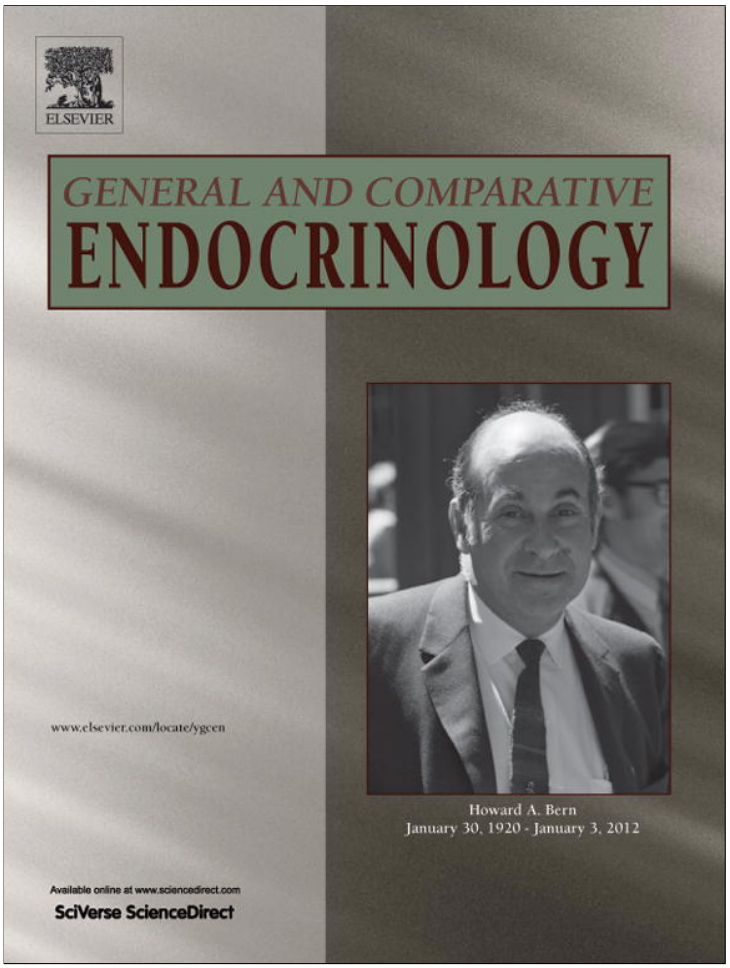

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Sexually dimorphic expression of receptor-alpha in the cerebral cortex of neonatal *Caiman latirostris* (Crocodylia: Alligatoridae)

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ABSTRACT

In mammals, estrogens have been described as endocrine and paracrine modulators of neuronal differentiation and synapse formation. However, the functional role of circulating estrogens and the distribution of estrogen receptors (ERs) in the cerebral cortex of reptiles have not been clearly established. *Caiman latirostris* (*C. latirostris*) is a South American species that presents temperature-dependent sex determination (TSD). By using immunohistochemistry, we have studied the distribution of ERα in the cerebral cortex of neonatal caimans. We studied brain samples from ten-day-old TSD-females and TSD-males and from female caimans that were administered estradiol during embryonic development (hormone-dependent sex determination, HSD-females). ERα was detected in the medial (MC), dorsal (DC) and lateral (LC) cortices. ERα expression in the MC showed sex-associated differences, being significantly greater in TSD-females compared to TSD-males. Interestingly, the highest ERα expression in the MC was exhibited by HSD-females. In addition, the circulating levels of estradiol were significantly higher in females (both TSD and HSD) than in TSD-males. Double immunostaining showed that ERα is expressed by neural precursor cells (as detected by ERα/doublecortin or ERα/glial fibrillary acidic protein) and mature neurons (ERα/neuron-specific nuclear protein). Our results demonstrate that the expression of ERα in the neonatal caiman cortex is sexually dimorphic and is present in the early stages of neuronal differentiation.

1. Introduction

Over the last three decades, strong evidence has emerged demonstrating that the cellular actions of estrogens in the brain are not restricted to areas related to reproduction but rather have widespread effects throughout the developing and adult brain [24]. Estrogens orchestrate cellular mechanisms that are involved in the development and differentiation of various neuronal populations, the modulation of synaptic plasticity and neuronal excitability, the induction of neuronal survival and axonal outgrowth, and adult neurogenesis [23].

Classically, the pleiotropic effects of estrogens are mediated in large part via estrogen receptors (ERs), which are members of the nuclear receptor superfamily and function as hormone-dependent transcription factors [5]. Ligand-binding induces conformational changes in these receptors, leading to dimerisation, protein–DNA interaction, the recruitment of co-regulator proteins and other transcription factors and ultimately the formation of the pre-initiation complex. ERs regulate gene expression by binding to their cognate response element or through protein–protein interactions with other transcription factors [20].

The ontogeny and the sexual differences of ER-containing cells have been described for various brain regions [18,22]. The presence of ERs in brain structures that are not directly related to sexual behaviour suggests that the biological effects of gonadal hormones in the central nervous system (CNS) extends beyond reproduction-related functions and may affect and modify the motor behaviour in a sex-specific manner. In the human and rodent cerebral cortex, differential expression of both ER variants (ERα and ERβ) was detected at different stages of development [29,11]. However, little is known about the expression of ERs in the reptilian brain.

The reptilian cerebral cortex is formed by three principal areas: the medial, dorsal and lateral cortices. In all of the cortical areas,

Abbreviations: ERs, estrogen receptors; TSD, temperature-dependent sex determination; MC, medial cortex; DC, dorsal cortex; LC, lateral cortex; HSD, hormone-dependent sex determination; DCX, doublecortin; GFAP, glial fibrillary acidic protein; Neun, neuron-specific nuclear protein; CNS, central nervous system; CL, cell layer; IPL, inner plexiform layer; OPL, outer plexiform layer; GAM, Gonadal–Adrenal–Mesonephros complexes; IOD, integrated optical density; DAB, diaminobenzidine; Ep, epithelium; LV, lateral ventricle.

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most neuronal cell bodies are grouped to form a principal cell layer (CL) sandwiched between the inner and outer plexiform layers (IPL, OPL), which are populated by scarce interneurons and are where the afferent connections terminate in a highly laminated fashion [33].

Caiman latirostris (*C. latirostris*) is widely distributed in the wetlands and rivers of north-eastern Argentina, southern Brazil, Paraguay, Uruguay and Bolivia. *C. latirostris* is a species with temperature-dependent sex determination (TSD) and is one in which the exposure to 17β-estradiol (E_2), at the sensitive embryonic stage for gonadal sex determination, overrides the effects of male incubation temperatures and produces phenotypical females (a process referred to as hormone-dependent sex determination or HSD) [40]. In addition, caiman reproductive tissues are highly sensitive to the effects of endocrine disrupter compounds [38–40,34,35,6].

In the present study, we evaluated the immunohistochemical localisation of ERα in the cerebral cortex of neonatal *C. latirostris*. Our aims were as follows: to compare the expression of ERα of TSD-females, HSD-females and TSD-males; to assess whether ERα expression exhibits sexually dimorphic patterns; and to determine the immuno-phenotype of ERα-expressing cells.

2. Material and methods

2.1. Animals

All laboratory and field work was conducted according to the published guidelines for the use of live amphibians and reptiles in field and laboratory research [3] and in full compliance with the Universidad Nacional del Litoral Institutional Bioethics in Animal Care and Use Committee. The animals used for this study were also used for previous studies, and the detailed methods concerning egg collection, incubation conditions and treatment procedures can be found in [38,34]. Briefly, *C. latirostris* eggs were collected shortly after oviposition from five nests randomly selected from regions of the Chaco and Entre Ríos Provinces in Argentina during the 2004 and 2005 reproductive seasons. Eggs were transported to the laboratory and placed separately into two groups: half of the eggs from each clutch were incubated at 30 °C (female-producing temperature) and the other half at 33 °C (male-producing temperature). At stage 20 of embryonic development, a subgroup of eggs that were incubated at the male-producing temperature (33 °C) received 1.4 ppm of 17β-estradiol dissolved in absolute ethanol (Sigma Chemical, St. Louis, MO, USA). As the average egg weight was 65.1 ± 6.2 g, the selected dose represents approximately 90 μg/egg [40]. The treatment consisted of applying on the eggshell, at the embryo implantation zone, 50 μl of estradiol solution. The treatment was applied at stage 20 of embryo development, period identified as the window for sex determination in several members of Crocodylia, which was also confirmed for *C. latirostris* [40]. Before treatment, stage 20 was confirmed by opening an egg from the incubator. As expected, following the 17β-estradiol application, we obtained 100% females from eggs incubated at 33 °C. These females were referred to as HSD females.

2.2. Tissue collection and sex determination

At birth, all hatchlings were individually identified, weighed and measured. Ten days after hatching, five caimans of each group (TSD-females, TSD-males and HSD-females) were euthanised with sodium pentobarbital. Blood samples were collected from the trunk, and serum samples were stored at -20 °C for hormone assays. Gonadal–Adrenal–Mesonephros complexes (GAM) and brains were dissected and fixed at room temperature by immersion in 4% phosphate-buffered formalin (pH 7.4) for 6 and 24 h, respectively. Fixed tissues were dehydrated in an ascending series of ethanol, cleared in xylene and embedded in paraffin. Five-micrometre GAM sections were stained with trichromic Picrosirius solution and counterstained with Harris haematoxylin (Biopur, Rosario, Argentina). Sex was determined histologically by the presence of characteristic structures as previously described [40]. Brain sections (5 μm) were stained using Nissl staining to visualise the caiman cerebral cortex.

2.3. Antibodies

2.3.1. Generation and characterisation of the ERα antibody

To detect ERα, we used the affinity-purified rabbit polyclonal antibody LETH-ER-202y that was generated in our laboratory using previously described protocols [35]. The antigens were expressed in E. coli JM109 (Stratagene Corporation, La Jolla, CA, USA) as gluthatione-S-transferase fusion proteins using a pGEX4T-3 vector (Stratagene). The ERα antigen was designed to include the last 74 aa of the C-terminal region corresponding to the *Caiman crocodilus* sequence (Accession Number BAB79436). For immunohistochemical analysis, the antibodies were purified using antigen-linked affinity chromatography (Hi-Trap NHS activated HP column, GE Healthcare, Buenos Aires, Argentina) and were used at a dilution of 1:250. For specificity validation tests, the antigenic peptide was used to preadsorb ERα antibodies by incubating 1 μg of the antibody with 10–20 μg of the peptide for 24 h at 4 °C. For negative controls, the antibody-antigen complexes (pre-adsorbed antibodies) were applied to control tissues in immunohistochemical assays. In addition, the specificity of anti-ERα (LETH-ER-202y) was tested by Western blot analysis of oviductal protein extracts from a juvenile *C. latirostris* female (bw 2115 g). The oviduct was dissected, immediately frozen in liquid nitrogen and maintained at −80 °C until the protein was extracted. For the extraction of protein, the tissue was homogenised in ice-cold lysis buffer [1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 2 mmol/l EDTA, 50 mmol/l sodium fluoride, 1 mmol/l sodium vanadate, 0.048 mg/ml aprotinin, 0.0086 mg/ml leupeptin, 0.008 mg/ml pepstatin and 0.1 mg/ml PMSF (all from Sigma, USA) in phosphate-buffered saline, pH 7]. The resulting homogenate was incubated on ice for 30 min and centrifuged at 4 °C for 20 min at 14,000 g, and the supernatant was collected. The concentration of the extracted protein was determined by using a BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). Fifty milligrams of protein per lane along with a molecular weight marker (Promega Corporation, Madison, WI, USA) were resolved on an SDS–polyacrylamide gradient gel (10–17%) for 1.5 h at 25 mAmp and transferred onto a nitrocellulose membrane (Protoblot; Schleicher and Schuell, USA). Nitrocellulose membranes were blocked with 5% w/v non-fat dry milk in Tris-buffered saline-Tween (TTBS; 25 mM Tris, 0.14 M NaCl and 0.05% v/v Tween-20, pH 7.4) for 3 h at 37 °C to prevent non-specific binding. Membranes were rinsed with TTBS (0.5 M NaCl) and incubated with anti-ERα (LETH-ER-202y) diluted in 2% non-fat dry milk in TTBS 1:200 overnight at 8 °C. After incubation with the primary antibody LETH-ER-202y, the membrane was rinsed again with TTBS (0.5 M NaCl) as previously described and incubated with a peroxidase-labelled anti-rabbit secondary antibody (diluted 1:250 in 2% non-fat dry milk in TTBS) for 1 h and 30 min at 37 °C. The membrane was rinsed again in TTBS (0.5 M NaCl) and developed using 0.5 mg/ml diaminobenzidine (DAB; Sigma) for 10 min. Negative controls were performed with lanes not exposed to the primary antibodies.

2.3.2. Antibodies to establish a cortical cell immunophenotype

Commercially-available antibodies and morphological features were used to confirm the phenotype of ERα expressing cells. NeuN,

GFAP and DCX proteins were used as markers of mature neurons, glial cells and young migrating neurons, respectively. NeuN antibody (clone MAB377, 1:400 dilution. Chemicon International, Temecula, CA, USA) specifically recognises the DNA-binding region of neuron-specific protein NeuN, which is present in most central and peripheral nervous system postmitotic neurons of all vertebrates tested (avian, chicken, ferret, human, mouse). The anti-glial fibrillary acidic protein antibody (clone GFP/6F2, 1:300 dilution) was purchased from Novocastra (Newcastle upon Tyne, UK), this antibody was generated against the highly conserved intermediate filament protein of astrocytes and glial cells. The anti-doublecortin (DCX, sc-8066) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and is recommended for detection of DCX of mouse, rat and human origin, but also reactive with additional species, including equine, canine, bovine, porcine and avian.

The anti-rabbit and anti-mouse secondary antibodies (biotin conjugate, 1:200 dilution) were purchased from Zymed (San Francisco, CA, USA) and Sigma, respectively, and the anti-goat secondary antibody (biotin conjugate, sc-2042, 1:200 dilution) was purchased from Santa Cruz Biotechology. For dual immunofluorescence staining, the secondary antibodies used were AlexaFluor 488 goat anti-rabbit (green) (A-11034, 1:100 dilution, Invitrogen) and TRITC-conjugated anti-mouse (115-025-003, red, 1:200 dilution) (Jackson ImmnoResearch, West Grove, PA, USA).

2.4. Immunohistochemistry

Brain and GAM sections (5 μm in thickness) were removed from paraffin and dehydrated in a graded ethanol series. Microwave pretreatment for antigen retrieval was performed as described previously and according to routine immunohistochemical procedures [27]. Endogenous peroxidase activity and non-specific binding sites were blocked. Primary antibodies were incubated overnight at 4 °C. After incubation with biotin-conjugated secondary antibodies for 1 h, the reactions were developed using a streptavidin–biotin peroxidase method and diaminobenzidine (Sigma) as a chromogen substrate. Samples were mounted with permanent mounting medium (PMyR, Buenos Aires, Argentina). Negative controls that replaced the primary antibody with non-immune goat serum (Sigma) were performed for each immunohistochemical staining.

2.5. Quantification of ERα expression by image analysis

Tissue sections were evaluated using an Olympus BH2 microscope (illumination: 12 V halogen lamp, 100 W, equipped with a stabilised light source) with a Dplan 40X objective (numerical aperture = 0.65) (Olympus). To measure the integrated optical density (IOD) of ERα immunostaining, image analysis was performed using the Image Pro-Plus 4.1.0.1® system (Media Cybernetics, Silver Spring, MA, USA) as previously described [30,31]. In brief, the images were recorded with a Spot Insight V3.5 colour video camera attached to the above-mentioned microscope. The evaluation of expression was performed individually on the medial, dorsal and lateral cortices of brain sections (at least 5 fields from each cortical region were recorded). From each caiman, at least three 5-μm sections obtained 150 μm apart from each other were evaluated. The microscope was set up properly for Koehler illumination. Correction of unequal illumination (shading correction) and the calibration of the measurement system were performed with a reference slide. The images of immunostained slides were converted to greyscale. Using the Auto-Pro macro language, an automated standard sequence operation was created to measure the IOD as a linear combination between the average grey intensity and the relative area occupied by positive cells. Because IOD is a dimensionless parameter, the results are expressed as arbitrary units.

2.6. Dual immunohistochemistry

To establish the immunophenotype of ERα-positive cells, dual immunohistochemistry was performed. Young migrating neurons were detected with antibodies against DCX, a microtubule-associated protein present in migrating neuroblasts and during the maturation of developing neurons [21]. NeuN protein was used to evaluate post-mitotic neurons, and anti-GFAP was used to detect astrocytes and neuronal precursors.

2.6.1. ERα/GFAP and ERα/DCX

After ERα immunostaining, sections were microwave heated (1 min at the maximum power setting, followed by 4 min at 40% of full power and 10 additional min after the power was turned off to wash out any remaining antibody from the previous assay). Sections were rinsed with phosphate buffered saline (PBS) and pre-incubated again with normal goat antiserum for 30 min. The samples were incubated overnight at 4 °C with the second primary antibody: either anti-GFAP or anti-DCX. Reactions were developed using a streptavidin–biotin–peroxidase method. Visualisation of these antigens was achieved using the nickel-intensified DAB technique [43]. The DAB solution (2.3 mg DAB, 4 ml 0.05 M Tris–HCl buffer [pH 7.5], 15 μl 30% H_2O_2, and 460 μl 1% nickel chloride) was added to the samples. The slides were counterstained with Mayer haematoxylin (Biopur, Rosario, Argentina) and mounted with permanent mounting medium. ERα-positive cells exhibited brown-stained nuclei and black-stained cytoplasm (GFAP or DCX).

2.6.2. ERα/NeuN

A procedure based on immunofluorescence was used as follows: brain sections were removed from paraffin, rehydrated and submitted to microwave antigen retrieval [42]. To minimise non-specific background staining, sections were blocked for 1 h with normal goat serum (Sigma). The incubation with primary antibodies was performed overnight at 4 °C. The secondary antibodies (1:200 dilution, Jackson ImmunoResearch Laboratories, Inc.) were incubated for 1 h, and the sections were washed for a total of 45 min with three rinses of PBS. Finally, all sections were washed in PBS, mounted with Vectashield fluorescent mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA) and stored in the dark at 4 °C. Controls included sections incubated using primary antibody buffer solution (3% BSA, 0.1% Tween 20 in PBS) in place of the primary antibody to control for non-specific staining. All immunostained sections were examined using an Olympus BX-51 microscope equipped for epifluorescence detection and with the appropriate filters (Olympus). Images were recorded using a high-resolution USB 2.0 digital colour camera (QImaging® Go-3, QImaging, Surrey, BC, Canada).

2.7. Hormone assays

Serum levels of estradiol were determined by a radioimmunoassay (RIA) using E_2, [2,4,6,7,16,17–3H (N)] (PerkinElmer Life And Analytical Sciences Inc., Boston, USA) and specific antibodies provided by Dr. G.D. Niswender [8]. Three successive extraction steps with 2 ml of ethyl ether as solvent (Merck, Buenos Aires, Argentina) from 600 μl of serum were made (Lacau-Mengido et al., 2000; Kass et al., 2004). The organic fractions (rich in steroid) of each sample were separated, pooled, and evaporated to dryness. The dry extracts were resuspended in PBS with the addition of sodium azide and gelatin (Sigma) and the level of E_2 was assayed with the labelled $E_2(^3H)$ and the antiserum using an standard curve made with calibrators with different known concentrations of the steroid. Radioactivity was read in a scintillation counter (WinSpectral, PerkinElmer). Furthermore, to determine the extraction efficiency, a known amount of tritium labelled hormone was added to 600 μl of dextran coated charcoal stripped caiman serum, and

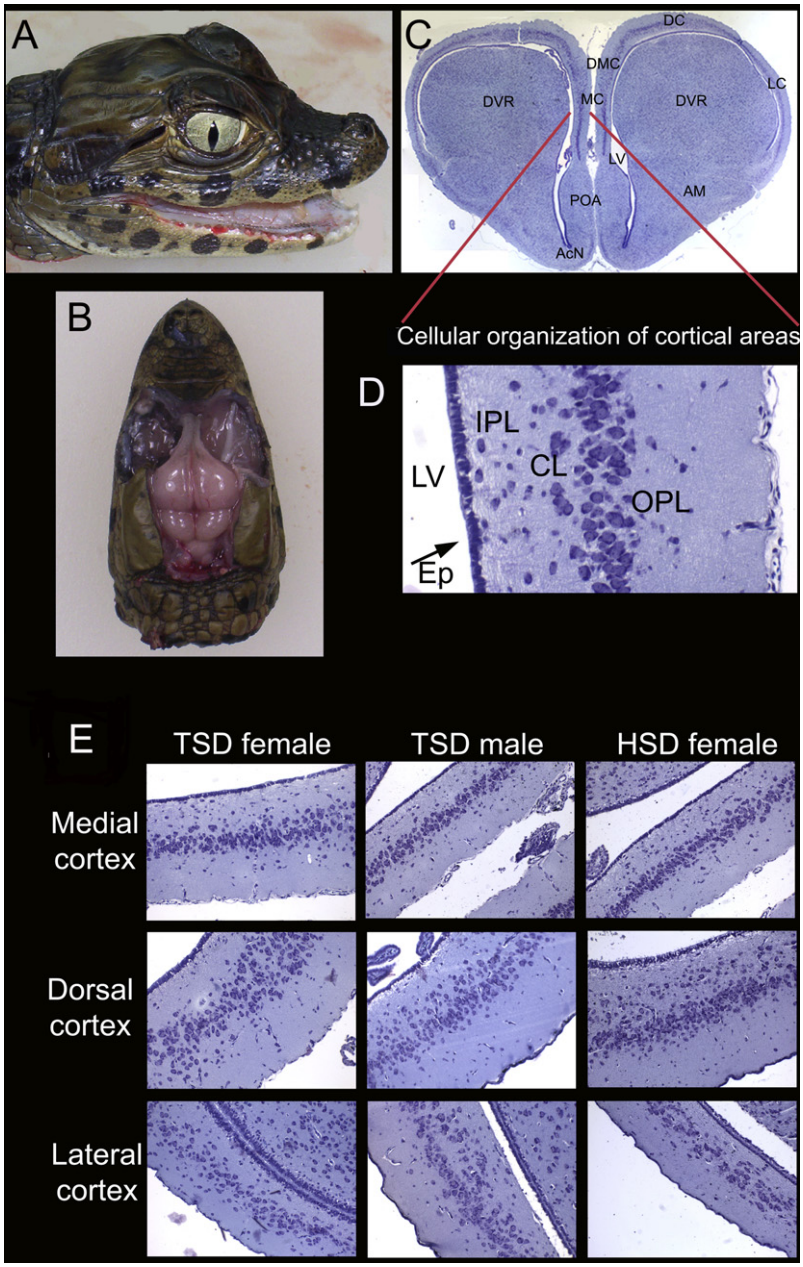

Fig. 1. The cerebral cortex of neonatal *C. latirostris*. Macroscopic images of the head: (A) lateral and (B) dorsal views (Snout-to-first cervical vertebra length: 3 cm). (C) Low magnification image of a 5 μm transversal section of the brain. Note the different cortical areas lining the lateral ventricle (LV). (D) High magnification image showing the cellular organisation of the medial cortex. Most neuronal nuclei are grouped in a principal cell layer (CL) between the inner and the outer plexiform layers (IPL, OPL, respectively). The arrow points to the ependymal epithelium (Ep). (E) Representative photomicrographs from the medial, dorsal and lateral cortices of TSD-females, TSD-males, and HSD-females. MC: medial cortex; DMC: dorso-medial cortex; DC: dorsal cortex; LC: lateral cortex; DVR: dorsal ventricular ridge; POA: preoptic area; AM: amygdala; AcN: accumbens nucleus. Magnification: (C) X40, (D) X600, (E) X400.

then was subjected to the extraction process and resuspended. This solution was then mixed with scintillation fluid and the amount of radioactivity recovered was measured. The percent recovery was 90.2%. The sensitivity was 1.6 pg/ml and the coefficients of variation intra-and inter-assay were 2.89% and 3.85%, respectively.

2.8. Statistical analysis

The data are reported as the mean ± SEM. Kruskal–Wallis analysis was performed to obtain the overall significance followed by Dunn's post-test to establish whether the ERα protein expression was different between experimental groups. $P < 0.05$ was accepted as significant.

3. Results

3.1. Cerebral cortex structure of C. latirostris on postnatal day 10

The structure of the cerebral cortex is shown in Fig. 1. As with other reptiles, the cerebral cortex of *C. latirostris* has a single, dense layer of neurons (CL) close to the lateral ventricle. The cortex is divided into different areas: the medial, dorso-medial, dorsal and lateral cortices (MC, DMC, DC and LC, respectively) (Fig. 1C). In these areas, the neuronal cell bodies are grouped in a main CL between inner and outer plexiform layers (IPL and OPL, respectively), where axonal afferents end in a highly laminated fashion (Fig. 1D). In addition, a scarce but complex population of interneurons are

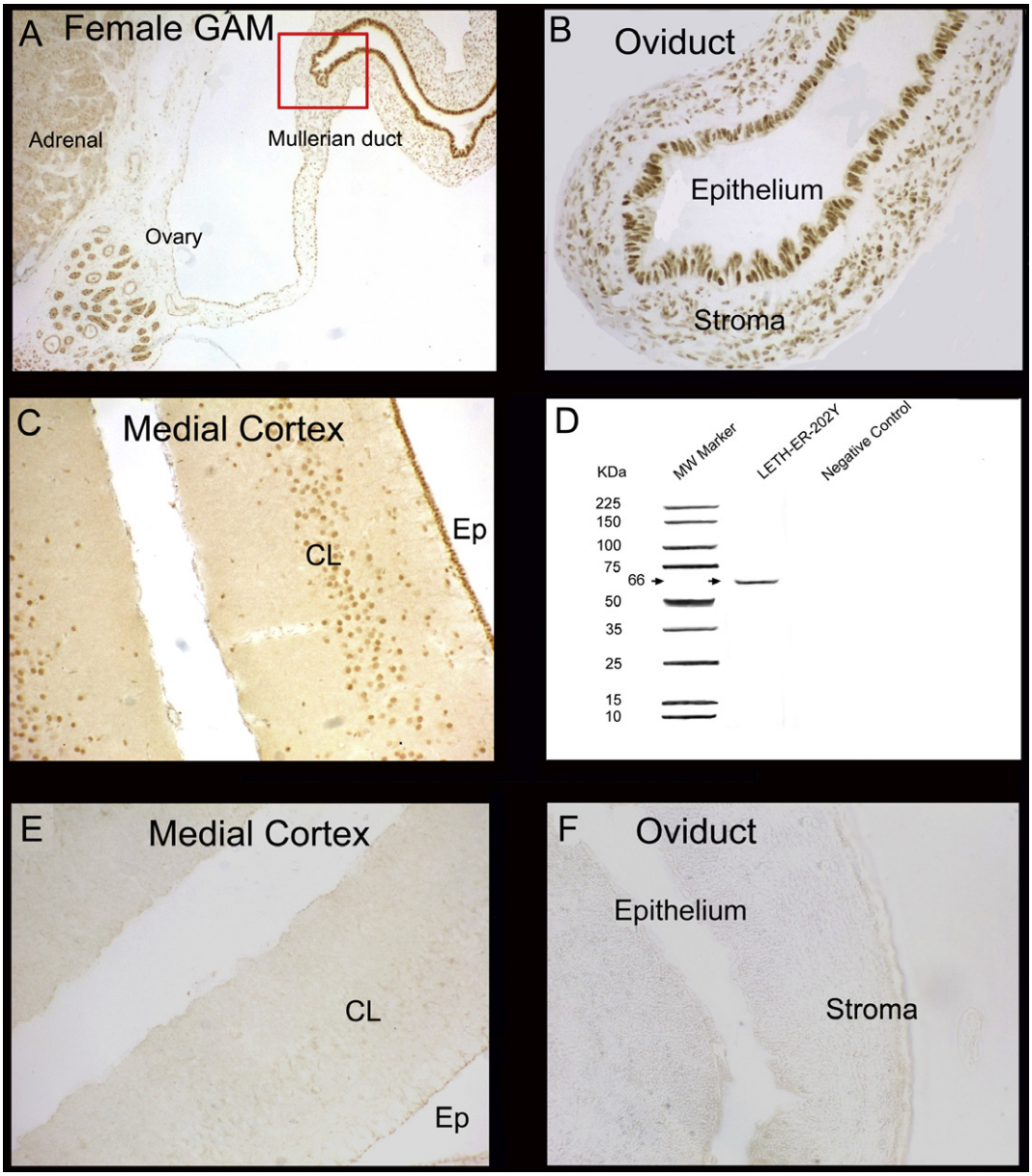

Fig. 2. Characterisation of the ERα antibody. (A) The neonatal female oviduct was used as a control tissue. The oviduct is attached dorsolaterally to the gonad by the fibrous mesosalpinx. (B) ERα was detected as a nuclear protein in oviductal epithelial and stromal cells. (C) In the medial cortex of a TSD-female, the ERα protein is abundantly expressed in the cellular layer (CL) and the ependymal epithelium (Ep). In the CL both nuclear and perinuclear patterns of ERα staining were observed in most of the cells. (D) Western blot assay shows that the antibodies specifically detect a protein of 66 kDa. (E) and (F) Negative control, samples were incubated with the pre-adsorbed primary antibody. Magnification: (A) ×100, (B and F) ×600, (C and E) ×200.

distributed in the CL. All areas have an ependymal epithelium (Ep) lining the lateral ventricle (LV). Representative photomicrographs of the MC, DC and LC in TSD-females, TSD-males and HSD-females are shown in Fig. 1E, with a similar structure in all animals.

3.2. Characterization of the ERα antibody

The polyclonal antibody generated against ERα (LETH-ER-202y) was successfully used in Western blot and immunohistochemistry studies (Fig. 2). As shown in Fig. 2A and B, the LETH-ER-202y antibody was suitable for detecting ERα expression in paraffin-embedded caiman oviducts, a tissue known to be ERα-rich. ERα nuclear protein was detected in oviductal epithelial and stromal cells. The oviductal luminal epithelium exhibited both a strong intensity and a high percentage of ERα-positive cells. As shown in Fig. 2D, an immuno-positive band of approximately 66 kDa was detected in the *C. latirostris* oviduct using the anti-ERα (LETH-ER-202y) antibody; this band was absent when the primary antibody was omitted (negative control). In addition, this specific staining was absent when the primary antibody was pre-incubated with the peptide used as an immunogen (Fig. 2E and F).

3.3. Sex-associated differences in ERα expression in the cerebral cortex of C. latirostris

The expression of ERα was studied using an immunohistochemical approach. Our results showed that ERα is present in all of the cortical areas of the neonatal caiman brain. While all layers (CL, IPL and OPL) contained ERα-positive cells, the CL showed the highest level of expression. Moreover, most of the epithelial nuclei were

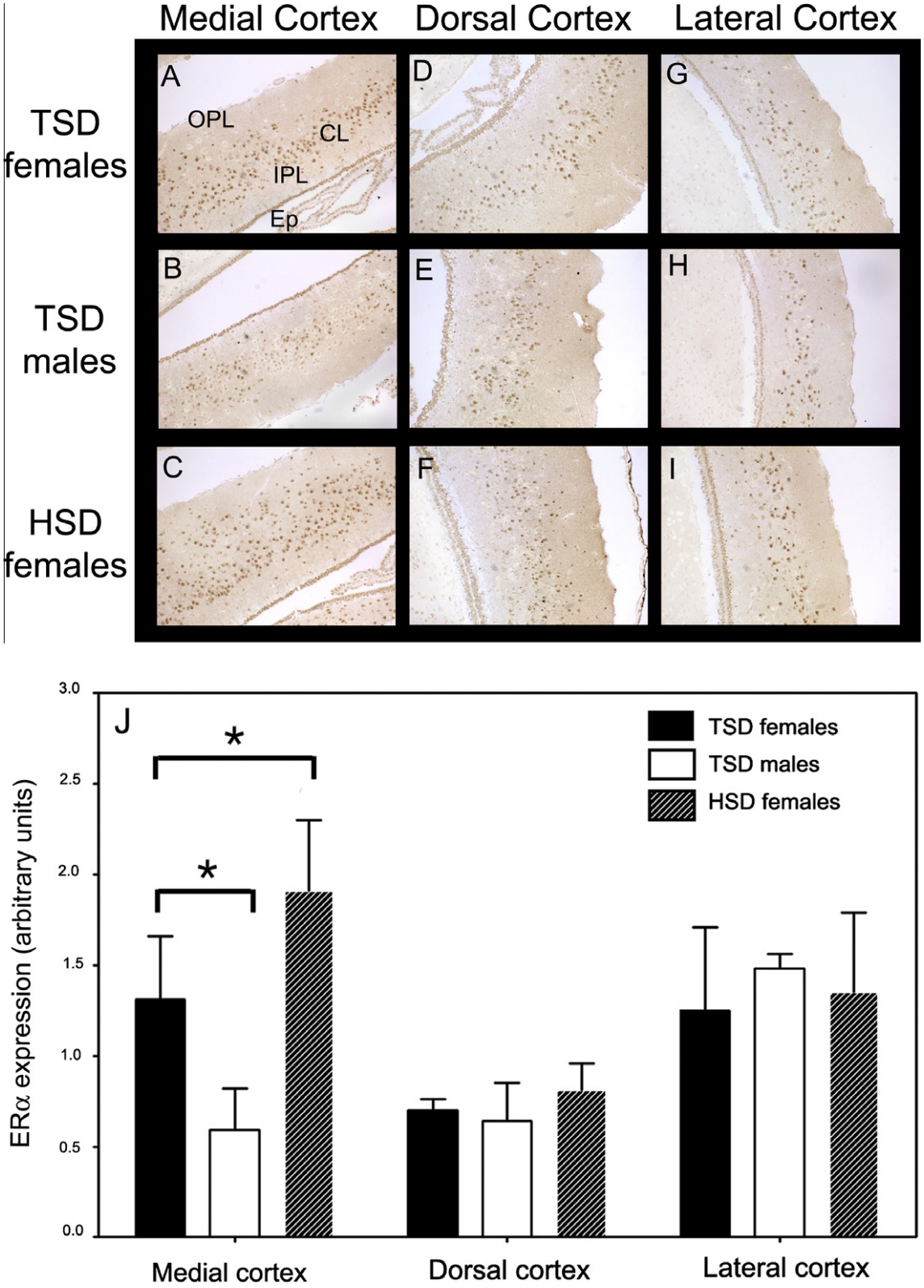

Fig. 3. Sex-associated differences in ERα expression. Each panel shows representative immunostained sections of the medial (A–C), dorsal (D–F) and lateral cortices (G–I) in TSD-females, TSD-males and HSD-females. Positively stained cells were observed in all cellular compartments (CL, cellular layer; IPL, inner plexiform layer; OPL, outer plexiform layer; Ep: ependymal epithelium). Magnification: ×400. Immunohistochemical quantification of ERα was performed in the cellular layer (CL) of medial, dorsal and lateral cortices. In the medial cortex, the ERα staining was greater in TSD-females compared to males. Interestingly, HSD-females showed the maximal amount of ERα immunostaining in the MC when compared with both TSD groups. Asterisk denotes $p < 0.05$ vs. TSD-females. ($n = 5$ brains/group).

positively stained with the anti-ERα antibodies. ERα expression was primarily found in the nuclei of the different cell types, while in the CL, both nuclear and perinuclear staining were observed in most of the cells (Fig. 2C). Immuno-positive cell nuclei in the cortex displayed strong immunolabeling, easing their detection and quantification.

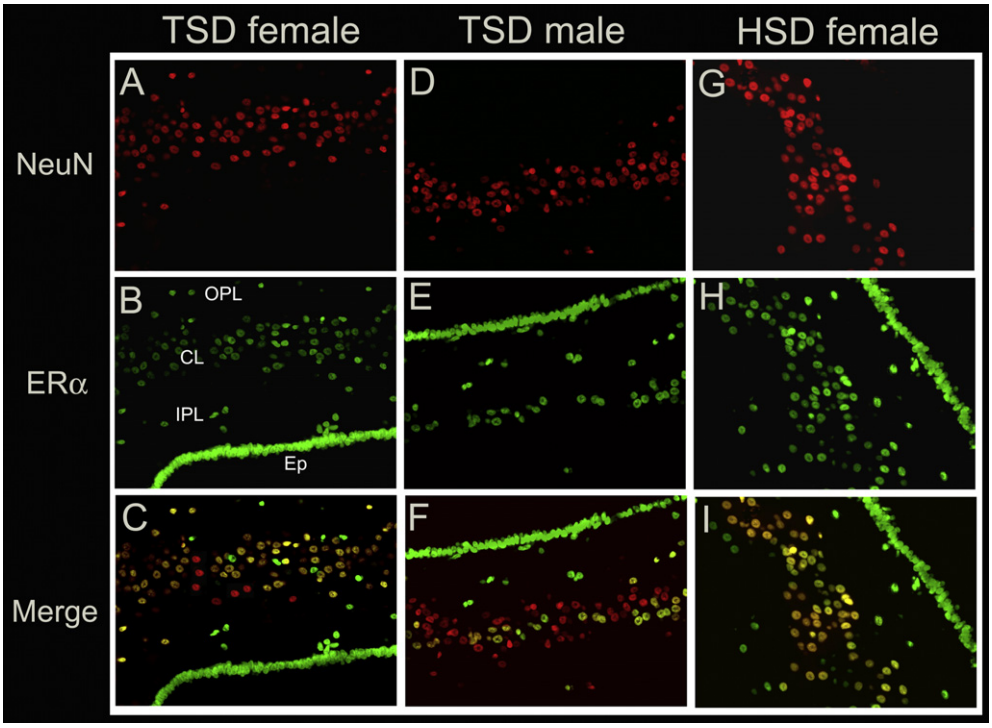

Fig. 4. ERα expression in mature neurons of the medial cortex. Dual-immunofluorescence staining for ERα/NeuN was performed in the MC of TSD-females (A–C), TSD-males (D–F) and HSD-females (G–I). Substantial co-expression of both proteins in the nuclei of cells comprising the CL is evidenced by yellow nuclei in the merged images; the percentage of double-immunostained cells was determined to be larger in both female groups than in males. CL, cellular layer; IPL, inner plexiform layer; OPL, outer plexiform layer; Ep: ependymal epithelium. Magnification: ×400.

The comparison of ERα expression between TSD-females, TSD-males and HSD-females was achieved by image analysis. Quantification was performed individually in the three main cortical areas: MC, DC and LC. Only the expression of ERα in the MC showed sex-associated differences at postnatal day 10. The expression of ERα was significantly stronger in TSD-females compared to males ($p < 0.05$) (Fig. 3). Interestingly, HSD-females exhibited a stronger ERα expression in the MC compared to both males and TSD-females ($p < 0.05$).

3.4. Immunophenotype of ERα-expressing cells

To assess whether ERα is expressed in mature neurons of the MC, we performed dual immunofluorescence with the vertebrate NeuN protein. Our results revealed that the ERα protein was abundantly expressed in the nuclei of mature neurons of the MC in all experimental groups (Fig. 4). The level of ERα/NeuN co-expression was higher in female than in male caimans.

To evaluate whether precursor cells expressed ERα, we combined ERα labelling with the immuno-detection of GFAP or DCX, which are known markers of glial and young migrating neurons, respectively. The precursor nature of ERα-positive cells located in the Ep was demonstrated by the observation of double-labelled precursor somata, i.e., with the cytoplasm GFAP immuno-reactive, and the nucleus labelled with the ERα antibody (Fig. 5). In addition, ERα/DCX double-immunostained cells were clearly detected in the IPL and CL throughout the different cortical areas (Fig. 5). No differences were found in the distribution pattern of precursor cells expressing ERα among HSD females, TSD-females and TSD-males.

3.5. Serum estradiol levels in neonatal caimans

The serum levels of estradiol in neonatal caimans are shown in Table 1. The highest estrogen serum levels were found in females; no differences were observed between TSD- and HSD-females. In addition, we found that the circulating levels of estradiol were significantly lower in TSD-males than in TSD- and HSD-females ($p < 0.001$).

4. Discussion

This study shows that the cerebral cortex of *C. latirostris* presents the general histoarchitecture described for other reptiles [33,1]. Our findings reveal that the expression of ERα in the neonatal caiman cortex is sexually dimorphic and is present in the early stages of neuronal differentiation.

ERα was abundantly expressed in different cellular layers of the neonatal caiman cerebral cortex. Furthermore, the levels of expression of ERα in the MC exhibited a sexually dimorphic pattern. When we compared TSD animals, a greater level of ERα expression in the MC was observed in females than in males. Likely, this pattern of expression is related to the differences in circulating E2 profiles during the early postnatal period.

Different mechanisms for the control of the expression of ERs could also explain the sex differences observed for the neonatal *C. latirostris* cerebral cortex. In mammals, differential transcriptional promoter usage accounts for the dimorphic ERα expression observed in females and males. In the rat, it has been described that a system of untranslated first exons is associated with the promoter selection for the initiation of ERα transcription [14,15,28]. In a previous study, we observed that the expression of ERα in the hypothalamic preoptic area showed sex-associated differences, with a greater level of expression in female compared to male rats at postnatal day 8 [26]. In addition, we reported that ERα promoter use exhibited a strong sexual dimorphism, as control females showed greater levels of the more active promoter transcripts than male pups [26].

The sexual dimorphic ERα expression detected in the MC of *C. latirostris* is in accordance with findings observed in the prefrontal

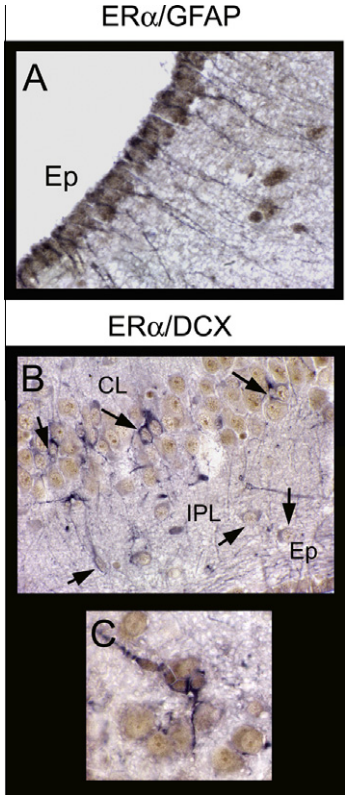

Fig. 5. ERα expression in precursor cells in the brain cortex. GFAP and DCX were used as markers of glial and young migrating neurons, respectively (A) double labelling with ERα/GFAP revealed the occurrence of co-labelled cells that form part of the ependymal epithelium. (B) and (C) ERα/DCX immunostained cells were clearly detected in the IPL and CL within the different cortical areas. Arrows indicate ERα/DCX-positive cells. CL, cellular layer; IPL, inner plexiform layer; OPL, outer plexiform layer; Ep: ependymal epithelium. Magnification: (A and B) ×400; (C) ×1000.

Table 1
Serum levels of estradiol in neonatal *Caiman latirostris*.

Experimental group	Estradiol (pg/ml)[a]
TSD-females	40.5 ± 3.70
TSD-males	1.79 ± 1.34*
HSD-females	37.0 ± 6.70

[a] Values represent means ± SEM. Asterisks denote significant differences versus TSD-females ($p < 0.001$).

cortex of mice. Female mice show a higher expression of ERα mRNA than males at PND10. In addition a sexual dimorphism in the methylation pattern of ERα promoter region was observed, suggesting potential sex differences in modulation of ERα gene by epigenetic mechanisms [44].

ERα mRNA expression is also dramatically regulated following brain injury. Middle cerebral artery occlusion (MCAO) is a well-established model of focal ischemia. Studies in rats and mice have demostrated a gender differences in neuronal cell death and ERα expression following MCAO. In this model of injury, males have a larger MCAO-induced cell loss and a significant decrease in ERα positive cells than females. In this context, ERα is associated with a neuroprotective function making that female brains are consistently more protected against cell death [2]. The differences observed in this work between male and female ERα expression in the MC could be interpreted under this hypothesis, but more research is necessary to confirm these results.

Surprisingly, the HSD-females (females produced by the *in ovo* exposure to exogenous estradiol) displayed greater ERα expression in the MC than TSD-females, despite both groups having similar E_2 circulating levels. This differential expression of ERα in the cerebral cortex could be an indication of estrogenic perturbation in neonatal HSD-females. In a previous work, we observed that neonatal HSD-females show alterations in the follicular development lacking type III follicles, the most advanced follicular stage seen in neonatal caimans. This observation is associated with a higher incidence of MOFs (multi-oocyte follicles) in HSD-juvenile caimans. MOF pathogenesis is unknown, but it is postulated to be a consequence of oocyte clusters that did not separate and became enclosed individually in a follicle [16]. It was suggested that MOF formation due to exposure to estrogenic compounds involves a disruption in the gonadotropin-estrogen/activin signaling pathway [12]. The up-regulation of the ERα expression observed in the MC of HSD-females could be related with this hormone disruption.

Caimans have been shown to be a good sentinel species for active estrogenic compounds [38–40,34,35,6]. Research on this species is of particular interest, to both assess the impact of endocrine disruptors on caiman populations and to further characterise *C. latirostris* as a sentinel of ecosystem health, which may enable the characterisation of regions with different levels of contamination burdens.

Using double immunohistochemistry, we determined that ERα-positive cells are young migrating neurons (ERα/DCX double-positive cells), post-mitotic neurons (ERα/NeuN double-positive cells) and neuronal precursors and/or astrocytes (ERα/GFAP double-positive cells).

The immunohistochemical images showed that the nuclear localisation of ERα in the cerebral cortex and confirmed the fact that it may act as a nuclear receptor that influences gene transcription by interacting with specific regulatory DNA sequences [17]. In previous studies, the extra-nuclear localisation (cytoplasm and/or plasma membrane) of ERs has been demonstrated in the immature and adult brains of various species [7,9,25]. The extra-nuclear distribution of ERs is supported by data showing that can rapidly affect neuronal cells by stimulating different transductional pathways such as phosphatidylinositol [36] or cAMP-dependent pathways [4]. A potential role for estrogens in the attenuation of hypoxic–ischaemic and oxidative injuries in the neonatal rat brain has been recently demonstrated [10,13]. Because caimans begin to dive during the early postnatal period, the high expression of ERα in the cerebral cortex could represent a protective mechanism against hypoxia. Previously, we demonstrated a positive correlation between total erythrocyte counts, haematocrit and haemoglobin concentrations within the juvenile caiman mass [45]. Furthermore, adult *Crocodylus palustris* showed a greater red blood cell count compared to juveniles [37]. The relatively low erythrocyte counts during the early postnatal period could intensify hypoxia in the neonatal brain. The high expression of nuclear ERα observed in young migrating neurons and GFAP-positive cells clearly suggests a possible neuroprotective role against hypoxic injury during the early periods of neuronal differentiation. Furthermore, it has been demonstrated that sex steroids can act as trophic factors for developing neurons [41]. In the rat hippocampus, the population dynamics of neural precursor cells were modified by estrogen during the early postnatal period [32]. In the present study, both types of neural precursor cells (ependymal epithelial cells and migrating neurons, which were detected by anti-GFAP and anti-DCX antibodies, respectively) showed strong ERα expression. In the lizard, it has been shown that the Ep is an area of proliferative cells that gave rise to migratory immature neurons that are further recruited to the nervous system parenchyma [19]. Here we showed that, in all cortical areas of neonatal female and male caimans, neuronal precursor cells expressed high levels of ERα. New experiments have been designed to assess the roles of ERα and E_2 levels in the control of the proliferation of neural precursor cells in the *C. latirostris* cerebral cortex.

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References

[1] F. Aboitiz, J. Montiel, D. Morales, M. Concha, Evolutionary divergence of the reptilian and the mammalian brains: considerations on connectivity and development, Brain Res. Brain Res. Rev. 39 (2002) 141–153.
[2] N.J. Alkayed, I. Harukuni, A.S. Kimes, E.D. London, R.J. Traystman, P.D. Hurn, Gender-linked brain injury in experimental stroke, Stroke 29 (1998) 159–165. discussion 166.
[3] American Society of Ichthyologists and Herpetologists (ASIH), Guidelines for Use of Live Amphibians and Reptiles in Field and Laboratory Research Revised by the Herpetological Animal Care and Use Committee, second ed., 2004.
[4] S.M. Aronica, W.L. Kraus, B.S. Katzenellenbogen, Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription, Proc. Natl. Acad. Sci. USA 91 (1994) 8517–8521.
[5] M. Beato, P. Herrlich, G. Schutz, Steroid hormone receptors: many actors in search of a plot, Cell 83 (1995) 851–857.
[6] P.M. Beldoménico, F. Rey, W.S. Prado, J.C. Villarreal, M. Muñoz-de-Toro, E.H. Luque, In ovum exposure to pesticides increases the egg weight loss and decreases hatchlings weight of *Caiman latirostris* (Crocodylia: Alligatoridae), Ecotoxicol. Environ. Saf. 68 (2007) 246–251.
[7] J.D. Blaustein, Cytoplasmic estrogen receptors in rat brain: immunocytochemical evidence using three antibodies with distinct epitopes, Endocrinology 131 (1992) 1336–1342.
[8] V.L. Bosquiazzo, J.G. Ramos, J. Varayoud, M. Muñoz-de-Toro, E.H. Luque, Mast cell degranulation in rat uterine cervix during pregnancy correlates with expression of vascular endothelial growth factor mRNA and angiogenesis, Reproduction 133 (2007) 1045–1055.
[9] C.H. Clarke, A.M. Norfleet, M.S. Clarke, C.S. Watson, K.A. Cunningham, M.L. Thomas, Perimembrane localization of the estrogen receptor alpha protein in neuronal processes of cultured hippocampal neurons, Neuroendocrinology 71 (2000) 34–42.
[10] B. Gerstner, J. Lee, T.M. DeSilva, F.E. Jensen, J.J. Volpe, P.A. Rosenberg, 17Beta-estradiol protects against hypoxic/ischemic white matter damage in the neonatal rat brain, J. Neurosci. Res. 87 (2009) 2078–2086.
[11] M. Gonzalez, A. Cabrera-Socorro, C. Perez-Garcia, J.D. Fraser, F.J. Lopez, R. Alonso, et al., Distribution patterns of estrogen receptor α and β in the human cortex and hippocampus during development and adulthood, J. Comp. Neurol. 503 (2007) 790–802.
[12] L.J. Guillette, B.C. Moore, Environmental contaminants, fertility, and multioocytic follicles: a lesson from wildlife?, Semin Reprod. Med. 24 (2006) 134–141.
[13] J. Guo, D.N. Krause, J. Horne, J.H. Weiss, X. Li, S.P. Duckles, Estrogen-receptor-mediated protection of cerebral endothelial cell viability and mitochondrial function after ischemic insult in vitro, J. Cereb. Blood Flow Metab. 30 (2010) 545–554.
[14] S. Hirata, T. Koh, N. Yamada-Mouri, K. Hoshi, J. Kato, The untranslated first exon 'exon 0S' of the rat estrogen receptor (ER) gene, FEBS Lett. 394 (1996) 371–373.
[15] S. Hirata, T. Koh, N. Yamada-Mouri, J. Kato, The novel untranslated first exon 'exon 0N' of the rat estrogen receptor gene, Biochem. Biophys. Res. Commun. 225 (1996) 849–854.
[16] T. Iguchi, N. Takasugi, Polyovular follicles in the ovary of immature mice exposed prenatally to diethylstilbestrol, Anat. Embryol. (Berl.) 175 (1986) 53–55.
[17] C.M. Klinge, Estrogen receptor interaction with estrogen response elements, Nucleic Acids Res. 29 (2001) 2905–2919.
[18] S. Kuhnemann, T.J. Brown, R.B. Hochberg, N.J. MacLusky, Sex differences in the development of estrogen receptors in the rat brain, Horm. Behav. 28 (1994) 438–491.
[19] C. Lopez-Garcia, A. Molowny, J.M. Garcia-Verdugo, F.J. Martinez-Guijarro, A. Bernabeu, Late generated neurons in the medial cortex of adult lizards send axons that reach the Timm-reactive zones, Brain Res. Dev. Brain Res. 15 (1990) 249–254.
[20] A. Maggi, Liganded and unliganded activation of estrogen receptor and hormone replacement therapies, Biochim. Biophys. Acta 2011 (1812) 1054–1060.
[21] N. Matsuo, S. Kawamoto, K. Matsubara, K. Okubo, Cloning and developmental expression of the murine homolog of doublecortin, Biochem. Biophys. Res. Commun. 252 (1998) 571–576.
[22] M.D. McAbee, L.L. DonCarlos, Ontogeny of region-specific sex differences in androgen receptor messenger ribonucleic acid expression in the rat forebrain, Endocrinology 139 (1998) 1738–1745.
[23] B.S. McEwen, Stress, sex, and neural adaptation to a changing environment: mechanisms of neuronal remodeling, Ann. NY Acad. Sci. 1204 (Suppl.) (2010) E38–E59.
[24] B.S. McEwen, S.E. Alves, Estrogen actions in the central nervous system, Endocr. Rev. 20 (1999) 279–307.
[25] T.A. Milner, B.S. McEwen, S. Hayashi, C.J. Li, L.P. Reagan, S.E. Alves, Ultrastructural evidence that hippocampal alpha estrogen receptors are located at extranuclear sites, J. Comp. Neurol. 15 (2001) 355–371.
[26] L. Monje, J. Varayoud, E.H. Luque, J.G. Ramos, Neonatal exposure to bisphenol A modifies the abundance of estrogen receptor α transcripts with alternative 5′-untranslated regions in the female rat preoptic area, J. Endocrinol. 194 (2007) 201–212.
[27] M. Muñoz de Toro, M.V. Maffini, R.H. Giardina, E.H. Luque, Processing fine needle aspirates of prostate carcinomas for standard immunocytochemical studies and in situ apoptosis detection, Pathol. Res. Pract. 194 (1998) 631–636.
[28] N. Osada, S. Hirata, T. Shoda, K. Hoshi, The novel untranslated exon 'exon 0T' encoded between the exon 0 and exon 1 of the rat estrogen receptor alpha (ER alpha) gene, Endocr. J. 48 (2001) 465–472.
[29] A.K. Prewitt, M.E. Wilson, Changes in estrogen receptor-alpha mRNA in the mouse cortex during development, Brain Res. 1134 (2007) 62–69.
[30] J.G. Ramos, J. Varayoud, V.L. Bosquiazzo, E.H. Luque, M. Muñoz-de-Toro, Cellular turnover in the rat uterine cervix and its relationship to estrogen and progesterone receptor dynamics, Biol. Reprod. 67 (2002) 735–742.
[31] J.G. Ramos, J. Varayoud, L. Kass, H. Rodriguez, L. Costabel, M. Muñoz-de-Toro, et al., Bisphenol a induces both transient and permanent histofunctional alterations of the hypothalamic–pituitary–gonadal axis in prenatally exposed male rats, Endocrinology 144 (2003) 3206–3215.
[32] J.G. Ramos, J. Varayoud, L. Monje, G. Moreno-Piovano, M. Muñoz-de-Toro, E.H. Luque, Diethylstilbestrol alters the population dynamic of neural precursor cells in the neonatal male rat dentate gyrus, Brain Res. Bull. 71 (2007) 619–627.
[33] A. Reiner, A comparison of neurotransmitter-specific and neuropeptide-specific neuronal cell types present in the dorsal cortex in turtles with those present in the isocortex in mammals: implications for the evolution of isocortex, Brain Behav. Evol. 38 (1991) 53–91.
[34] F. Rey, M. González, M.A. Zayas, C. Stoker, M. Durando, E.H. Luque, et al., Prenatal exposure to pesticides disrupts testicular histoarchitecture and alters testosterone levels in male *Caiman latirostris*, Gen. Comp. Endocr. 162 (2009) 286–292.
[35] F. Rey, J.G. Ramos, C. Stoker, L.E. Bussmann, E.H. Luque, M. Muñoz-de-Toro, Vitellogenin detection in *Caiman latirostris* (Crocodylia: Alligatoridae): a tool to assess environmental estrogen exposure in wildlife, J. Comp. Physiol. B. 176 (2006) 243–251.
[36] T. Simoncini, A. Hafezi-Moghadam, D.P. Brazil, K. Ley, W.W. Chin, J.K. Liao, Interaction of estrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase, Nature 28 (2000) 538–541.
[37] B.A. Stacy, N. Whitaker, Hematology and biochemistry of captive mugger crocodiles (Crocodylus Plustris), J. Zoo Wildl. Med. 31 (2000) 339–347.
[38] C. Stoker, P.M. Beldoménico, V.L. Bosquiazzo, M.A. Zayas, F. Rey, H. Rodríguez, et al., Developmental exposure to endocrine disruptor chemicals alters follicular dynamics and steroid levels in *Caiman latirostris*, Gen. Comp. Endocrinol. 156 (2008) 603–612.
[39] C. Stoker, M.R. Repetti, S.R. García, M.A. Zayas, G.H. Galoppo, H.R. Beldoménico, et al., Organochlorine compound residues in the eggs of broad-snouted caimans (*Caiman latirostris*) and correlation with measures of reproductive performance, Chemosphere 84 (2011) 311–317.
[40] C. Stoker, F. Rey, H.A. Rodriguez, J.G. Ramos, P. Sirosky, A. Larriera, et al., Sex reversal effects on *Caiman latirostris* exposed to environmentally relevant doses of the xenoestrogen bisphenol A, Gen. Comp. Endocrinol. 133 (2003) 287–296.
[41] C.D. Toran-Allerand, The estrogen/neurotrophin connection during neural development: is co-localization of estrogen receptors with the neurotrophins and their receptors biologically relevant?, Dev Neurosci. 18 (1996) 36–48.
[42] J. Varayoud, J.G. Ramos, V.L. Bosquiazzo, M. Muñoz-de-Toro, E.H. Luque, Developmental exposure to bisphenol A impairs the uterine response to ovarian steroids in the adult, Endocrinology 149 (2008) 5848–5860.
[43] J. Varayoud, J.G. Ramos, P.P. Joazeiro, G.S. Montes, M.M. Muñoz De Toro, E.H. Luque, Characterization of fibroblastic cell plasticity in the lamina propria of the rat uterine cervix at term, Biol. Reprod. 65 (2001) 375–383.
[44] M.E. Wilson, J.M. Westberry, A.L. Trout, Estrogen receptor-alpha gene expression in the cortex: sex differences during development and in adulthood, Horm. Behav. 59 (2011) 353–357.
[45] M.A. Zayas, H.A. Rodríguez, G.H. Galoppo, C. Stoker, M. Durando, E.H. Luque, et al., Hematology and blood biochemistry of young healthy broad- snouted caimans (*Caiman latirostris*), J. Herpetol. 45 (2011) 516–524.