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Distribution and sex differences in aromatase-producing neurons in the brain of Japanese quail embryos

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ABSTRACT

The biochemical properties, neuroanatomical location, and function of aromatase (ARO), the enzyme that converts testosterone to 17β-estradiol, have been studied extensively in the adult quail brain. Conversely, very little is known about ARO in quail embryos. This study investigated the distribution of ARO in quail prosencephalon at embryonic days (E) 9, 11, and 15 by immunocytochemistry. ARO-immunoreactive cells were observed within the walls of the cerebral ventricles, the ventral striatum, medial preoptic nucleus (POM), medial part of the bed nucleus of the stria terminalis (BSTM), lateral part of the BST, and in the tuberal region. The BSTM and to a lesser extent the POM showed transient, female-biased sex-differences. In the BSTM, the number of the ARO-immunoreactive cells, the fractional area covered by ARO-immunoreactive structures, and the overall extension of ARO-immunoreactivity were greater in females at E9 and E11, but these differences largely disappeared at E15 and post-hatch day 1. The sex differences were confirmed at the transcriptional level by *in situ* hybridization. In the lateral part of the POM, females showed slightly more ARO-immunoreactivity than males at E11. Treatment of E9 male embryos with estradiol completely feminized ARO-immunoreactivity at E11. The origins and the functional significance of these sex differences remain unknown.

1. Introduction

Aromatase (ARO) converts testosterone to 17β-estradiol and mediates many of the physiological effects of this androgen in the brain, including neuroprotection, brain repair, and the activation and differentiation of brain circuits that regulate reproductive behavior (Cooke et al., 1998; Forlano et al., 2006; McCarthy, 2008; Garcia-Segura, 2008; Balthazart et al., 2009a; Saldanha et al., 2009).

This last role of ARO has been studied intensely in adult Japanese quail, a bird that has long been a model for studying the endocrine regulation of male copulatory behavior (Balthazart et al., 2003b, 2009a for extensive literature). In this species, the remarkably high ARO activity and expression in the adult brain made it possible to correlate the data collected at morphological, biochemical, and physiological levels.

ARO-producing neurons are present in preoptic-hypothalamic and pallial regions that have been implicated, to various extents, in the control of many sexually differentiated components of male quail sexual behavior (Riters et al., 1998; Thompson et al., 1998; Taziaux et al., 2006, 2008). In particular, ARO-producing neurons characterize the medial preoptic nucleus (POM), medial part of the bed nucleus of the stria terminalis (BSTM), the nucleus teniae of the amygdala, and the tuberal region (Balthazart et al., 1990; Foidart et al., 1995; Aste et al., 1998b; Voigt et al., 2007).

Neuronal networks that are implicated in the control of reproductive functions are irreversibly differentiated toward a male or a female phenotype by gonadal hormones during a restricted window of time termed "the critical period" (Cooke et al., 1998; McCarthy, 2008; Negri-Cesi et al., 2008; Balthazart et al., 2009a for comprehensive reviews). Unlike in rodents, where estradiol produced by brain ARO defeminizes male brain circuits (McCarthy, 2008), in quail estradiol promotes demasculinization of the female brain (Balthazart et al., 2009a). Indeed, treatment of male quail embryos with estradiol, before E12, results in adult quail that have lost sensitivity to the activating effects of testosterone on copulatory behavior (Adkins, 1979; Adkins-Regan et al., 1982), whereas, exposure of females

Abbreviations: ARO, aromatase; E, embryonic day; EB, 17β-estradiol 3-benzoate; CA, anterior commissure; Cb, cerebellum; CPa, pallial commissure; POM, medial preoptic nucleus; BSTL, lateral part of the bed nucleus of the stria terminalis; BSTM, medial part of the bed nucleus of the stria terminalis; Hp, hippocampus; LFB, lateral forebrain bundle; M, mesopallium; N, nidopallium; OM, occipito-mesencephalic tract; P1, post-hatch day 1; ROT, nucleus rotundus; S, septal region; OTu, olfactory tubercle; OT, optic tectum; TnA, nucleus teniae of the amygdala; TSM, septopallial-mesencephalic tract; VMH, ventro-medial hypothalamus.

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embryos to anti-estrogens or ARO-inhibitors results in adult quail with a male-typical copulatory sequence (Adkins, 1976; Adkins-Regan et al., 1982; Balthazart et al., 1992). The developing ovary is thought to be the main source of differentiating estradiol in quail because a higher plasma concentration of this estrogen is present in female than male embryos during the critical period (Schumacher et al., 1988b; Ottinger et al., 2001).

This observation, together with earlier biochemical studies that showed a lack of sex differences in the activity of embryonic ARO (Schumacher et al., 1988a; Hutchison et al., 1990), lowered the interest in this enzyme during development. Therefore, in spite of the large amount of information available in adult quail, very little is known about this enzyme in embryos.

However, it is now well established that the brain, beside metabolizing circulating steroids, expresses all the enzymes that are needed to synthesize androgens and estrogens *de novo* from cholesterol (Mellon et al., 2001; Tsutsui et al., 2006; Tomy et al., 2007; Do Rego et al., 2009; London et al., 2009). This notion also applies to the quail embryonic brain (Ukena et al., 2001; Aste et al., 2008). Therefore, the developing brain may be the site of an autonomous and complex hormonal environment in cross talk with steroids from other endocrine organs.

Given the complete lack of information on the general distribution of ARO-producing cells in the developing brain, this study analyzed, at a detailed neuroanatomical level, the immunocytochemical distribution of this enzyme during a window of time spanning the critical period for brain differentiation.

Moreover, this study determined the presence of female-biased, transient sex differences in ARO expression within the BSTM and, to a much lesser extent, the POM. Treatment of male embryos with estradiol at E9 reversed these sex differences, showing that the estrogenic hormonal milieu plays a part in the development of the aromatase-immunoreactive (ARO-ir) system.

2. Materials and methods

2.1. Subjects

Fertilized Japanese quail (*Coturnix japonica*) eggs were obtained from our laboratory's colony. The eggs were incubated at 37 °C with 50–60% relative humidity and the day when the eggs were put into the incubator was considered as E0. Embryos were sacrificed at E9, E11, and E15. In addition to embryos, quail of both sexes were sacrificed at post-hatch day 1 (P1). The sex of the embryos was determined by visual inspection of the gonads or by PCR analysis of genomic DNA extracted from blood, according to the method of Fridolfsson and Ellegren (1999). PCR analysis was performed using primers complementary to the CHD1 gene sequence located on the sex chromosomes (5′-GTT ACT GAT TCG TCT ACG AGA-3′ and 5′-ATT GAA ATG ATC CAG TGC TTG-3′). The length of the CHD1 gene sequence is different on the W and Z chromosomes. Therefore, two PCR products of different length are amplified from female birds (ZW), whereas, only one PCR product is amplified from males (ZZ). The PCR products were separated by electrophoresis in 1.5% agarose gels.

In a separate experiment, forty eggs were injected at E9 with 17β-estradiol 3-benzoate (EB) (5 μg/10 μl/egg; Sigma–Aldrich Co., St. Louis, MO, USA) diluted in corn oil or only with vehicle (Wako Pure Chemical Co., Japan). This dosage had been shown previously to induce behavioral, neuroanatomical, and neurochemical demasculinization in male quail embryos (Adkins, 1979; Aste et al., 1991; Panzica et al., 1998). A small hole was opened into the air chamber of the egg and injections were done by introducing the tip of a pipette through a small hole. The hole was sealed with tape and eggs were returned to the incubator. EB- and oil-treated embryos were sacrificed at E11.

Animal care and experiments were in accordance with the Guide for the Animal Care and Use in Nagoya University.

2.2. Brain fixation and sectioning

Embryos were placed on ice to induce hypothermal anesthesia and perfused transcardially with 0.01 M PBS, followed by 4% paraformaldehyde fixative buffered with 0.1 M phosphate (pH 7.4). Brains were post-fixed overnight at 4 °C in the same fixative solution, cryoprotected with 0.1 M phosphate-buffered 20% sucrose until they sunk and then frozen in dry ice. Brains were stored at −80 °C until use. Sixteen to twenty embryos were sacrificed for each age and sex group and, 5 female and 5 male quail were sacrificed at P1.

Brains were cut into 12 μm thick coronal sections and collected on gelatin-coated slides (for immunocytochemical investigations) or 3-(2-aminoethylamino) propyldimethoxymethylsilane-coated slides (for *in situ* hybridization investigations), in four series. One series of sections was stained with cresyl violet and the others were preserved at −20 °C or at −80 °C until used for immunocytochemistry or *in situ* hybridization, respectively.

2.3. Immunocytochemistry

All the incubations and washes were performed at room temperature. Sections were washed with 0.01 M PBS containing 0.2% Triton-X (0.01 M PBSTX) for 30 min and incubated with 0.01 M PBS containing 0.3% H_2O_2 for 20 min to inhibit endogenous peroxidases. Sections were incubated with normal horse serum (ImmPRESS, Vector Laboratories Inc., CA, USA) for 20 min and then overnight with a rabbit anti-quail recombinant ARO polyclonal antibody (1:500 in 0.01 M PBSTX). After a 1.5 hours incubation with peroxidase-conjugated anti-rabbit IgG (ImmPRESS, Vector Laboratories Inc., CA, USA) diluted 1:1 in 0.01 M PBS, the peroxidase reaction was developed in 0.05 M TRIS pH 7.6 containing 0.2 mg/ml DAB, 0.0045 M H_2O_2, and 4.13 mg/ml nickel ammonium sulfate. Extensive washes in 0.01 M PBS were performed between steps of the immunocytochemical procedure.

With the exclusion of P1 brains, sections were always processed in matched series, including subjects from each experimental group to minimize variations linked to the experimental procedure.

The anti-ARO antibody used in this study was prepared against a recombinant protein produced in *Escherichia coli* and corresponded to a 230 amino acid sequence from the quail CYP19 gene (Harada et al., 1992). This sequence included the catalytic site of the enzyme (Balthazart et al., 2003a for comparison of this sequence with ARO in other species). The validation of the anti-ARO antibody was described by Foidart et al. (1995) and further detailed by Carere et al. (2007). The validation included Ouchterlony double diffusion tests, Western blotting, and absorption of the anti-ARO antibody with recombinant antigen.

The antibody used in this study belongs to the same lot as that used by Carere et al. (2007). In the present study, the specificity of labeling in embryos was further confirmed by omitting the primary or the secondary antibody and by absorption of the anti-ARO antibody with a 10-fold molar excess of recombinant antigen.

2.4. In situ hybridization

Total-mRNA was extracted from the brain of an E15 embryo using an RNAspin Mini kit (GE Healthcare, UK Ltd., UK) and reverse transcribed using oligo-dT primers and a ExScript RT reagent kit (Takara Bio Inc., Japan) in accordance with the protocols provided by the manufacturers. CYP19-cDNA fragments were amplified by PCR using specific primers (forward primer: 5′-CTG TTG AAA AGT TGG ATG AGC AC-3′; reverse primer: 5′-CAT TCG GTT TTG GGA AGA ATT CAA GCT TGT GCA TGC CTG C-3′) that were designed against chicken CYP19, the gene encoding for ARO (GenBank accession no. J04047). The obtained PCR fragments of 432 bp (nucleotides 146-577, GenBank accession no. S46949) were cloned into pGEM-T easy vectors (Promega Co., WI, USA). Plasmids were purified using Plasmid Midiprep kit (Bio-Rad Laboratories, Inc., CA, USA) and confirmed by sequencing.

Sense and antisense CYP-19-mRNA probes were transcribed *in vitro* from linearized pGEM-T easy vectors using digoxigenin (DIG)-labeled UTP (DIG RNA labeling kit, Roche Diagnostics GmbH, Germany) in accordance with the manufacturer's protocol. The probes were submitted for alkaline hydrolysis (60 mM Na_2CO_3 and 40 mM $NaHCO_3$) to shorten them to an average size of 150 nucleotides according to the method described by Cox et al. (1984).

Sections were washed with 0.01 M PBSTX for 30 min at room temperature, rinsed twice in 0.01 M PBS, and treated with 2.5 ng/ml proteinase K (Sigma–Aldrich Co., MO, USA) in 0.1 M PBS for 30 min (E15 embryos) or for 5 min (E9 embryos) at 37 °C. Sections were fixed in 4% paraformaldehyde fixative buffered with 0.1 M phosphate (pH 7.4) for 5 min and rinsed with 0.01 M PBS. Sections were incubated with hybridization buffer (50% formamide, 10% dextran sulphate, 1 mg/ml *E. coli* tRNA, 0.002 M Tris–HCl pH 8.0, 2.5 mM EDTA, 0.3 M NaCl, 1× Denhardt's solution) for 30 min at room temperature and then overnight at 45 °C with hybridization buffer containing digoxigenin-labeled CYP19-sense or antisense probes. Sections were rinsed twice for 30 min with 2× SCC (1× SCC = 0.15 M NaCl, 0.015 M sodium citrate) in 50% formamide at 45 °C, incubated with RNase A (20 μg/ml) at 37 °C for 30 min and rinsed twice for 30 min in 0.5× SCC. After extensive washes in 0.1 M TBS (0.1 M Tris–HCl, 1.5 M NaCl, pH 7.5), sections were incubated for 30 min at room temperature with 0.5% blocking buffer in 0.1 M TBS (Roche Diagnostics, Penzberg, Germany) and then with alkaline-phosphatase conjugated anti-digoxigenin-Fab (Roche Diagnostics, Penzberg, Germany) diluted at 1:4000 in 0.1 M TBS, overnight at 4 °C. The alkaline-phosphatase reaction was detected using nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate-toluidine in accordance with the manufacturer's protocol. No hybridization signal was detected in sections incubated with CYP19-sense probe.