

Gene expression profiles of estrogen receptors α and β in the fetal bovine hypothalamus and immunohistochemical characterization during development

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Abstract Steroid hormones intervene in the structural and functional regulation of neuronal processes during development and thus determine brain differentiation. The effects of estrogens are mediated by two transcription factors, namely estrogen receptor α (ER- α) and estrogen receptor β (ER- β), that regulate the expression of target genes through their binding to specific DNA target sequences. We describe the mRNA expression of ER- α and ER- β in the hypothalamus of developing male and female bovines as revealed by quantitative real-time polymerase chain reaction, and the distribution of the two ERs in hypothalamic sections of all fetal stages as shown by immunohistochemistry. The expression profiles of the mRNAs of both ERs are mutually correlated throughout the gestation period, and their levels increase significantly in the last stages of gestation. No sexual differences in the mRNA expression of either ER- α or ER- β have been found in our fetal specimens. The use of specific antisera against ER- α and ER- β has allowed us to characterize and confirm the distribution of these receptors in the hypothalami of all fetal stages considered. Our results offer detailed information concerning the distribution of ER- α and ER- β in the developing bovine hypothalamus and provide additional insights into the processes involved in the hypothalamic development of a mammal with a long gestation and a highly gyrencephalic brain.

Keywords ER- α · ER- β · Hypothalamus · Expression profile · Bovine

Introduction

During ontogenesis, the neurosteroid 17 β -estradiol (E2) plays an important role in the differentiation of several brain structures and is responsible for the induction of sexual differentiation of the central nervous system (CNS; MacLusky and Naftolin 1981; Arnold and Gorski 1984). As has long been established, androgens in the male CNS are converted to estrogens by the enzyme aromatase, a common feature shared by all mammalian and avian models studied so far (Lephart 1996). In the female CNS, the effects of estrogens are prevented by circulating α -fetoprotein, which sequesters most plasma estrogens, preventing their conversion by aromatase (Lenz and McCarthy 2010). Recent evidence, however, indicates that estradiol also participates directly in the regulation of brain sexual differentiation in females (Bakker and Brock 2010; Brock et al. 2011). The action of E2 is mediated mainly by nuclear estrogen receptors (ERs) called ER- α and ER- β (Mermelstein and Micevych 2008). Like other steroid hormone receptors, ERs regulate transcriptional activation, binding as dimers to specific DNA sequences called EREs (estrogen-responsive elements; Sharma and Thakur 2006). Upon estradiol binding, the activated ERs induce gene expression and modulate the development of an array of targets within the brain. Both ERs are expressed in many brain areas during ontogeny (Zhao et al. 2008), and the local estrogen production intervenes in the regulation of several morpho-functional aspects of the CNS, influencing the organization of neural circuits.

Numerous studies have demonstrated that E2 is trophic and protective during embryogenesis, and that it regulates the maturation of distinct brain structures (Fan et al. 2010). At

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the cellular level, the activation of ERs can induce the growth of axons and the proliferation of dendritic arborizations, stimulate the formation of new synapses, influence cell migration, and regulate the apoptotic process (Asimiadou et al. 2005). Evidence suggests that distinct pathways exist by which estrogens can regulate biological processes via ERs (Hall et al. 2001). In the classical model, ligand-activated ERs bind to DNA specifically at the EREs and bring co-regulators to the transcription start site. In addition, estrogens might elicit effects through non-genomic mechanisms by binding to ERs localized on the plasma membrane of target cells (Razandi et al. 2004; Vasudevan and Pfaff 2008).

In general, estradiol and its ERs have a profound effect on the nervous system, influencing the sexual differentiation of the brain (Gorosito et al. 2008; Mouriec et al. 2009; Schaub et al. 2008; Yamada et al. 2009; Peruffo et al. 2008b) and the central control of reproductive behavior (Pfaff et al. 2000) at the level of the hypothalamus, the most sensitive brain area involved in sexual dimorphism. During pregnancy, the undifferentiated nervous system is sexually bipotential, and exposure to estrogens during a specific perinatal temporal window determines the sexual phenotype of some hypothalamic nuclei and circuits, promoting gender-specific behavioral patterns in adults (Lephart 1996). Structural variations include differences in the volume and shape of some nuclei and the different neurochemical features of neural populations. For example, prenatal estradiol in male rats promotes apoptosis in the anteroventral periventricular nucleus and inhibits it in the sexually dimorphic nucleus of the preoptic area (Lenz and McCarthy 2010). In both the arcuate nucleus and the preoptic area, E2 induces a higher morphological complexity of glial cells inhibiting neuronal spinogenesis in the former but promoting it in the latter (Arai et al. 1996). In females, E2 instead exerts its effects mostly in the postnatal period on the same nuclei of the preoptic area (Arai et al. 1996). The different behavior of male and female individuals is based on the neuroanatomical and neurochemical organization of key areas of the brain. In the hypothalamus, the presence of sexually dimorphic nuclei has been reported in all vertebrates studied so far (Panzica et al. 2001), and groups of sexually dimorphic neurons have been found in the preoptic area (Bleier et al. 1982; Panzica et al. 1995, 1996) in which the influence of steroids on the fetal brain eventually promotes gender-specific behavior in adults.

Most knowledge concerning the molecular mechanisms of sexual differentiation in the brain relies upon rodent models, with little (if any) information being available for other mammals. In a previous work, we have characterized the expression and localization of ERs in the fetal bovine frontal cortex (Peruffo et al. 2011), and a few data are available on the expression and distribution of ER- α and ER- β in the developing ovine brain (Schaub et al. 2008). However, no

information has been presented on the ontogenesis of the bovine hypothalamus.

In the present work, we have used the bovine (*Bos taurus*) as an alternative model to laboratory rodents. Our animal model might be convenient for explaining the role of steroids during brain differentiation because of its long duration of gestation (9 months) and its large and highly gyrified cerebral cortex. Bovine tissues can be obtained at commercial slaughterhouses and thus allow a considerable saving of experimental animal lives.

Whereas little doubt remains regarding the existence of ER- α and ER- β , their specific roles in the nervous system remain difficult to explain in a physiological context. Here, we have compared the expression profiles of ER- α and ER- β mRNAs in the fetal bovine hypothalamus of both genders throughout prenatal development. We have also investigated the localization of both receptors by immunohistochemistry in representative individuals of all developmental stages.

Materials and methods

Tissue sampling

Fetuses were obtained at nearby commercial abattoirs upon the accidental slaughtering of pregnant cows. Animals were treated according to the European Communities Council directive 86/609/EEC concerning animal welfare during the commercial slaughtering process and were constantly monitored under mandatory official veterinary medical care.

Hypothalami were collected under sterile conditions from a series of bovine fetuses (for details, see Table 1). The fetal age of the specimens was determined based on accurate measurements of the fetal crown-rump length by using widely accepted reference tables (McGeady et al. 2006). Specimens were grouped into four fetal stages (four quarters), each corresponding to approximately 10 weeks (total duration of gestation is 38 weeks). The presence of the anterior commissure, the shape of the third ventricle, and the enlargement of the single mammillary body were used as landmarks for uniform tissue sampling.

For the real-time polymerase chain reaction (PCR) analysis, samples from the hypothalami were stored in RNAlater (Qiagen, Hilden, Germany) at -20°C in order to preserve mRNA for subsequent retrotranscription. For the immunohistochemical analysis, the bovine hypothalami were fixed by immersion in 4 % buffered formalin and maintained at 4°C for at least 1 week. Each sample was then washed in 0.1 M phosphate-buffered saline (PBS), pH 7.4, processed for paraffin embedding, and cut into a series of coronal sections at a thickness of 7–8 μm . As a topographical reference, every tenth section was Nissl-stained to improve the localization of the hypothalamic boundaries.

Table 1 Fetal stages

Fetal stage (weeks of gestation)	Number of fetuses	Crown-rump length (cm)	Male fetuses	Female fetuses
1 (0–10)	6	<9.5	3	3
2 (10–20)	11	9.5–3.1	7	4
3 (20–30)	7	31–61	4	2
4 (30–40)	7	>61	3	4

Quantitative real-time PCR

To quantify gene expression, we performed relative qRT-PCR (quantitative real-time PCR). Total RNA extraction from hypothalamic samples was performed with Trizol (Invitrogen, Carlsbad, Calif., USA) following the manufacturer's instructions. The isolated RNA was resuspended in diethylpyrocarbonate-treated water, treated with DNase I (Invitrogen), and stored at -80°C . The quality of the RNA was assessed by ethidium-bromide-stained agarose gel electrophoresis, and the amount of total RNA was quantified by UV absorbance at 260 nm. Reverse transcription was performed by using Reverse Transcriptase (SuperScript III, Invitrogen) on 2 μg total RNA with random hexamers in accordance with the manufacturer's instructions. qRT-PCR was carried out on a LightCycler 124 480 Instrument (Roche Applied Science, Mannheim, Germany). The primer sequences (Table 2) were designed by using ProbeFinder software (Roche Diagnostics). This software automatically provides the best primer sequences and couples them to the appropriate Universal Probe Library (UPL) probe (Roche; Bakker 2006).

The reaction mixture for qRT-PCR (Lightcycler DNA Master Hybridization Probes mixture, Roche Molecular Biochemicals), containing reaction buffer, nucleotides, and *Taq* polymerase, was prepared according to the manufacturer's instructions. The following components were then added to the reaction mixture: UPL probes at a final concentration of 0.1 μM ; sense primers (200 nM); antisense primers (200 nM); 2 μl cDNA (80 ng/ μl); H_2O to a final volume of 20 μl . The PCRs were cycled as follows: 95°C for 10 min (1

Table 2 Oligonucleotide primers used for quantitative real-time polymerase chain reaction (*ER* estrogen receptor, *Fwd* forward, *Rev* reverse)

Target	Primer sequence	Universal probe library
ER- α	Fwd–GAGCGCGAGGAGTACGTT	81
	Rev–TGTGCACAGAGTCTGAATTGG	
ER- β	Fwd–CGTGACATCAAGGAGAAGCTC	8
	Rev–CCCGTCAGGAAGCTCGTAG	
β -Actin	Fwd–CGTGACATCAAGGAGAAGCTC	81
	Rev–CCCGTCAGGAAGCTCGTAG	

cycle), followed by 40 cycles composed in turn of a denaturation phase (95°C for 10 s), an annealing phase (55°C for 30 s), followed by an extension phase (72°C for 30 s). Relative quantification values of expression were determined for each sample by using the standard curve method, and these values were normalized to the Ct values of β -actin, the “housekeeping” control gene. At least three independent extractions, reverse transcriptions, amplifications, and quantifications were performed.

Immunohistochemistry

Serial coronal sections of fetal hypothalami (two from each embryonic age group) were used for immunohistochemical procedures according to a well-established laboratory protocol based on the avidin-biotin technique (Cozzi et al. 2010). Briefly, after hydration of the sections in graded alcohols, antigen retrieval was carried out in citrate buffer (pH 6) at 120°C in a pressure cooker for 5 min. Inactivation of endogenous peroxidases for 30 min in 3 % H_2O_2 in absolute methanol was followed by saturation with 10 % normal goat serum for 20 min. A primary monoclonal anti-ER- α antiserum raised in mouse (Thermo Scientific, Rockford, Ill., USA; dilution 1:100) or a polyclonal anti-ER- β antiserum raised in rabbit (Thermo Scientific; dilution 1:100) was applied overnight at 4°C . Sections were incubated with a biotinylated anti-rabbit (for ER- β) or anti-mouse (for ER- α) IgG secondary antibody for 1 h (Vector Laboratories, Burlingame, Calif., USA; dilution 1:200) and then with avidin–biotin complex (Vector Laboratories) for another hour. Finally, peroxidase activity was revealed with a diaminobenzidine solution (Vector Laboratories) containing H_2O_2 in TRIS buffer. Sections were dehydrated, cover-slipped with balsam, observed, and photographed via an Olympus Vanox AH-3 photomicroscope. Negative controls were performed by replacing the primary antibodies with normal swine serum. Under these conditions, no immunostaining was observed.

Statistical analysis

Statistical analysis was performed with the following statistical methods. A preliminary correlation analysis followed by two-way analysis of variance (ANOVA) was used to test for significant influences of gestational time and gender on gene expression of ER- α , and ER- β . The Pearson correlation coefficient was calculated to evaluate a possible linear relationship between time and gene expression profiles or between pairs of expression profiles. Post-hoc ANOVA pair-wise comparisons were performed by using Bonferroni's test (Montgomery 2004). The statistical software Minitab (release Minitab 14.13) was employed for all analyses. A significance level of 5 % was used to reject the null hypothesis. Values are reported as means \pm SEM.

Results

Analysis by qRT-PCR

The expression profiles of ER- α and ER- β were charted by using qRT-PCR (Fig. 1) and by grouping fetal bovine hypothalami into four fetal stages (4 quarters), each corresponding to approximately 10 weeks (as reported by Peruffo et al. 2008b; for an overall gestation time of 9 months, see Table 1). The results demonstrated that both ER- α and ER- β mRNAs were expressed in the fetal hypothalamus throughout gestation.

First, on considering gestation time and gene expression, the Pearson correlation index (r) showed that the expression patterns of both ERs were significantly correlated with gestation time (for ER- α , $r=0.65$, $P \sim 0$; for ER- β , $r=0.38$, $P=0.038$), underlining a direct association between gestational time and ER expression during fetal hypothalamic development.

The ANOVA F-test results confirmed the significant effect of fetal stages on the expression of both ERs (for ER- α , $P=0.008$; for ER- β , $P=0.025$), whereas no difference was shown between genders (for ER- α , $P=0.846$; for ER- β , $P=0.231$), and no interaction was found between fetal stage and gender (for ER- α , $P=0.603$; for ER- β , $P=0.439$; Fig. 1).

Post-hoc comparisons, performed in order to determine those fetal stages that were relevant for the variations in ER expression and with Dunnett simultaneous tests as controls, indicated that the increase was significant on comparing the first stage (as control level) *versus* the third and fourth fetal stage; specifically, the P -values for ER- α were $P_{1vs3}=0.025$ and $P_{1vs4}=0.005$; and for ER- β , they were $P_{1vs3}=0.008$ and $P_{1vs4}=0.014$ (see Fig. 2a, b). These data therefore indicated that the mRNA expression of both ER- α and ER- β in the bovine fetal hypothalamus increased in the last stages of gestation.

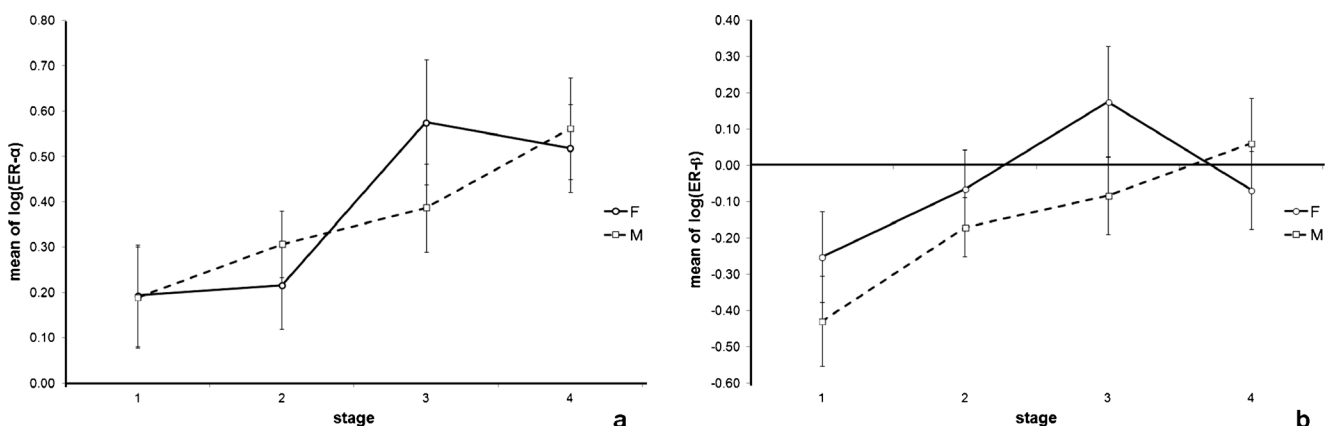


Fig. 1 Expression profile trend (mean values) of estrogen receptors (ER) by gender (F female, M male). **a** ER- α , **b** ER- β (y-axis male and female relative mRNA levels of ERs, x-axis fetal stages of development as

Immunohistochemical results

The topography and the cytoarchitectonic organization of the fetal bovine hypothalami described here was based on the analysis of Nissl-stained coronal sections (Fig. 3a, d, g, j).

The immunohistochemical analysis showed immunoreactivity for both ER- α and ER- β in the specimens from all the four fetal stages (Fig. 3).

In the first stage, immunoreactivity for both ERs was relatively faint, with ER- α apparently being limited to a small perinuclear region, both in the para- and the periventricular zones, whereas ER- β labeling seemed to be more diffuse and weaker in the cytoplasm and in the suprachiasmatic area (Fig. 3b, c).

In the second and third stages, immunoreactivity for both ERs was stronger than in less advanced specimens. In the basal hypothalamus, many ER- α -positive radial fibers were noted extending across the whole thickness, during the second stage (Fig. 3f), in the paraventricular, periventricular and suprachiasmatic areas. Immunopositivity in the medial preoptic area or in the medio-lateral hypothalamus was limited to single isolated cells. In the third stage, we observed few but strongly labeled ER- α cell bodies (Fig. 3i), and ER- β bipolar neurons could be recognized in the periventricular region (Fig. 3h). The number of positive elements in the medial preoptic area remained minimal and allowed no clear identification of a possible sexually dimorphic nucleus.

In the fourth stage, positive cells were more abundant than in the previous stages for both ERs. ER- α -positive cell bodies were clearly evident, as were some fibers, and in the dorsal hypothalamic region, the labeling for ER- β seemed to be localized to a distinct nucleus and in the ependyma of the third ventricle.

gestation quarters, bars standard error of the means calculated from the mean square error by analysis of variance)

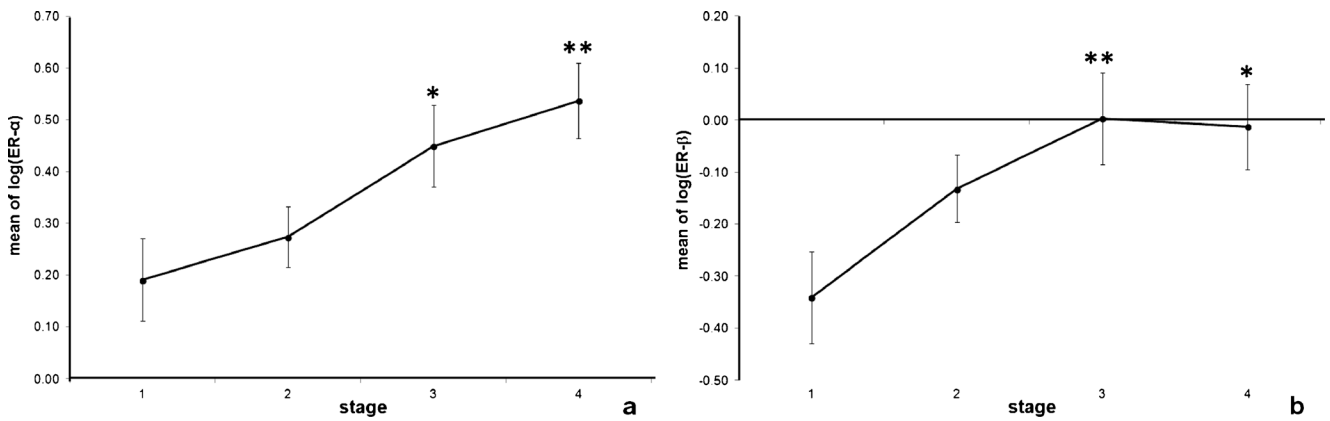


Fig. 2 Expression profile trend (mean values) of ERs. **a** ER- α , **b** ER- β (*y*-axis relative mRNA levels of ERs, *x*-axis fetal stages of development in gestation quarters, *bars* standard error of the means calculated from the

mean square error by analysis of variance). *Statistically significant increase at 5 %. **Statistically significant increase at 1 %

Discussion

The present investigation confirms that ER- α and ER- β are expressed in the bovine fetal hypothalamus during development. The expression patterns of both ERs are

correlated with gestational time, and their levels increase significantly during the last fetal stages, if both sexes are pooled together. Quantitative analyses of mRNA expression have demonstrated no differences between the sexes with regard to both ERs. The immunohistochemical

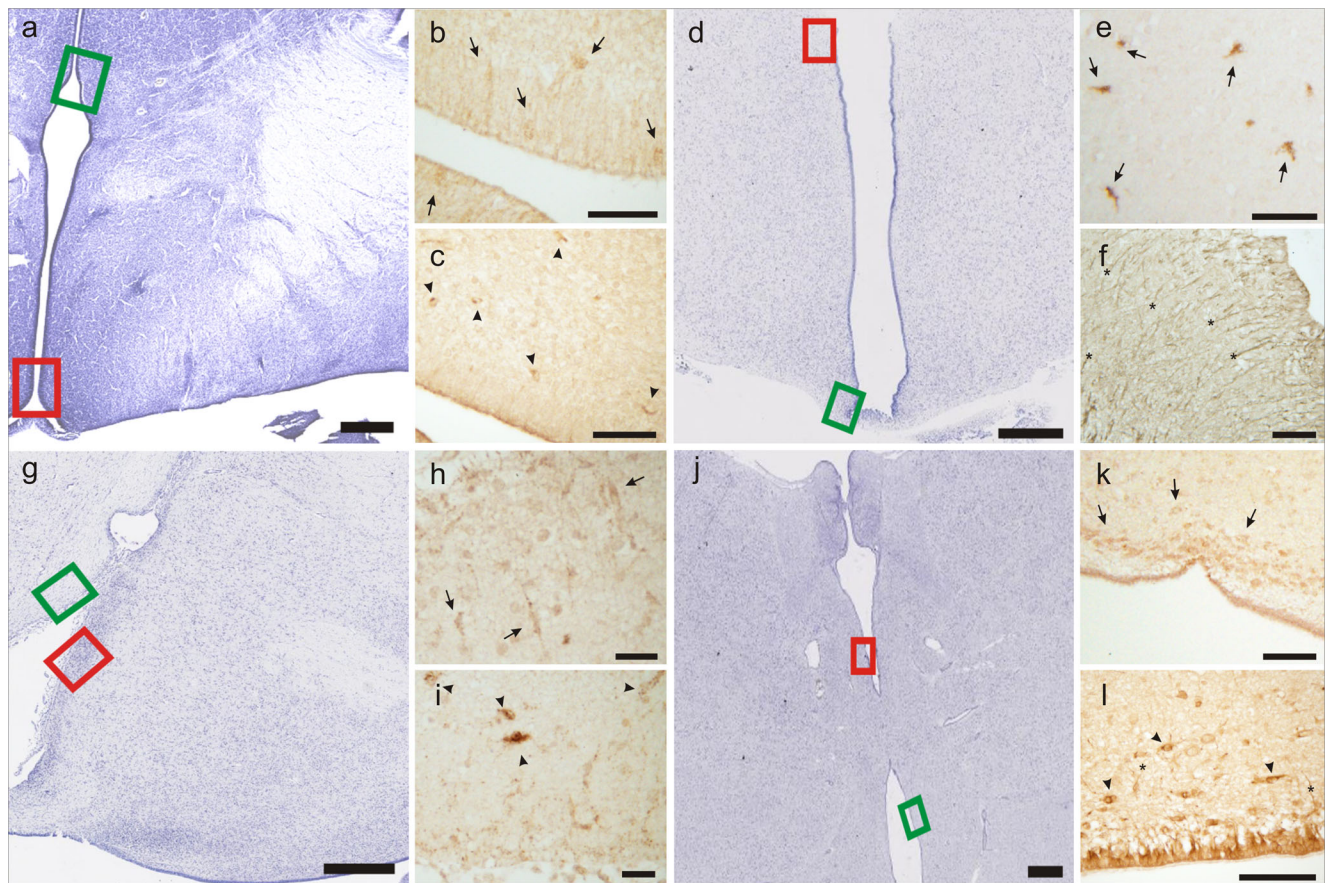


Fig. 3 Histology and immunohistochemistry of bovine fetuses. Representative images for the first (*top left*), second (*top right*), third (*bottom left*), and fourth (*bottom right*) fetal stage, showing a Nissl-stained section of the hypothalamic region (**a, d, g, j**) and corresponding images of immunostaining against ER- β (**b, e, h, k**) and ER- α (**c, f, i, l**). In the

Nissl images, *green and red rectangles* indicate, respectively, the corresponding fields of ER- α - and ER- β -immunostained images (*arrowheads* ER- α -immunoreactive cell bodies, *arrows* ER- β -immunoreactive cell bodies, *stars* ER- β -ir cytoplasmic extensions). *Bars* 20 μ m (**h, i**), 50 μ m (**b, c, e, f, k, l**), 500 μ m (**a, d, g**), 1 mm (**j**)

analysis of the ERs revealed many positive neurons in the hypothalami of all fetal stages, and the labeling of the first stages appeared consistently lower than that in later stages, with a marked increase in the abundance of positive cells in the fourth stage. These findings provide evidence that the co-expression of ERs is important in determining the correct responses of neural populations to estrogen action during the development of the hypothalamus, supporting the importance of ERs in coordinating both classical and rapid actions of estrogen (Ábrahám et al. 2004; Mani et al. 2012). The faintness of the immunostaining in the early fetal stages might be related to the immature organization of the CNS, a finding emphasized by immersion fixation.

As is well known, the action of E2 through its nuclear receptors is a key factor for the development and differentiation of the hypothalamic area and other brain structures (McEwen 2002; Ohtani-Kaneko 2006; Morissette et al. 2008; Melcangi et al. 2011). Most data in the literature refer to the presence of ERs in the hypothalamus of adult mammals, whereas ontogenetic characterizations of ERs in fetal hypothalami are scarce.

A few studies in rodents (Fan et al. 2006; Friedman et al. 1983) have revealed that ER- α is the predominant receptor in the hypothalamus, and that it controls reproduction, whereas ER- β influences non-reproductive processes such as cortical layering, interneuron migration, and brain morphogenesis in general (Fan et al. 2010). In rats, single-cell RT-PCR has revealed the expression of both ER α and ER β isoforms in cultured fetal and adult rat hypothalamic neurons (Hu et al. 2008).

Studies performed in sheep (a species with a relatively long gestation period of 150 days) have indicated that both ERs are expressed in the hypothalamus, and that their expression is developmentally regulated (Schaub et al. 2008). The expression of ERs has also been detected and measured in the fetal brain of primates (Pomerantz et al. 1985). Their levels have been measured in male and female rhesus monkeys at days 70, 100, and 160 post-conception. In this species, no sex differences have been found at any stage of development, but a progressive increase in ER levels has been found to occur in the hypothalamus between the fetal and adult stages (Sholl and Kim 1989). The only data available concerning the expression of ERs in the human fetal brain have revealed that both receptors are present in the cortex (Fried et al. 2004; González et al. 2007).

In a previous work, we characterized the presence of ER- α and ER- β in primary cell cultures from the hypothalamus of fetal bovines (Peruffo et al. 2008a), and in a recent study, we analyzed the profile expression of ERs in the fetal frontal cortex of the same species, showing that both ERs peak at the last fetal stage (Peruffo et al. 2011). However, we had not previously assessed the presence of ERs in the fetal bovine hypothalamus.

A comparison with other species is difficult, since newborn calves and lambs are relatively mature and precociously able to stand, move, and relate to the external world, thus suggesting that the brain of ruminants is more mature at birth than those of neotenic mammals, including experimental rodents. In the fetal brain of rodents, the mRNAs of both ERs increase perinatally (Al-Bader et al. 2008; Prewitt and Wilson 2007); however, no complete ontogenetic data are available on the ER profiles in the fetal hypothalamic area. The distribution of both ER- α and ER- β protein in the brain of adult rats is documented (Perez et al. 2003), but ontogenetic data are lacking.

Immunohistochemical observations in the hypothalamus of postnatal rats have revealed that neurons of supraoptic, paraventricular, suprachiasmatic, and tuberous nuclei exclusively contain ER- β mRNA (Shughrue et al. 1997). Studies on the ovine brain have demonstrated that both ER- α and ER- β are expressed throughout the latter half of gestation, but ER- α protein levels increase throughout the latter half of gestation, whereas ER- β protein levels rise during postnatal days 1 and 7 (Schaub et al. 2008).

Despite the lack of ontogenetic data, the distribution of ERs has been documented in the adult hypothalamus of the domestic pig (*Sus scrofa*). In female pigs, the number of cells showing ER immunoreactivity in this area is higher than that in male pigs (Van Leeuwen et al. 1995).

In long gestation species such as humans, primates, pigs, ovines, and bovines, only a limited number of studies have described the profile expression of ERs in specific brain region during ontogenesis, possibly because of the difficulty in obtaining fresh tissue samples of fetal brain. Here, the immunostaining for both receptors has been shown to be present in the classical neurosecretory nuclei (paraventricular, periventricular, suprachiasmatic), and positivity for ER- β receptor has also been localized in the ependyma of the third ventricle in older individuals. However, immunostaining in the medial preoptic area is too weak or irregular to allow the definition of a possible sexually dimorphic nucleus. The lack of a specific precise atlas for the bovine hypothalamus (for representations, see Yoshikawa 1967) and the relative differences from other species (for the sheep: Richard 1967; Welento et al. 1969; for the goat: Tindal et al. 1968; Zuccolilli et al. 1995) have made comparisons problematic. An explanation of the presence of ER- β receptors in the ependyma requires further investigation in fetuses at the late stages of development.

Interestingly, in the long gestation species, such as ovine and bovine, the significant increase of both the ERs during the last fetal stage overlaps with the increase of the circulating concentrations of estrogens in late gestation (Nathanielsz et al. 1982; Yu et al. 1983; Gabai et al. 2004). This increase in plasma concentrations, combined with the increase of ERs, suggests an amplified estrogen action in estrogen target-

tissues, such as the hypothalamus, during a specific phase of fetal maturation.

Studies on the hypothalamic expression of ERs during development are scarce, and the precise relationships between the co-existence of both receptors and their function remain ill-defined, at least in fetal stages. The present study has been designed to determine the profiles of ER- α and ER- β expression during all fetal stages in the bovine, a mammal in which some ontogenetic characteristics (the long gestation), morphological features of the nervous system (large gyrencephalic brain), and reproductive peculiarities (polyestrous cycle) suggest its suitability for use as a translational model for aspects of sexual dimorphism of the brain. We wish to underline the importance of the bovine as an experimental model, since tissues are readily available in large quantities at slaughterhouses, thus contributing to a reduction of the sacrifice of laboratory animals.

In conclusion, we report data obtained from a comparison of the ontogenetic expression and distribution of two ERs in the fetal bovine hypothalamus during pregnancy. This information provides an anatomic foundation for future studies and advances our understanding of estrogen action in the brain in an alternative animal model used in neuroscience.

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