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Female-biased sex difference in vasotocin-immunoreactive neural structures in the developing quail brain

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ABSTRACT

The bed nucleus of the stria terminalis pars medialis (BSTM), medial preoptic nucleus (POM), and lateral septal region (LS) exhibit more vasotocin-immunoreactive (VT-ir) neural structures in male than in female adult quail. VT-ir cells and fibers in these regions are sensitive to gonadal steroids only in males. The insensitivity of adult female VT-ir neural structures to sex steroids is attributed to estradiol exposure during a critical period in embryonic life.

Although the VT-ir system has been intensively examined in adult quail, information is limited in embryos and juveniles. Therefore, we herein investigated the development of VT-immunoreactive neural structures from embryonic day (E) 9 to adulthood with a particular focus on the BSTM, POM and LS of both sexes.

VT-ir neural structures were more evident in female than in male embryos from E9 (BSTM and POM) and E11 (LS). This sex difference disappeared between E15 and post-hatch day 1 in the BSTM and POM, and during the first week of life in the LS. Male-biased sex differences in VT-ir structures appeared at puberty. Female-biased sexual dimorphism in the density of the VT-ir structures of BSTM was reflected by the stronger expression of VT mRNA in females than in males. However, the density of VT mRNA somata was comparable in the two sexes.

The exposure of male embryos to estradiol resulted in the feminization of VT-ir neural structures in the BSTM, but not in the POM or LS at E11.

Collectively, these results suggest that sex differences in VT-ir neural structures changes drastically throughout quail life. In embryos, endogenous estradiol may stimulate the expression of VT in females, resulting in a robust sex difference in VT-ir cells and fibers in favor of this sex.

1. Introduction

Two major neural systems in birds produce VT (the avian homolog of vasopressin): the magnocellular and parvocellular systems. The former consists of large neurons mainly located in the paraventricular and supraoptic nuclei that innervate the neurohypophysis through the hypothalamo-neurohypophysial tract. This system is involved in osmoregulation and water balance. The latter consists of small vasotocinergic neurons mainly located in the paraventricular nucleus and BSTM, and to a lesser extent, in the preoptic area (De Vries and Panzica, 2006; Goodson et al., 2012).

The vasotocinergic cells of the BSTM are sexually dimorphic, sensitive to gonadal steroids in seasonal birds and, these characteristics are associated to similar sex differences and sensitivity to gonadal steroids of VT-ir fibers in the LS and preoptic area (De Vries and Panzica, 2006; Goodson and Bass 2001; Panzica et al., 2001).

VT-ir somata and/or fibres in the BSTM, LS and preoptic area are thought to be part of a complex neural network that regulates multiple aspects of social behavior in context- and species-specific manners (Albers, 2015; Goodson et al., 2012)

In the quail, VT-immunocytochemistry combined with tracing methods revealed that the VT-ir cells in the BSTM project to the medial preoptic nucleus (POM) (Absil et al., 2002). In contrast, the source of VT-ir fibers in the LS has not yet been investigated.

Abbreviations: BSTM, bed nucleus of the stria terminalis; D, post-hatch day; E, embryonic day; EB, 17β-estradiol benzoate; FA, fractional area; POM, medial preoptic nucleus; LS, lateral septal region; VT, vasotocin; VT-ir, vasotocin-immunoreactive.

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Nevertheless, VT-ir neurons in the quail BSTM are commonly considered to innervate the LS, and this is based on similarities in the neuroanatomical location, sex differences, and sensitivity to steroids of the VT-ir system in this bird and of vasopressin-ir neural elements in the homologous nuclei of the rat (De Vries and Panzica, 2006).

In adult quail, the BSTM, POM, and LS exhibit more VT-ir somata and/or fibers in males than in females. This dimorphism results from the stimulatory effects of circulating testosterone in males and from insensitivity to sex steroids in females (Panzica et al., 2001).

Gonadectomy has been shown to decrease, while the administration of testosterone increases VT-ir neural structures in the BSTM, POM, and LS of males (Viglietti-Panzica et al., 1992, 1994).

The effects of testosterone on VT-ir cells and fibers in male quail are known to be mediated by its conversion into estradiol by the enzyme aromatase in the brain (Balthazart et al., 2009; Viglietti-Panzica et al., 2001).

Conversely, treatment with testosterone or estradiol has no effect on the VT-ir neural structures of females (Viglietti-Panzica et al., 1992, 1994).

The insensitivity of VT-ir neural elements to sex steroids in female quail results from the exposure to estradiol, probably of an ovarian origin, during a "critical period for brain sexual differentiation" that ends by E12 (Adkins, 1979; Panzica et al., 1998). The exposure of male embryos to exogenous estradiol before E12 results in adults showing feminized vasotocinergic phenotype in the BSTM, POM, and LS. On the other hand, adult females show male-typical VT-ir cells and fibers in these regions when the production of estradiol is blocked *in ovo* by an inhibitor of aromatase (Panzica et al., 1998). These results are in line with the feminizing effects of estradiol on brain structures and sexual behavior in birds (De Vries and Panzica, 2006).

However, recent findings suggest that the insensitivity of VT-neural structures to sex steroids in the female BSTM is due to impaired translational mechanisms, because the acute treatment of adult quail with testosterone increased VT mRNA-expression to similar levels in both sexes. This same stimulatory effect was elicited by estradiol in females (Aste et al., 2013).

Although the sex differences and steroid sensitivity of quail VT-ir and VT mRNA expression have been extensively examined in adults (Aste et al., 2013; Panzica et al., 1999), information in developing quail is limited. Therefore, the aim of the present study was to address this issue by studying developmental changes and sex differences in VT-ir neural structures in quail embryos and juveniles, with a focus on the BSTM, POM, and LS.

2. Materials and methods

2.1. Incubation and animal housing conditions

Fertilized Japanese quail (*Coturnix japonica*) eggs obtained from our breeding colony were incubated at 37 °C with 50–60% relative humidity. The day when the eggs were placed into the incubator was regarded as E0.

Hatching occurred on E17, the birds were housed in groups until the age of three weeks, and were then placed in individual cages. Throughout their life, the birds were exposed to a photoperiod of 16 h light and 8 h dark and food and water were available *ad libitum*.

2.2. Treatment with 17β-estradiol 3-benzoate (EB)

At E9, eggs were injected with EB diluted in corn oil at 5 μg/10 μl/egg or only vehicle in the air chamber by introducing the tip of a pipette through a small hole (EB: Sigma-Aldrich Co., St. Louis, USA; corn oil: Wako Pure Chemical Co., Japan). This dosage has been shown to induce the demasculinization of VT-ir neural structures in male quail (Panzica et al., 1998). The hole was sealed with tape and eggs were returned to the incubator.

2.3. Brain fixation and sex determination

Birds were sacrificed at E9, E11, E15, post-hatch days (D) 1, D7, D28, and at adulthood. All birds were perfused transcardially with 0.01 M PBS, followed by 4% paraformaldehyde fixative buffered with 0.1 M phosphate (pH 7.4) after hypothermal (embryos) or pharmaceutical (Pentobarbital sodium; Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) anesthesia.

The sex of the embryos was determined by a visual inspection of the gonads or a PCR analysis of genomic DNA extracted from blood, according to the method of Fridolfsson and Ellegren (1999).

Brains were post-fixed overnight at 4 °C in fixative solution, cryoprotected with 0.1 M phosphate-buffered 20% sucrose, and frozen in dry ice. Brains were stored at −80 °C until used.

Brains were cut into 14 μm thick (E9-E15) or 20 μm (post-hatch ages) coronal sections and collected on 3-(2-aminoethylamino) propyldimethoxymethylsilane-coated slides in four (embryos) or five series (post-hatch ages). One series of sections was stained with cresyl violet while the others were preserved at −20 °C or at −80 °C until used for immunocytochemistry or *in situ* hybridization, respectively.

2.4. Immunocytochemistry

All incubations and washes were performed at room temperature. Sections were permeabilized with 0.01 PBS-TX (0.01 M PBS, 0.2% Triton-X) for 30 min, and endogenous peroxidase was inhibited with 0.01 M PBS containing 0.3% H_2O_2 for 20 min. Sections were incubated with normal goat serum (Vectastain Elite, Vector Laboratories Inc., Burlingame, USA) for 20 min and then overnight with a guinea pig anti-vasopressin polyclonal antibody (lot #A03607, Bachem Immunochemicals, Torrance, USA. 1:5000 in 0.01 M PBSTX). After a 1.5 h incubation with biotinylated rabbit IgG (1:400 in 0.01 M PBS-TX, Vectastain Elite, Vector Laboratories Inc., Burlingame, USA), sections were incubated with the avidin-biotin complex (Vectastain Elite, Vector Laboratories Inc., Burlingame, USA) diluted 1:600 in PBS for 2 h. The peroxidase reaction was developed in 0.05 M TRIS pH 7.6 containing 0.2 mg/ml DAB, 0.0045% H_2O_2, and 4.13 mg/ml nickel ammonium sulphate. Extensive washes in 0.01 M PBS or 0.01 M PBS-TX were performed between each step of the immunocytochemical procedure.

Sections were always processed in matched series including subjects from each age group in order to minimize variations linked to the experimental procedure.

The specificity of the antibody was determined in consecutive sections of E11 and D7 females by preabsorption with 5–50 μM of VT or 5–150 μM of mesotocin at 4 °C overnight (VT, mesotocin: Bachem Immunochemicals, Torrance, USA). A previous study reported the specificity of the VT-antibody used in adult quail (Aste et al., 2013). Preabsorption with 5 μM of VT completely eliminated VT-like immunoreactivity, and lower concentrations of VT completely eliminated VT-ir cells and fibers in the BSTM, POM, and LS, but not in magnocellular neurons. Conversely, preabsorption with 150 μM of mesotocin did not affect VT-like immunoreactivity.

2.5. VT mRNA in situ hybridization

The procedure used for mRNA extraction, the preparation of VT mRNA probes, and *in situ* hybridization was described in Aste et al. (2013). Briefly, total mRNA was extracted from the brain of an E15