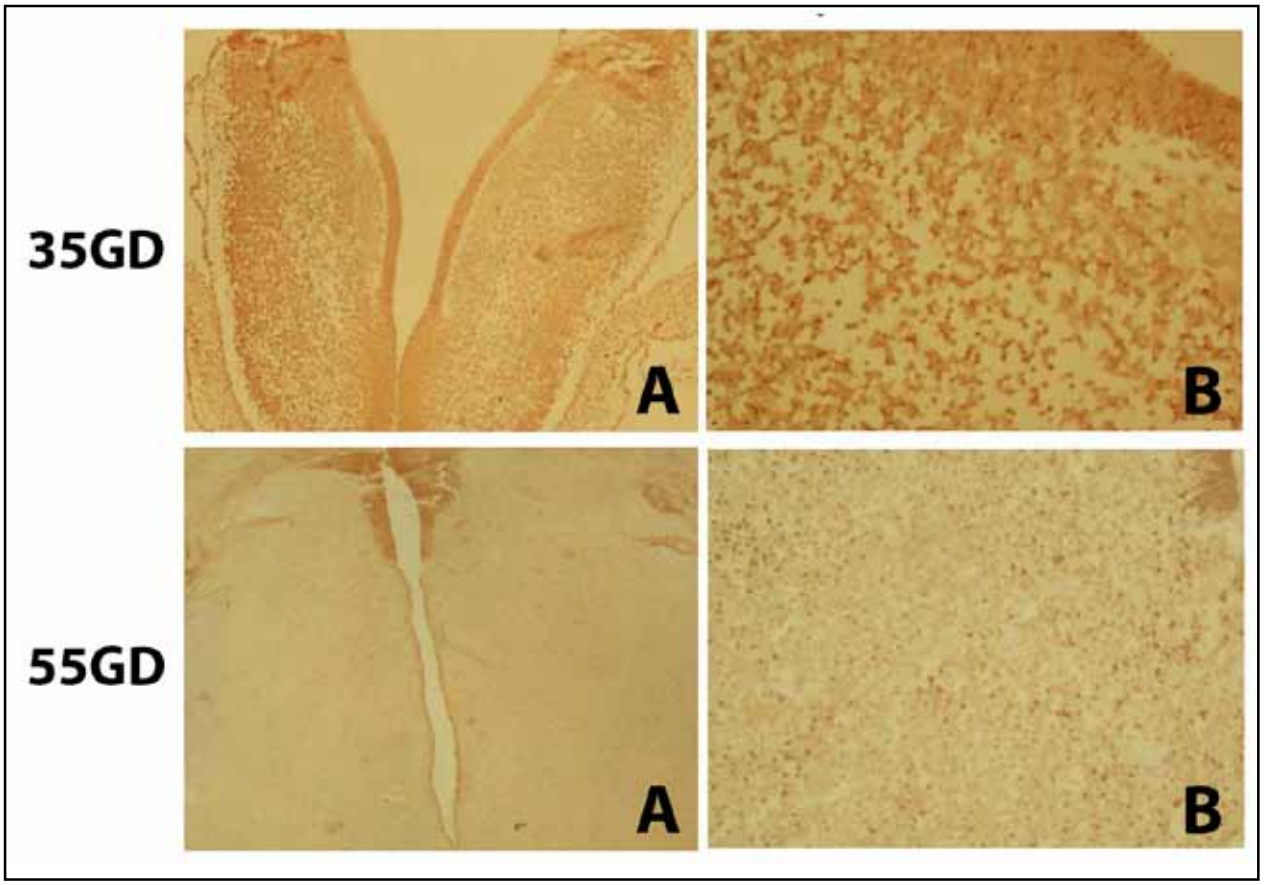

Fig. 4. Aromatase gene expression. *In situ* hybridization of fetal sheep brain at early stages of development. **A**=4×, **B**=20×.

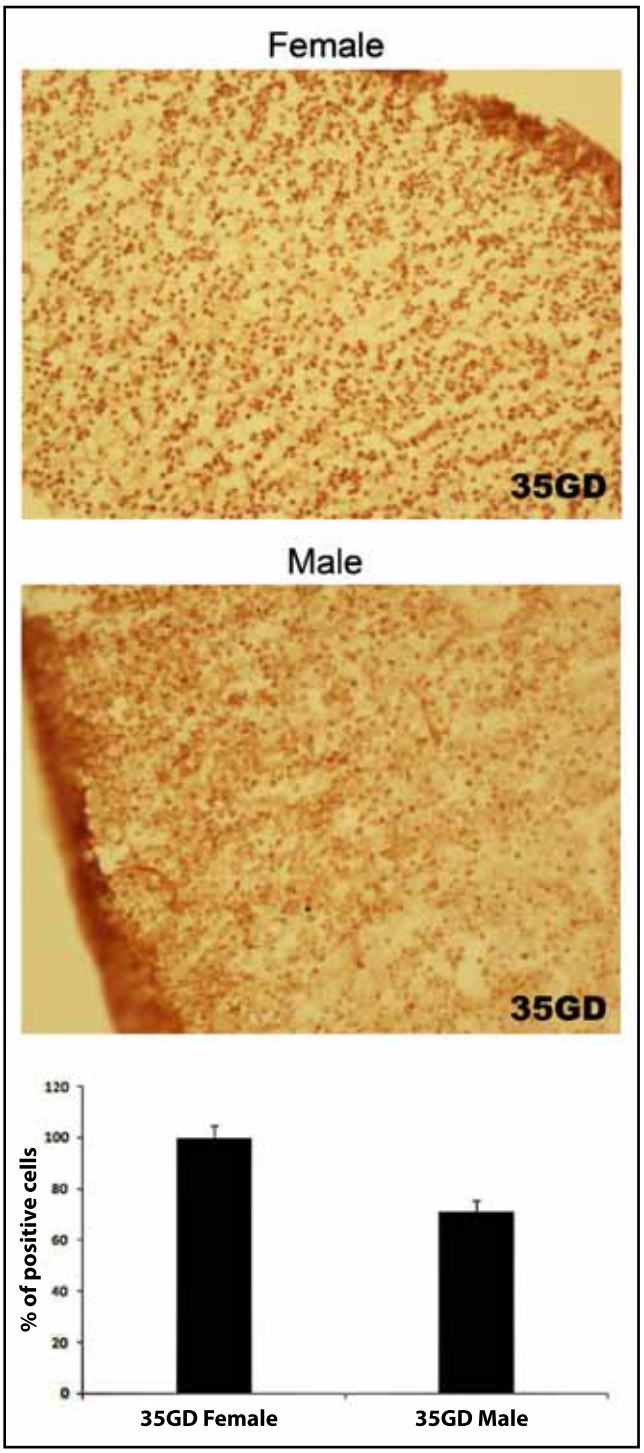

Fig. 5. Sex differences in aromatase expression. Female and male 35GD fetuses. Aromatase immunoreactivity is stronger in females. 20×. The diagram indicates the percent differences in immunopositive cells between sexes. $p<0.05$.

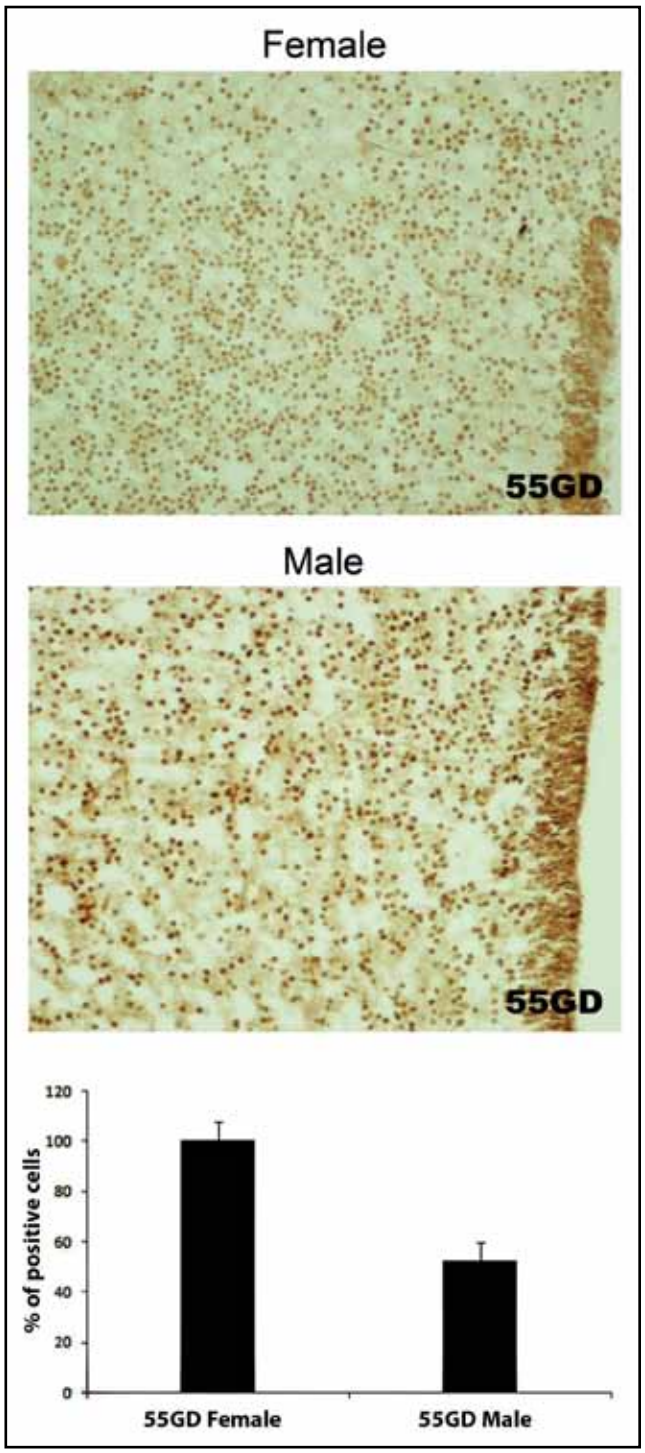

Fig. 6. Sex differences in aromatase expression. Female and male at 55GD fetuses. Aromatase immunoreactivity is stronger in females. 20×. The diagram indicates the percent differences in immunopositive cells between sexes. $p<0.05$.

Androgen receptor immunoexpression in the diencephalic periventricular area displayed a marked immunoexpression at 35GD which gradually declined at the following stages, showing a trend similar to that found for aromatase immunoexpression (Figure 8).

DISCUSSION

As initially determined by Short (1974) and later by Clarke *et al.* (1976), brain sexual differentiation in fetal sheep occurs prenatally, approximately from 30GD to 100GD. Aromatase is a fundamental enzyme involved in brain sexual differentiation and studies on

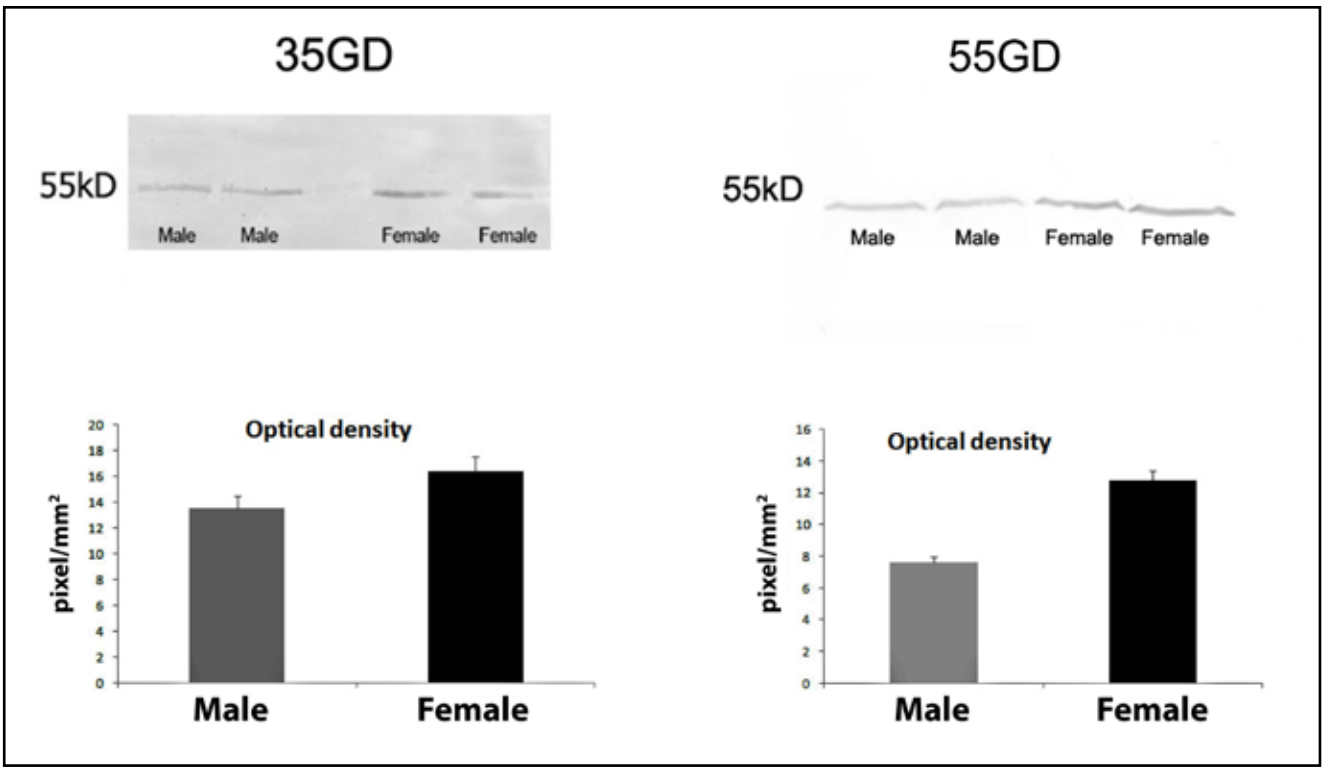

Fig. 7. Sex differences in aromatase expression. Western blot analysis at G35D and 55GD. A single band migrates at 55kD. The protein amount is higher in females than in males in both gestation days. The diagram represents the optical density of the bands.

the sheep reported that its activity can be detected at 64GD (Roselli *et al.* 2003). Our work pointed out for the first time that aromatase immunoexpression can be detected along the whole period of sexual differentiation in the diencephalic periventricular area of fetal sheep brain. The higher aromatase expression in the early stages (35GD and 55GD) than in the late stages (80GD and 115GD) is in agreement with what reported by Connolly *et al.* (1994), who demonstrated aromatase activity in guinea pig at early stages of sexual differentiation (35GD–40GD) showing a steady decline through development. Such higher amount in the early stages was also found in the present investigation on the sheep, suggesting that this enzyme is essential in the first half of the sexual differentiation period representing an input for the masculinization processes of the brain. Testosterone exposure of female sheep fetuses from 30GD to 86GD led to the masculinization of the external genitalia and defeminization with the elimination of LH surge (Wood *et al.* 1991). In addition, ewes showed a modification of LH surge mechanism leading to sporadic ovulation. In contrast, exposure to testosterone between 89GD and 135GD was without effects (Clarke & Scaramuzzi 1978; Short 1974). Variability in phenotypes was observed in female lambs from mothers undergone 20-day androgen treatment at the early (G30D to 50GD) or late (65GD to 85GD) stages of the critical period. The first group possessed a penis and an empty scrotum, whereas the second one was phenotypically normal (Wood *et al.* 1995). In addition, female fetuses exposed to androgens at early stage of brain sexual differentiation showed a delayed puberty timing (Wood & Foster 1998). A continued exposure to estrogens during gestation is needed to complete masculinization and defeminization processes in the fetal sheep brain (Foster *et al.* 2006), and the high aromatase immunoexpression detected here at the early stages (35GD and 55GD) likely suggests that this enzyme is involved in the onset of ovine brain sexual differentiation.

In our work aromatase expression was widely distributed in different brain regions, especially in the diencephalic periventricular area. The distribution pattern of aromatase in ovine fetal brain is consistent with the idea that the enzyme takes part in the crucial neuronal mechanisms involved in the central regulation of reproductive behavior and neuroendocrine functions.

Androgen receptor immunoreactivity revealed the same trend as aromatase immunoexpression, being marked in the early (35GD) and decreased in the late (115GD) period of brain sexual differentiation. Thus, a correlation may be suggested between androgen and aromatase activity, as documented in rodents (Abdelgadir *et al.* 1994; Connolly *et al.* 1990). However, data about the relationship between testosterone and brain aromatase are conflicting since some authors reported that in the rat testosterone can inhibit the expression and activity of aromatase (Lephart *et al.* 1992; Negri-Cesi *et al.* 2001). In details, Lephart *et al.* showed a significant dose-dependent decrease in aromatase activity

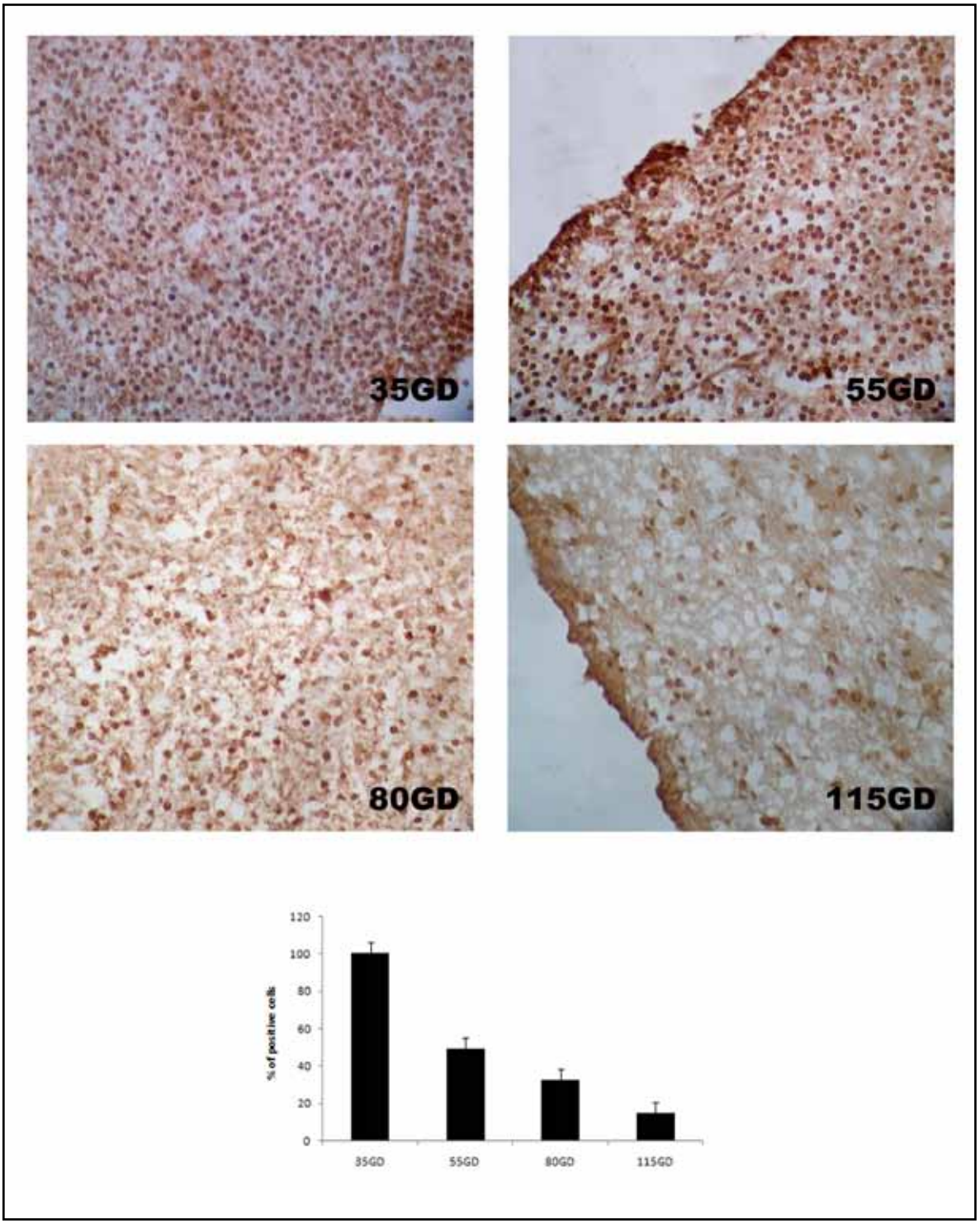

Fig. 8. Androgen receptor immunoreactivity. Fetal sheep brain at different developmental stages. 20×. The diagram below indicates percentage of immunopositive cells. Values are statistically significant ($p<0.05$).

caused by testosterone and dihydrotestosterone. In contrast, it has been demonstrated that androgens have a stimulatory role on aromatase expression both in rat and mouse brain (Karolczak *et al.* 1998; MacLusky *et al.* 1985). Moreover, studies on monkeys suggested that aromatization is not essential for androgen regulation during the development (Roselli & Resko 1986; Resko & Elliwood 1985). This mismatching evidence could be due to interspecific differences in the regulatory mechanisms of sex behavior.

In the present investigation higher aromatase immunoexpression was detected in female than in male sheep fetal brain. The existence of differences in aromatase expression between sexes is however still matter of debate. Indeed, Roselli *et al.* (2003) demonstrated that sex differences were not present in fetal sheep hypo-

thalamic areas at 64GD. This finding is consistent with other studies on the rat (George & Ojeda 1982; Lauber *et al.* 1997; Weisz *et al.* 1982), rabbit (George *et al.* 1978), and guinea pig (Connolly *et al.* 1994), all performed during the critical period of sexual differentiation. In contrast, Roselli and Resko (1986) observed in the monkey a little but significant sex difference in aromatase activity in the amygdala and cerebral cortex but not in the hypothalamus and preoptic areas. In addition, Tobet *et al.* (1985) showed sex differences in the ferret fetal brain where aromatase activity was high in the preoptic area, but there were no sex differences at any postnatal stage examined. Moreover, age-dependent sex differences were demonstrated in aromatase immunoreactivity during the critical period of sexual differentiation in the rat (from 18GD to post-natal day 10) and shortly after (Lauber *et al.* 1997; Negri-Cesi *et al.* 2008). The rats did not display sex differences prenatally, but at days 2 and 6 after birth, males expressed significantly higher levels of aromatase mRNA than females, although at weaning (post-natal day 21) aromatase activity was higher in females than in males. This trend could be due to the fact that rat aromatase is involved in the onset of female puberty and is required for the normal expression of female sexual behavior (Negri-Cesi *et al.* 2008).

In conclusion, our study showed changes in aromatase immunoexpression in the diencephalic periventricular area throughout the whole period of brain sexual differentiation of fetal sheep. Aromatase expression was higher in the early stages (35GD and 55GD) than in the late stages (80GD and 115GD). Sex differences in aromatase immunoexpression at early stages were also detected, more precisely females showed higher aromatase expression than males. Androgen receptor and aromatase expression were similar as to their occurrence during the whole period of brain sexual differentiation and their upregulation at early stages (35GD and 55GD). The correlation between androgen receptor and aromatase suggests a possible synergy of these two molecules in the neuronal tuning of reproduction and sex behavior.

REFERENCES

1 Abdelgadir SE, Resko JA, Ojeda SR, Lephart ED, McPhaul MJ, Roselli CE (1994). Androgens regulate aromatase cytochrome P450 messenger ribonucleic acid in rat brain. Endocrinology. **135**: 395–401.
2 Balthazart J, Ball GF (1998). New insights into the regulation and function of brain estrogen synthase (aromatase). Trends Neurosci. **21**: 243–249.
3 Clarke IJ, Scaramuzzi RJ, Short RV (1976). Effects of testosterone implants in pregnant ewes on their female offspring. J Embryol Exp Morphol. **36**: 87–99.
4 Clarke IJ, Scaramuzzi RJ (1978). Sexual behaviour and LH secretion in spayed androgenized ewes after a single injection of testosterone or oestradiol-17beta. J Reprod Fertil. **52**: 313–320.
5 Connolly PB, Roselli CE, Resko JA (1990). Aromatase activity in adult guinea pig brain is androgen dependent. Biol Reprod. **43**: 698–703.
6 Connolly PB, Roselli CE, Resko JA (1994). Aromatase activity in developing guinea pig brain: ontogeny and effects of exogenous androgens. Biol Reprod. **50**: 436–441.
7 Foster DL, Jackson LM, Padmanabhan V (2006). Programming of GnRH feedback controls timing puberty and adult reproductive activity. Mol Cell Endocrinol. **25**: 109–119.
8 Garcia-Segura LM, Wozniak A, Azcoitia I, Rodriguez JR, Hutchison RE, Hutchison JB (1999). Aromatase expression by astrocytes after brain injury: implications for local estrogen formation in brain repair. Neuroscience. **89:** 567–578.
9 Garcia-Segura LM (2008). Aromatase in the brain: not just for reproduction anymore. J Neuroendocrinol. **20**: 705–712.
10 George FW, Tobleman WT, Milewich L, Wilson JD (1978). Aromatase activity in the developing rabbit brain. Endocrinology. **102**: 86–91.
11 George FW, Ojeda SR (1982). Changes in aromatase activity in the rat brain during embryonic, neonatal, and infantile development. Endocrinology. **111**: 522–529.
12 Herbosa CG, Foster DL (1996). Defeminization of the reproductive response to photoperiod occurs early in prenatal development in the sheep. Biol Reprod. **54:** 420–428.
13 Karolczak M, Küppers E, Beyer C (1998). Developmental expression and regulation of aromatase and 5alpha-reductase type I mRNA in the male and female mouse hypothalamus. J Neuroendocrinol. **10:** 267–274.
14 Lauber ME, Sarasin A, Lichtensteiger W (1997). Transient sex differences of aromatase (CYP19) mRNA expression in the developing rat brain. Neuroendocrinology. **66:** 173–180.
15 Lephart ED, Simpson ER, McPhaul MJ, Kilgore MW, Wilson JD, Ojeda SR (1992). Brain aromatase cytochrome P-450 messenger RNA levels and enzyme activity during prenatal and perinatal development in the rat. Brain Res Mol Brain Res. **16**: 187–192.
16 Lephart ED (1996). A review of brain aromatase cytochrome P450. Brain Res. **22**: 1–26.
17 Lepore G, Gadau S, Mura A, Zedda M, Farina V (2009). Aromatase immunoreactivity in fetal ovine neuronal cell cultures exposed to oxidative injury. Eur J Histochem. **53**: 233–238.
18 MacLusky NJ, Philip A, Hurlburt C, Naftolin F (1985). Estrogen formation in the developing rat brain: sex differences in aromatase activity during early post-natal life. Psychoneuroendocrinology. **10**: 355–361.
19 Mara L, Pilichi S, Sanna A, Accardo C, Chessa B, Chessa F, Dattena M, Bomboi G, Cappai P (2004). Sexing of in vitro produced ovine embryos by duplex PCR. Mol Reprod Dev. **69**: 35–42.
20 Martínez-Cerdeño V, Noctor SC, Kriegstein AR (2006). Estradiol stimulates progenitor cell division in the ventricular and subventricular zones of the embryonic neocortex. Eur J Neurosci. **24**: 3475–3488.
21 McGeady TA, Quinn PJ, FitzPatrick ES, Ryan MT (2006). Veterinary Embryology. In: Chapter 23: Age determination of the embryo and foetus. Oxford: Blackwell Publishing. p. 331–336.
22 Moody SA, Miller V, Spanos A, Frankfurter A (1996). Developmental expression of a neuron-specific beta-tubulin in frog (Xenopus laevis): a marker for growing axons during the embryonic period. J Comp Neurol. **364**: 219–230.
23 Naftolin F, Ryan KJ, Petro Z (1971). Aromatization of androstenedione by the diencephalon. J Clin Endocrinol Metab. **33**: 368–370.
24 Negri-Cesi P, Melcangi RC, Celotti F, Martini L (1992). Aromatase activity in cultured brain cells: difference between neurons and glia. Brain Res. **589**: 327–332.
25 Negri-Cesi P, Colciago A, Motta M, Martini L, Celotti F (2001). Aromatase expression and activity in male and female cultured rat hypothalamic neurons: effect of androgens. Mol Cell Endocrinol. **178**: 1–10.
26 Negri-Cesi P, Colciago A, Pravettoni A, Casati L, Conti L, Celotti F (2008). Sexual differentiation of the rodent hypothalamus: hormonal and environmental influences. J Steroid Biochem Mol Biol. **109**: 294–299.
27 Resko JA, Ellinwood WE (1985). Negative feedback regulation of gonadotropin secretion by androgens in fetal rhesus macaques. Biol Reprod. **33**: 346–352.

28 Roselli CE, Resko JA (1986). Effects of gonadectomy and androgen treatment on aromatase activity in the fetal monkey brain. Biol Reprod. **35**: 106–112.
29 Roselli CE, Stormshak F, Resko JA (2000). Distribution of aromatase mRNA in the ram hypothalamus: an in situ hybridization study. J Neuroendocrinol. **12**: 656–664.
30 Roselli CE, Resko JA (2001). Cytochrome P450 aromatase (CYP19) in the non-human primate brain: distribution, regulation, and functional significance. J Steroid Biochem Mol Biol. **79**: 247–253.
31 Roselli CE, Resko JA, Stormshak F (2003). Estrogen synthesis in fetal sheep brain: effect of maternal treatment with an aromatase inhibitor. Biol Reprod. **68**: 370–374.
32 Roselli CF (2007). Brain aromatase: roles in reproduction and neuroprotection. J Steroid Biochem Mol Biol. **106**: 143–150.
33 Senger PL (2003). Pathway to pregnancy and parturition. In: Chapter 6: Puberty. 2nd rev. ed. Washington: Current Conception, Inc. p. 128–143.
34 Simerly RB (2002). Wired for reproduction: organization and development of sexually dimorphic circuits in the mammalian forebrain. Annu Rev Neurosci. **25**: 507–536.
35 Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshelwood MM, Graham-Lorence S, et al. (1994). Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocr Rev.**15**: 342–355.
36 Short RV (1974). Sexual differentiation of the brain of the sheep. INSERM. **32**: 121–142.
37 Tabori NE, Stewart LS, Znamensky V, Romeo RD, Alves SE, McEwen BS, et al. (2005). Ultrastructural evidence that androgen receptors are located at extranuclear sites in the rat hippocampal formation. Neuroscience. **130**: 151–163.
38 Tobet SA, Shim JH, Osiecki ST, Baum MJ, Canick JA (1985). Androgen aromatization and 5 alpha-reduction in ferret brain during perinatal development: effects of sex and testosterone manipulation. Endocrinology. **116**: 1869–1877.
39 Von Schassen C, Fester L, Prange-Kiel J, Lohse C, Huber C, Böttner M, Rune GM (2006). Oestrogen synthesis in the hippocampus: role in axon outgrowth. J Neuroendocrinol. **18**: 847–856.
40 Weisz J, Brown BL, Ward IL (1982). Maternal stress decreases steroid aromatase activity in brains of male and female rat fetuses. Neuroendocrinology. **35**: 374–379.
41 Wood RI, Ebling FJ, l'Anson H, Bucholtz DC, Yellon SM, Foster DL (1991). Prenatal androgens time neuroendocrine sexual maturation. Endocrinology. **128**: 2457–2468.
42 Wood RI, Mehta V, Herbosa CG, Foster DL (1995). Prenatal testosterone differentially masculinizes tonic and surge modes of luteinizing hormone secretion in the developing sheep. Neuroendocrinology. **62**: 238–247.
43 Wood RI, Foster DL (1998). Sexual differentiation of reproductive neuroendocrine function in sheep. Rev Reprod. **3**: 30–40.