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Expression of sex hormone receptors in the brain of male and female newly hatched chicks

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ABSTRACT

Chromosomal sex and steroid hormones play a determining role in brain sexual differentiation during chick embryonic development. Hormone effects on the brain are associated with the expression pattern of their intracellular receptors, which is sexually dimorphic in many species. We determined by Western blot the content of progesterone, estrogen, and androgen receptors (PR-A and PR-B, ERα, and AR, respectively) in the cortex, cerebellum, tectum, and hypothalamus of female and male newly hatched chicks. Males presented a higher content of PR-B in the tectum whereas females exhibited a higher content of PR-A in the hypothalamus. ERα was only detected as a band of 66 kDa, and it showed a higher content in the cerebellum and tectum of females as compared to these regions in males. Besides, males exhibited a higher content of AR in the tectum than females. Our study suggests that newly hatched chicks show a sexual dimorphism in the expression of sex hormone receptors in brain regions involved in sexual behavior such as the hypothalamus, and in non-sexual behavior such as the optic tectum and the cerebellum.

1. Introduction

Sex steroid hormones (progesterone, estradiol, and testosterone) participate in the regulation of several functions of the brain such as the control of reproductive behavior and the modulation of the neuroendocrine system in birds and mammals (Gahr, 2001; Zubeldia-Brenner et al., 2016).

Progesterone, estradiol and testosterone exert their effects through their intracellular receptors (PR, ER, and AR, respectively), which are ligand-dependent transcription factors. These receptors regulate many physiological processes by interacting with DNA-specific sequences called hormone-responsive elements (McEwan, 2004).

The intracellular PR in chickens is encoded by a single gene, which is transcribed and translated into two isoforms: the N-terminally truncated PR-A (79 kDa) and PR-B (110 kDa) (Conneely et al., 1987; Gronemeyer et al., 1987). It has been shown that PR isoforms regulate the expression of different genes and exert distinct functions (Tora et al., 1988). In both male and female chickens, PR isoforms have been detected in several brain regions of the adult (Camacho-Arroyo et al., 2007; Gahr, 2001; Sterling et al., 1987) and immature animals (Gasc and Baulieu, 1988), as well as in the embryos (Camacho-Arroyo et al., 2003; Guennoun et al., 1987).

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Besides, two ER subtypes have been described (ER-α of 66 kDa and ER-β of 54 kDa), which are codified by two different genes, and regulate diverse functions (Lindberg et al., 2002; MacGregor and Jordan, 1998). Interestingly, in chicken tissues, two forms of ER-α protein have been reported: ER-α form I (66 kDa), and ER-α form II (61 kDa). The two chicken ER-α isoforms differ in their ability to modulate the transcription of estrogen target genes in a promoter and cell type-specific manner (Griffin et al., 1999).

The expression of ER-α has been reported in the hypothalamus and adenohypophysis pars distalis of female and male chick embryos (Woods et al., 1995). Also, it has been detected in the whole brain of Japanese quail and chicken during the embryonic development (Brunström et al., 2009; Camacho-Arroyo et al., 2003). This receptor has also been found in the hypothalamus and anterior pituitary gland of adult cockerels and hens (Griffin et al., 2001).

Regarding AR, a single gene has been identified in birds and appears to be highly conserved among bird taxa in its hormone-binding and DNA-binding domains (Gahr and Metzdorf, 1997; Nastiuk and Clayton, 1995). AR was first cloned in *Gallus domesticus* and exhibits a high affinity for testosterone and 5α-dihydrotestosterone (Gahr, 2001). Both AR mRNA and protein have been detected in chicken embryos (Katoh et al., 2006). AR distribution has been described in the hypothalamus and adenohypophysis pars distalis of the male chick embryo (Woods et al., 1994). Also, its expression has been detected in the cerebellum, bed nucleus of the stria terminalis, amygdala, preoptic area, hypothalamus, and midbrain of young chicks (Pfannkuche et al., 2011).

However, the expression pattern of sex hormone receptors in different brain regions of newly hatched male and female chicks has not been determined. Therefore, we studied PR, ERα, and AR content in the cerebellum, hypothalamus, optic tectum and cerebral cortex of male and female newly hatched chicks by Western blot analysis.

2. Materials and methods

2.1. Animals

Fertile white Leghorn eggs (Babcock-B300) were incubated at 38 °C in a forced-draft incubator until hatching. Chicks were killed by decapitation within 24 h after hatching, and the skin and feathers were gently pushed to expose the skull. Brains were carefully removed, and four regions were dissected: cerebral cortex, optic tectum, cerebellum, and hypothalamus. First, the brains were placed in a dorsal position, and the outer portion of the two cerebral hemispheres was cut out to obtain the cerebral cortex. Then, the two optic lobes (optic tectum) of the midbrain and the cerebellum of the hindbrain were dissected. Subsequently, the remaining part of the brain was placed in a ventral position to obtain the hypothalamus, which forms the ventral portion of the diencephalon on either side of the third ventricle. The hypothalamus samples were carefully dissected: initial incisions were made below the optic chiasma (which is a well-defined and large structure in the midline) and above the hypophysis. Then, two lateral cuts were made forming a rectangle, and a final cut was made parallel to the surface of the hypothalamus. The dissected brain regions were immediately processed for protein extraction. The sex of the newly hatched chicks was determined by an in situ microscopic examination of the gonads. The experiments were performed under the guidelines of the Mexican Law of Animal Protection.

2.2. Protein extraction and western blotting

Tissue samples were homogenized with a Polytron PCU11 homogenizer (Kinematica, CHE) using RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton and 0.1% SDS) supplemented with a protease inhibitor cocktail (p8340, Sigma-Aldrich, USA). Total proteins were obtained by centrifugation at 22,000*g*, at 4 °C for 15 min and quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

Protein samples (70 µg) were separated on a 7.5% SDS-PAGE at 80 V. Then, they were transferred to nitrocellulose membranes (Millipore, USA) in semi-dry conditions at room temperature at 60 mA for 1 h for ERα and AR, and at 35 mA for 7 h for PR. Membranes were blocked with 3% nonfat dry milk and 2% bovine serum albumin (BSA) at 37 °C under constant agitation for 2 h. Then, they were incubated with the following primary antibodies: rabbit polyclonal anti-PR (1 µg/mL; sc-539 [C-20]), rabbit polyclonal anti-ERα (0.7 µg/mL; sc-7207 [H-184]), and rabbit polyclonal anti-AR (0.7 µg/mL; sc-815 [C-19]) at 4 °C for 48 h. Blots were then incubated with a mouse anti-rabbit secondary antibody (1:7500; sc-2357) conjugated to horseradish peroxidase at room temperature under constant agitation for 45 min.

Chemiluminescence signals were detected exposing membranes to Kodak Biomax Light Films (Sigma-Aldrich, USA) using the Supersignal West Femto as peroxidase substrate (Thermo Scientific, USA). The band density for the antigen-antibody complex was calculated as the area under a peak (scale of 72 pixels/inch) in a semiquantitative way using a 14.1 megapixels digital Canon camera (SD1400IS, Canon, MEX) and the ImageJ 1.45S software (National Institutes of Health, USA).

To correct for differences in the total protein loaded in each lane, PR, ERα, and AR protein content was normalized to that of α-tubulin. Therefore, blots were stripped with a 0.1 M glycine solution (pH 2.5, 0.5% SDS) at room temperature for 30 min and incubated with 0.7 µg/mL of a mouse monoclonal anti-α-tubulin antibody (sc-5286 [B-7]) at 4 °C overnight. Blots were then incubated with a goat anti-mouse secondary antibody (1:5000; sc-2005) conjugated to horseradish peroxidase at room temperature for 45 min and under constant agitation. Immunoblot chemiluminescent signals were detected as described. All antibodies were purchased from Santa Cruz Biotechnology, USA.

2.3. Statistical analysis

Statistical analysis was performed using a two-way ANOVA for ERα and AR, and a three-way ANOVA for PR isoforms, both