Available online at www.sciencedirect.com
SCIENCE DIRECT®
General and Comparative Endocrinology 141 (2005) 66–75
GENERAL AND COMPARATIVE ENDOCRINOLOGY
www.elsevier.com/locate/ygcen

# Overlap and co-expression of estrogen synthetic and responsive neurons in the songbird brain—a double-label immunocytochemical study

Colin J. Saldanha*, Luckshman Coomaralingam

*Department of Biological Sciences, Lehigh University, 111 Research Drive, Bethlehem, PA 18015, USA*

Received 21 May 2004; revised 11 November 2004; accepted 29 November 2004
Available online 7 January 2005

**Abstract**

The songbird telencephalon exhibits the capacity to both synthesize and respond to estrogen. Several telencephalic loci in addition to those in the diencephalon express aromatase (estrogen synthase) and estrogen receptors (ER). Little is known about the interactions between cells that contain aromatase and those that contain ER, particularly at the level of protein expression. Consequently, we do not know if locally synthesized estrogens affect ER via autocrine and/or paracrine mechanisms. Here we have mapped the distributions, identified areas of overlap, and measured the degree of co-expression of aromatase and ERα in the zebra finch (*Taeniopygia guttata*). First, alternate sections were stained with antibodies against either aromatase or ERα, revealing the distributions and therefore, the overlap between these proteins. Subsequently, using double-label light microscopy we have measured the number of aromatase soma, ERα soma, and co-expressing soma in areas of overlap in adult males and females. In the preoptic area about 10% of aromatase-positive soma co-express ERα. In the bed nucleus of the stria terminalis, ventromedial nucleus, nucleus taeniae, and the caudomedial nidopallium, although cells containing either protein were easily detectable, the level of co-expression was minimal. The degree of co-expression and the number of aromatase-positive soma did not differ between sexes. However, the number of ERα cells was higher in the female preoptic area relative to that in the male. Conversely, ERα is more abundant in the male bed nucleus of the stria terminalis relative to the female. We conclude that while local aromatization in the preoptic area may modulate ERα-containing neurons via autocrine pathways, paracrine mechanisms may predominate in other areas of the songbird brain.


*Keywords:* Aromatase; Zebra fish; Testosterone; Sex difference

## 1. Introduction

Estrogens are critical organizational and activational effectors of the vertebrate brain (Arnold and Gorski, 1984). In most vertebrates, estrogens are made available to the central nervous system (CNS) via synthesis in the ovary and transport through the vasculature. Once in the brain, a diverse and exhaustive set of cellular events are modulated by this steroid (Callard et al., 1990; McEwen et al., 1991).

The songbird brain is modulated by estrogens during development and adulthood. Administration of estrogens to hatchling female zebra finches (*Taeniopygia guttata*), starlings (*Sturnus vulgaris*), and canaries (*Serinus canarius*) partially masculinizes some song nuclei (Arnold et al., 1986; Casto and Ball, 1996; DeVoogd and Nottebohm, 1981; Gurney, 1981; Gurney and Konishi, 1980; Konishi and Akutagawa, 1988). Although various perturbations of estrogen action have failed to block the putative masculinizing effect of estrogens on the male song system (reviewed in Arnold, 1997), recent studies point to the critical role of local encephalic estrogen synthesis as key in the developing male song circuit (Holloway and Clayton, 2001).

* Corresponding author. Fax: +1 610 758 4004.
*E-mail address:* colin@lehigh.edu (C.J. Saldanha).

0016-6480/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygcen.2004.11.013

Estrogen administration to hatchling females can also masculinize some aspects of partner preference during adulthood (Adkins-Regan and Wade, 2001). In adults, estrogens can activate copulatory and singing behaviors in males of several species in laboratory and field studies (DeVoogd, 1991; Harding et al., 1983; Soma et al., 2004; Tramontin et al., 2003; Walters and Harding, 1988). Taken together, the data suggest that the brain of male and female songbirds is capable of responding to estrogen at multiple stages of development and adulthood.

As in many vertebrates, in passerine songbirds, neural estrogen provision can also occur by the site-specific expression of the estrogen-synthetic enzyme aromatase. While several birds and mammals express aromatase at hypothalamic and some limbic loci (Balthazart et al., 1991; Callard et al., 1990; Hutchison and Steimer, 1986; Naftolin et al., 1996; Roselli and Resko, 1993; Schumacher and Balthazart, 1987; Schumacher et al., 1983), aromatase levels are comparable or higher at several telencephalic areas in the songbird (Schlinger and Arnold, 1991, 1992). Specifically, numerous forebrain areas including the hippocampus, nidopallium, and arcopallium contain neurons capable of synthesizing estrogen which is available to modulate local steroid-dependent processes (Freking et al., 2000; Saldanha et al., 1998, 1999, 2000; Shen et al., 1994, 1995; Soma et al., 1999, 2003).

As in other vertebrates, the songbird brain is responsive to estrogen, in part, due to the site-specific expression of estrogen receptors. Of these, both the classic ER α (ERα) and ER β (ERβ) have been cloned and their distributions mapped (Bernard et al., 1999; Perlman and Arnold, 2003). Although the expression patterns of ERα and ERβ overlap, qualitative examinations reveal a preponderance of ERα transcripts relative to ERβ in the starling (*S. vulgaris*) and zebra finch brain (Bernard et al., 1999; Gahr et al., 1993; Jacobs et al., 1996, 1999; Perlman and Arnold, 2003). Taken together, these data suggest that areas of the songbird telencephalon contain estrogen synthetic (aromatase-expressing) and estrogen responsive (ER-expressing) neurons.

The transcription of aromatase and ERα occurs in overlapping areas of the brain (Jacobs et al., 1999). Specifically, both transcripts are expressed in the preoptic (POA) and ventromedial (VMN) nuclei of the hypothalamus and in the telencephalic bed nucleus of the stria terminalis (BnST), nucleus taeniae (NT), and caudomedial nidopallium (NCM). These studies have identified potential areas of communication between aromatase and ERα clusters in the songbird brain. However, at the protein level, these interactions are not well understood.

Studies that have used antibodies to reveal the interactions between aromatase and ERα are limited to species that show little telencephalic aromatization (Balthazart et al., 1991; Veney and Rissman, 1998). Notably, with the exception of the tuberal area in the quail (Balthazart et al., 1991), there appears to be low co-localization of hypothalamic aromatase and ERα in avian and mammalian species. To the best of our knowledge the interactions between aromatase and ER proteins in the songbird telencephalon remain unknown.

Towards this end, we have used antibodies that specifically recognize aromatase (Saldanha et al., 2000) and ERα in songbird brain to identify where and how estrogen synthetic and responsive neurons may interact.

## 2. Materials and methods

### 2.1. Subjects and tissue preparation

Subjects were adult male ($N=5$) and female ($N=5$) zebra finches, from our colony at the Lehigh University animal facility. All housing and experimental protocols were carried out in accordance with IACUC requirements. Birds were housed in same-sex groups of 3–4 birds in visual isolation from the opposite sex in steel cages ($18\times12\times12$) cages in a room maintained at $20\pm2$ °C and LD 14:10 (lights on at 06:00 h). Food, water, and grit were available ad libitum.

Subjects were killed under deep anesthesia (ketamine/xylazine) and perfused trans-cardially with 5 ml of 0.1 M phosphate buffer (PB) followed by 30–40 ml of fixative. The fixative used was 15% saturated picric acid in 4% paraformaldehyde contained in 0.1 M PB (pH 7.35). The brains were removed and immersed in 4% paraformaldehyde (pH 7.4) overnight at 4 °C. The gonads and oviduct were removed immediately following the perfusion and the diameters of testes and the two largest ovarian follicles were measured. Following post-fixation, the brains were embedded in 8% gelatin and re-immersed in 4% paraformaldehyde for 48–72 h at 4 °C.

Coronal sections (50 μm) were cut on a vibratome and collected into a high-sucrose/ethylene glycol solution for storage at −20 °C (Watson et al., 1986). This antifreeze solution preserves the antigenicity of the songbird brain for extended periods (up to 3 years in our hands). Sections were collected into three separate sets (A–C). In four birds (two per sex), sections from Set A were processed for aromatase. In these same birds, adjacent sections (Set B) were processed for ERα. This permitted us to obtain the distributions and overlap between aromatase and ERα expression in the zebra finch brain. The remainder of Sets A and B were used for other experiments in the laboratory.

For all 10 birds, we performed double-label immunocytochemistry (ICC) for both antigens on sections from Set C using the protocols described below.

### 2.2. ICC—single label

Aromatase (Set A; $N=2$ birds per sex) was visualized using a polyclonal antibody as previously published