

# Subcellular Compartmentalization of Aromatase Is Sexually Dimorphic in the Adult Zebra Finch Brain

Kevin N. Rohmann,<sup>1</sup> Barney A. Schlinger,<sup>2</sup> Colin J. Saldanha<sup>1,3</sup>

<sup>1</sup> Department of Biological Sciences, Lehigh University, Bethlehem, Pennsylvania 18015

<sup>2</sup> Department of Physiological Science and the Laboratory for Neuroendocrinology, UCLA, Los Angeles, California 90095

<sup>3</sup> Program in Cognitive Science, Lehigh University, Bethlehem, Pennsylvania 18015

Received 27 January 2006; accepted 22 April 2006

**ABSTRACT:** The vertebrate brain is a source of estrogen (E) via the expression of aromatase (*E-synthase*). In the zebra finch (*Taeniopygia guttata*), despite documented dimorphisms in E-action, no differences are detectable in circulating E, or the neural levels of aromatase transcription, activity, or somal protein expression. Studies of aromatase expression at the light- and electron-microscope levels reveal greater numbers of fibers and presynaptic boutons in adult males relative to females. We assayed aromatase activity and content in synaptosomes and microsomes from the anterior [containing IMAN and Area X (males)] and posterior telencephalon (containing HVC and RA) of adult birds. In contrast to non-song birds and mammals, both cell fractions contain abundant aromatase measurable in terms of activity (enzyme assays) and content (Western blots) with minimal enrichment in microsomes. From brain homogenates of identical concentration, aroma-

tase activity was higher in the synaptosomal relative to the microsomal fraction, in males relative to females, and in the posterior compared to anterior telencephalon. These effects were driven by high levels of synaptosomal aromatase in the male posterior telencephalon. These data suggest that males possess more aromatase per presynaptic bouton, or a greater number of aromatase-containing presynaptic boutons than females in the posterior telencephalon. Further, the present report reveals synaptic aromatization as a considerable source of E in the zebra finch brain, and supports the idea that telencephalic synapses in and around the adult male song production nuclei may be exposed to higher levels of E compared to the female brain. © 2006 Wiley Periodicals, Inc. *J Neurobiol* 67: 1–9, 2007

**Keywords:** estrogen; testosterone; songbird; synapse; learning

## INTRODUCTION

Steroids like estrogen (E) are made available to the vertebrate brain by endocrine, paracrine, and autocrine mechanisms. These modes of E-provision are achieved,

in part, by the localized expression of the enzyme aromatase (*E-synthase*), which converts androgens to estrogens. In females, aromatase expression in ovarian steroidogenic cells is largely responsible for circulating E in several vertebrates. In males of many species, aromatase is expressed at specific neural loci such as the diencephalic preoptic area (POA), ventromedial nucleus (VMN), and tuberal hypothalamus (Tu). Aromatization in the brain results in locally elevated levels of E critical in the organization and activation of male-specific copulatory and aggressive behaviors (Callard et al., 1978; Lephart, 1996; Schlinger, 1997).

Correspondence to: C.J. Saldanha.

Contract grant sponsor: NIH; contract grant number: RO1 047267 (C.S.) and 61994 (B.A.S.).

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Published online 20 October 2006 in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/neu.20303

The neural expression of aromatase is a key mechanism of hormone provision to E-sensitive circuits across vertebrates (MacLusky and Naftolin, 1981; Balthazart, 1997; Goodson et al., 2005).

Aromatase belongs to the class of membrane-bound cytochrome P<sub>450</sub> enzymes traditionally found in rough endoplasmic reticula (RER) in many cell types including neurons. Aromatase has also been documented in presynaptic boutons of quail (*Coturnix coturnix japonica*), rat (*Rattus norvegicus*), monkey (*Cercopithecus aethiops*), human (*Homo sapiens*) (Schlinger and Callard, 1989; Naftolin et al., 1996; Hojo et al., 2004), and most recently in zebra finches (*Taeniopygia guttata*; Peterson et al., 2005). However, despite clear evidence of synaptic localization using biochemical measures of activity following differential centrifugation (Schlinger and Callard, 1989) and immunoelectron microscopy (Naftolin et al., 1996; Hojo et al., 2004; Peterson et al., 2005), little is known about the function of synaptic aromatase in any species.

Aromatase is particularly abundant and widespread in the zebra finch brain, including several diencephalic and telencephalic regions (Shen et al., 1994, 1995; Saldanha et al., 2000). In adults of this species, encephalic E-provision is believed to modulate dimorphic steroid-dependent processes including singing, courtship and aggression, and spatial memory performance in males (Walters and Harding, 1988; Oberlander et al., 2004). However, these well-documented effects are difficult to reconcile with several characteristics of E-provision. In the zebra finch, neither circulating E (Adkins-Regan et al., 1990; Schlinger and Arnold, 1992; but see Hutchison et al., 1984), neural aromatase transcription (Shen et al., 1995; Saldanha et al., 1998), nor neural aromatase activity (Vockel et al., 1988, 1990; Schlinger and Arnold, 1991, 1992; Saldanha et al., 1998) has been found to differ between males and females. Lastly, somal aromatase expression is not dimorphic (Saldanha et al., 2000; Peterson et al., 2005). Correspondingly, at the protein level, E-receptors (ER) are conspicuously absent in the zebra finch song system (Arnold, 1997; Schlinger, 1997; Jacobs et al., 1999; Perlman and Arnold, 2003; Wade and Arnold, 2004), suggesting that sensitivity to E in song nuclei is not associated with dimorphic E-action. Taken together, these findings do not explain the dimorphism of E-dependent physiology in the zebra finch.

In contrast, synaptic aromatization offers some reconciliation between localized E-provision and the dramatic sexual dimorphism in singing behavior in the zebra finch. The number of aromatase-positive fibers and presynaptic boutons is higher in males rela-

tive to females (Saldanha et al., 2000; Peterson et al., 2005). These data suggest that the compartmentalization of aromatase may be a critical component underlying sexually dimorphic E-provision in this species. To test this hypothesis, we measured aromatase activity (enzyme assay) and content (Western blot) across ultrastructural compartments, brain areas, and sexes in adult birds. We reasoned that dimorphisms in compartmentalization may be more noticeable in the posterior telencephalon (around HVC and RA) compared to elsewhere in the brain.

## METHODS

### Tissue Collection

Adult zebra finches ( $n = 4$  per sex) were decapitated and the brains rapidly dissected on ice. The anterior telencephalon (an area of low-moderate levels of aromatase) containing Area X (in males) and the magnocellular nucleus of the anterior nidopallium (MAN) were separated from the posterior telencephalon (an area of high levels of aromatase) containing the hippocampus (HP), caudomedial nidopallium (NCM), HVC, and the robust nucleus of the arcopallium (RA). Tissue was weighed, placed into ice-cold STM solution [10 mg wet tissue into 100  $\mu$ L 0.25 M sucrose, 20 mM TrisHCl, 5 mM magnesium sulfate, 100  $\mu$ M phenylmethylsulfonyl fluoride (pmsF), 0.02% sodium azide in diethyl pyrocarbonate (DEPC) water], and homogenized by  $4 \times 5$  s bursts of an electric homogenizer.

### Preparation of Subfractions

Subfractions were prepared as previously described for quail hypothalami (Schlinger and Callard, 1989) and zebra finch telencephalon (Schlinger and Arnold, 1992). Homogenates were centrifuged for 15 min at  $1034 \times g$  at  $4^\circ\text{C}$ . The resulting supernatant (S1) was removed and the pellet (P1) was discarded. For each brain area the S1 from two 100  $\mu$ L aliquots of homogenate were pooled and further centrifuged for 30 min at  $10,081 \times g$  at  $4^\circ\text{C}$ . The supernatant (S2) was removed and the pellet (P2), containing synaptosomes and mitochondria, was washed as follows. P2 was washed (2X) with 100  $\mu$ L of STM solution followed by a 10 min centrifugation at  $10,081 \times g$  at  $4^\circ\text{C}$ . The P2 wash solution was added to S2 and this mixture was centrifuged for 60 min at  $100,000 \times g$  at  $4^\circ\text{C}$ . The supernatant (S3) was removed and stored at  $-80^\circ\text{C}$ . The pellet resulting from this step (P3), containing microsomes, along with the P2 were both weighed wet and resuspended in STM solution at a concentration of 2  $\mu$ L STM per milligram of pellet and then stored at  $-80^\circ\text{C}$ . By simultaneously measuring marker enzymes, avian brain subfractions prepared using similar protocols have been fully validated (Schlinger and Callard, 1989), so we did not assay the subfractions produced in these experiments.

### Experiment 1: Aromatase Activity Time Course

Aromatase activity was measured in a standard enzyme assay as previously described (Schlinger and Callard, 1989; Saldanha et al., 1998). Homogenates of the entire telencephalon (anterior and posterior combined) were resuspended in 180  $\mu\text{L}$  of ice-cold sucrose-phosphate buffer and incubated with 100 nM [1,2,6,7- $^3\text{H}$ ]AE (specific activity 74 Ci/mmol; NEN) for 10, 30, or 90 min at 41°C in the presence of a NADH/NADPH-generating cofactor cocktail (20  $\mu\text{L}$ ) and 1  $\mu\text{g}$  radioinert  $\text{E}_1$  and  $\text{E}_2$  (Steraloids, Newport, RI). Radioinert estrogens were included to protect formed tritiated estrogens from further metabolism. Control tubes contained everything but tissue. Reactions were terminated by snap freezing. To correct for procedural losses, tubes containing a known amount of [ $^3\text{H}$ ]E<sub>1</sub> were processed in parallel. Steroids were extracted with diethyl ether (3X), and androgens were separated from estrogens by phenolic partition (2X; Schlinger and Callard, 1989). Radioinert  $\text{E}_1$  and  $\text{E}_2$  (Steraloids) were added as markers, and steroids were separated using thin-layer chromatography (Schlinger and Callard, 1989). Steroids were visualized under ultraviolet light. After scraping the bands, tritiated steroids were eluted from the silica and measured in a scintillation counter. Total estrogen (E) represents the sum of cpm in  $\text{E}_1$  and  $\text{E}_2$ . The cpm measures were corrected for background and procedural losses and are reported as fmoles per milligram of protein (or per milligram homogenate). Protein content of the homogenates was measured by the method of Bradford (1976) using bovine serum albumin standards.

### Experiment 2: Sex, Brain Area, and Compartment Differences in Activity

To determine if aromatase activity was differentially expressed across sex, brain area, and compartment, homogenates of equivalent concentration (200  $\mu\text{g}/\text{mL}$ ) were separated by centrifugation (see above) and the P2 and P3 pellets were processed in entirety for aromatase activity. The entire pellet was incubated with a saturating concentration of tritiated androstenedione (AE; 100 nM) for 5 min and formed estrogens were measured as described above. Resultant fmoles E were normalized for 200  $\mu\text{g}$  of homogenate or per milligram protein as described above (Bradford, 1976) and compared across sex, brain area, and compartment (see below for statistics).

### Experiment 3: Aromatase Content as a Function of Total Protein Loaded

Samples were thawed on ice. Protein content of the homogenate was determined by the method of Bradford (1976). P2 (synaptosomal; 14.3, 28.6, or 57.2  $\mu\text{g}$  protein) and P3 (microsomal; 4.06, 8.12, or 20.31  $\mu\text{g}$  protein) were mixed with 5% sodium dodecyl sulfate (SDS) and Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA), and heated for 4 min at 90°C before separation by SDS-poly-

acrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto nitrocellulose paper (Bio-Rad) and blocked for 1 h at room temperature in 5% Blotto [nonfat dry milk (Carnation) in Tris-buffered saline (TBS), pH 7.6]. The blot was incubated in a zebra-finch-specific primary antibody against aromatase [anti-zebra finch aromatase C-terminal (AZAC); Saldanha et al., 2000] at a concentration of 1:15,000 in 1% Blotto for 48 h at 4°C on a nutator. The blot was washed 2  $\times$  10 min in 0.1% v/v Tween 20 in Tris-buffered saline (TBST), pH 7.6, and 2  $\times$  5 min in TBS. It was then incubated in 1:2500 goat antirabbit IgG secondary with conjugated peroxidase (Jackson ImmunoResearch, West Grove, PA). The blot was then washed 2  $\times$  10 min in TBST and 2  $\times$  5 min in TBS. The blot was incubated in Super Signal West Pico (Pierce Biotechnology, Inc., Rockford, IL) and exposed to Kodak Biomax MS film (Eastman Kodak Company, Rochester, NY). Sample (1  $\mu\text{L}$ ) was used to determine total protein concentration using the method of Bradford (1976).

### Experiment 4: Sex, Brain Area, and Compartment Differences in Content

To assess differences in aromatase content across compartments, 5  $\mu\text{g}$  (P2) and 2  $\mu\text{g}$  (P3) were loaded onto gels and the relative optical density (ROD) of the resultant 55 kD band was quantified as described above. The concentration of each subfraction loaded corresponded to a portion in the linear portion of the detection curve from Experiment 3 above. ROD measures were compared across sex, brain area, and compartment as described below.

### Quantification of Western Blots

Film from Western blots was scanned with no adjustment of contrast or brightness, and the ROD of each  $\approx$  55 kD (aromatase) band was measured using Image J software (NIH; optical density of an adjacent nonspecific area subtracted from the optical density of the specific 55 kD band).

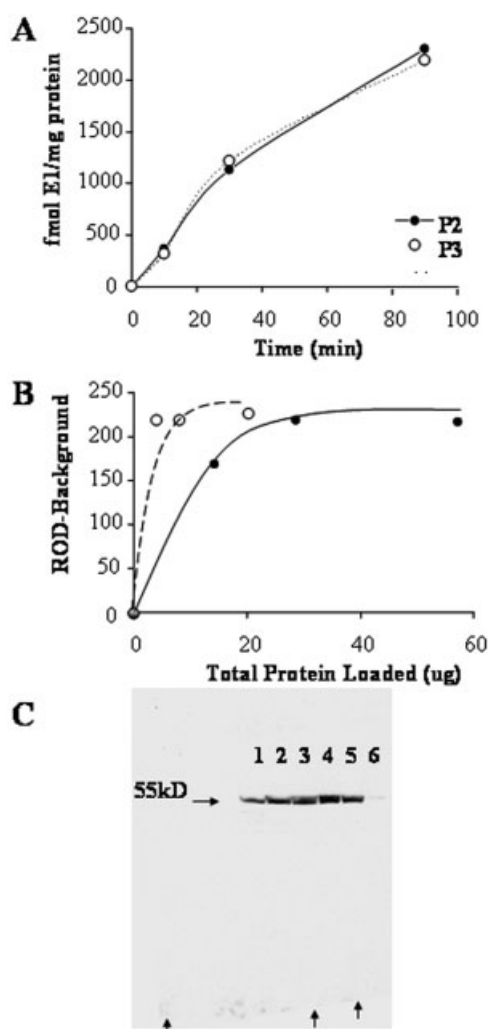
### Statistics

Fmoles E per 200  $\mu\text{g}$  homogenate, fmoles E per microgram protein, and ROD per microgram protein were analyzed by three-way ANOVA with sex as a between-subjects variable, but brain area and compartment as within-subject variables. Due to unequal variance all data were ln transformed prior to statistical analysis. Least square means comparisons were used to determine the source of significant differences in those main variables and interaction terms where  $p < 0.05$ . Although all statistical analyses were conducted on ln-transformed data, we present the untransformed means  $\pm$  SEM below.

## RESULTS

### Experiment 1

Aromatase activity was readily detectable in synaptosomal (P2) and microsomal (P3) pellets from adult zebra finch brain. The formation of E from radio-labeled androgen appeared linear until approximately 30 min. At greater time points, the formation of E appeared nonlinear perhaps due to exhaustion of substrate. As shown in Figure 1(A), the patterns of E-formation overlapped almost completely for P2 and



**Figure 1** Aromatase activity (A) and content (B) in synaptosomes (P2) and microsomes (P3) in the adult zebra finch brain (note: these data represent the entire telencephalon; see Methods). A representative Western blot (C) shows the detection of a single band at 55 kD in whole telencephalic homogenates (lane 1), synaptosomes (lanes 2 and 3), and microsomes (lanes 4 and 5) with minimal immunoprodukt observed in cytosol (lane 6). Arrows depict the bottom and left margin of the blot used in these studies.

P3, suggesting that: (a) the biochemical kinetics of the aromatase protein in these compartments appear very similar if not identical, and (b) aromatase activity is not enriched in the microsomal fraction (P3) relative to synaptosomes (P2) in the adult zebra finch brain.

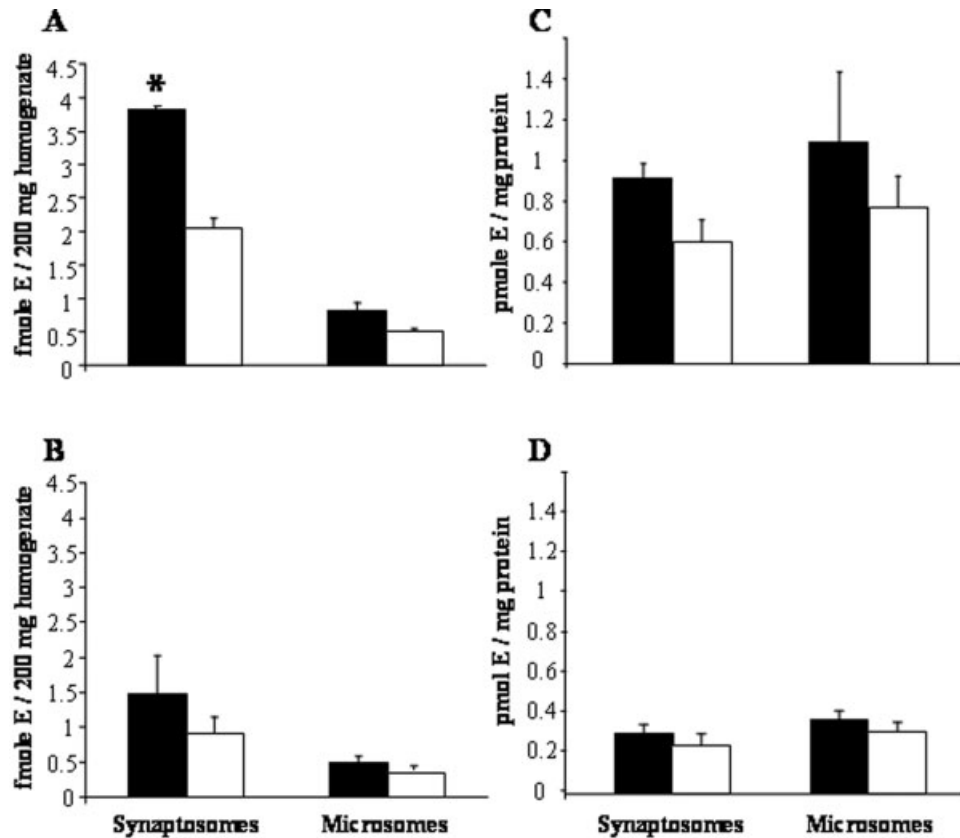
### Experiment 2

**Differences in Aromatase Activity per 200 mg Homogenate.** Aromatase activity per 200 mg homogenate was significantly different between sexes ( $p = 0.037$ ), brain areas ( $p = 0.004$ ), and compartments ( $p = 0.0001$ ), with males greater than females ( $1.66 \pm 0.36$  vs.  $0.95 \pm 0.18$  fmol E/200 mg homogenate/10 min), posterior greater than anterior telencephalon ( $1.80 \pm 0.34$  vs.  $0.81 \pm 0.18$  fmol E/200 mg homogenate/10 min), and synaptosomes greater than microsomes ( $2.06 \pm 0.32$  vs.  $0.54 \pm 0.06$  fmol E/200 mg homogenate/10 min) (Fig. 2). While the interaction of brain area with compartment was significant ( $p = 0.04$ ), neither the interactions of brain area with sex ( $p = 0.67$ ) nor compartment with sex ( $p = 0.28$ ) varied systematically.

**Differences in Aromatase Activity per Milligram Protein.** Normalization of activity per milligram protein maintained the significant differences in brain area and compartment. However, with this analysis, although the posterior telencephalon still shows higher fmoles E produced than the anterior telencephalon ( $0.841 \pm 0.10$  vs.  $0.311 \pm 0.025$  pmol E/mg protein/10 min;  $p = 0.001$ ), it is the microsomal compartment that contains higher activity than the synaptosome ( $0.638 \pm 0.118$  vs.  $0.514 \pm 0.076$  pmol E/mg protein/10 min;  $p = 0.007$ ). We were unable to detect significant variations across sex ( $p = 0.08$ ) or any interaction terms. These data are summarized in Table 1.

### Experiment 3

As a means of determining aromatase content in telencephalic subfractions, we measured the ROD of aromatase immunoreactive bands on Western blots. As shown in Figure 1(C), Western blots of whole telencephalon, synaptosomes, and microsomes showed a single band at approximately 55 kD, the predicted size of the zebra finch aromatase peptide (Shen et al., 1994). Minimal immunoreactive aromatase was detected in the S3 supernatant, indicating that the vast majority of detectable aromatase had been isolated in the synaptosomal and microsomal subfractions. As depicted in Figure 1(B), loading different amounts of total protein resulted in measurable differences in



**Figure 2** Histograms of aromatase activity in synaptosomes and microsomes from the posterior and anterior telencephalon of adult zebra finches. Aromatase activity is shown normalized per homogenate (A,B) and per milligram protein (C,D). Higher activity was detected in males (black bars) relative to females, and in the posterior telencephalon (A,C) relative to the anterior telencephalon (B,D). Least square means comparisons reveal higher aromatase activity in the synaptosomal compartment of males relative to females in the posterior telencephalon only (asterisk).

aromatase using ROD of the 55 kD band as an index of aromatase content. In the P2 pellet, aromatase content was linear relative to total protein loaded at lower concentrations but saturated at higher concentrations. In contrast to the pattern described above, aromatase content appeared to be slightly enriched in the P3 fraction relative to that in the P2 pellet. For microsomal (P3) aromatase content, ROD quickly saturated, and high levels were detectable at all concentrations of protein tested. In contrast, for synaptosomal (P2) content, ROD did not saturate until approximately 20  $\mu\text{g}$  of total protein loaded. These patterns suggest a two- to threefold enrichment of aromatase content in microsomes (P3) relative to synaptosomes (P2) as measured by Western blots.

#### Experiment 4

**Differences in Aromatase Content per Milligram Protein.** In general agreement with the above, aroma-

tase content normalized per milligram protein revealed significant main effects of sex ( $p = 0.039$ ), brain area ( $p = 0.023$ ), and compartment ( $p = 0.003$ ), with males greater than females ( $11.52 \pm 4.04$  vs.  $4.45 \pm 0.072$ ), posterior greater than anterior telencephalon ( $10.70 \pm 3.50$  vs.  $4.25 \pm 0.73$ ), and microsomes greater than synaptosomes ( $11.21 \pm 3.48$  vs.  $3.74 \pm 0.36$ ) (see Table 1). Importantly, both the interactions of compartment with sex and compartment with brain area were found to be significant ( $p = 0.016$  and  $0.04$ , respectively). Least square means analysis of ROD per microgram protein revealed that the microsomes in the male posterior telencephalon had higher aromatase content than that measured in any other compartment, sex, or brain area (see Table 1).

To insure that the observed differences in activity and content did not reflect differences in the propensity of subfractions to pellet, we analyzed sex, brain area, and compartment differences in the protein content of formed pellets and pellet weight (wet weight).

**Table 1** Aromatase Activity (fmoles E/mg/protein/10 min), Aromatase Content (ROD/mg Protein), and Protein Content of Pellets across Compartment, Brain Area, and Sex

	Male		Female	
<b>fmole E/mg protein/10min</b>	669.96 ± 117.96 <sup>a</sup>		481.87 ± 71.32 <sup>b</sup>	
Brain area (anterior/posterior)	342.01 ± 30.45 <sup>a</sup>	997.91 ± 167.24 <sup>b</sup>	279.04 ± 38.33 <sup>a</sup>	684.70 ± 92.61 <sup>b</sup>
Compartment (synapto/micro)	306.3 ± 43.2 <sup>a</sup>	377.6 ± 40.1 <sup>a</sup>	905.1 ± 80.9 <sup>b</sup>	1090 ± 343.8 <sup>b</sup>
			243.7 ± 62.3 <sup>a</sup>	314.4 ± 46.2 <sup>a</sup>
			599.3 ± 108.4 <sup>b</sup>	770.1 ± 152.9 <sup>b</sup>
<b>ROD/mg protein</b>	11.52 ± 4.04 <sup>a</sup>		4.45 ± 0.72 <sup>b</sup>	
Brain area (anterior/posterior)	5.41 ± 1.01 <sup>a</sup>	17.62 ± 7.47 <sup>b</sup>	4.25 ± 0.73 <sup>a</sup>	10.70 ± 3.51 <sup>b</sup>
Compartment (synapto/micro)	3.48 ± 0.55 <sup>a</sup>	7.34 ± 1.02 <sup>a</sup>	2.93 ± 0.39 <sup>a</sup>	3.74 ± 0.53 <sup>a</sup>
			3.82 ± 2.02 <sup>a</sup>	7.28 ± 1.47 <sup>a</sup>
<b>Protein content of pellet (mg)</b>	4.73 ± 0.61		6.02 ± 0.67	
Brain area (anterior/posterior)	6.2 ± 0.52	3.30 ± 0.84	6.03 ± 1.09	6.02 ± 0.86
Compartment (synapto/micro)	6.72 ± 0.74	5.61 ± 0.71	5.69 ± 0.84	6.13 ± 0.99
			6.37 ± 2.18	5.91 ± 1.57

All data are means ± sem. Within each row, numbers with different superscripts are significantly different from each other.

No differences across sex, brain area, or compartment were detected in the protein content of pellets following differential centrifugation ( $p = 0.09$ ,  $p = 0.21$ , and  $p = 0.25$ , respectively). In general agreement with the above, there was no difference between sex ( $p = 0.106$ ) or brain area ( $p = 0.366$ ) in the wet weight of synaptosomal or microsomal subfractions, but the synaptosomal subfraction wet weight was less than that of the microsomal subfraction ( $0.304 \pm 0.014$  vs.  $0.670 \pm 0.021$  g,  $p < 0.001$ ).

## DISCUSSION

The present data reveal three characteristics of E-provision in the zebra finch brain. Firstly, a significant portion of aromatase in the songbird brain appears localized within synaptosomal compartments. Further, this synaptosomal compartmentalization is strongly dimorphic with higher expression in males relative to females. Finally, the posterior telencephalon, including the HP, NCM, and areas around HVC and RA, most strongly contributes to sex, brain area, and compartment-specific influences on aromatase expression in this species. Taken together, these data underscore the potential contribution of aromatase within synaptic boutons as an important modulator of sex-specific, E-dependent behavior in the songbird brain.

Previous work has established the presence of this enzyme in presynaptic boutons of quail, rodents, primates, and songbirds (Schlinger and Callard, 1989; Schlinger and Arnold, 1992; Naftolin et al., 1996; Hojo et al., 2004; Peterson et al., 2005). In the diencephalon of non-song birds the activity of this P450 enzyme is enriched about 10-fold in microsomes relative to synaptosomes (Schlinger and Callard, 1989). A similar enrichment relative to whole tissue homogenates has also been reported in microsomes of the rodent brain where activity in other compartments was undetectable (Roselli, 1995). In contrast, the present measures of aromatase activity reveal a near-equivalent compartmentalization of aromatase activity per milligram protein in synaptosomes and microsomes [see Fig. 1(A)] or a minor (less than twofold) enrichment in microsomes [Fig. 2(C,D)]. Correspondingly, Schlinger and Arnold (1992) have reported a similar minor enrichment in microsomal aromatase activity relative to synaptosomes in the juvenile zebra finch telencephalon. Taken together, these data suggest an important difference between aromatase in the songbird brain relative to other species. A substantial portion of aromatase activity is apparent in the synaptosome of the songbird telencephalon.

Measures of aromatase content, however, suggest some enrichment in microsomes relative to synaptosomes, particularly in the male posterior telencephalon [Fig. 1(B) and Table 1]. We are unsure as to the reason for this apparent discrepancy. One possibility is that aromatase may be sequestered differently within microsomes and synaptosomes, rendering measures of content per milligram protein unpredictable. Differential sequestration may also affect epitopes on the aromatase protein, thereby altering measures of optical density on Westerns. Lastly, it is notable that the P2 fraction contains mitochondria, structures that almost certainly contribute to protein content, but not to aromatase activity (Schlinger and Callard, 1989). This suggests the possibility that we may be underestimating the aromatase activity in synaptosomes when the data are normalized with protein content. For the reasons above, we suggest that measures of aromatase per milligram homogenate [Fig. 2(A,B)] may be more reliable indices of aromatase compartmentalization in the zebra finch. Further, due to technical limitations, we were unable to load smaller concentrations of microsomal protein in the Western Blot experiments. This results in ROD measures in the saturated range [see Fig. 1(B)], and perhaps compromises interpretations as to microsomal aromatase (but not synaptosomal aromatase) content.

Among homeotherms, songbirds demonstrate the highest levels of telencephalic aromatase reported (Goodson et al., 2005), and the current data reveal that a substantial portion of this activity is contained within presynaptic boutons. Songbirds, therefore, emerge as a powerful animal model to study the ability of projection (and inter-) neurons to specifically alter the steroidal milieu at synapses. Indeed, “trans-synaptic” steroid effects have been reported in the songbird brain (Gurney and Konishi, 1980; Brenowitz and Lent, 2002), suggesting that synaptic steroid provision may represent a powerful mode of E-provision in members of this order.

As mentioned previously (see Introduction), ER has proven difficult to localize within several song nuclei in the zebra finch. In contrast, several song nuclei express abundant androgen receptor (AR; see Arnold, 1997). This pattern of steroid receptor expression combined with the current findings of synaptosomal aromatization raise an intriguing possibility. Aromatization may serve as an efficient sink for androgens, prohibiting their effects on neural targets. Alternatively (or in addition), locally synthesized E from synaptic boutons may function via ER-independent, nongenomic mechanisms. These hypotheses await experimental testing *in vivo* (see Peterson et al., 2005).

The dramatic dimorphisms in CNS structure and function in the zebra finch have provided a strong foundation for numerous studies on the relationships among hormones, brain, and behavior (Nottebohm and Arnold, 1976). To our knowledge, however, only a handful of studies have reported differences in E-provision towards explaining these dimorphisms (Saldanha et al., 2000; Peterson et al., 2005; present data). We have found that aromatase activity from homogenates of identical concentration (200 mg/mL) is strongly influenced by sex (males > females), brain area (posterior > anterior telencephalon), and compartment (synaptosome > microsome). This pattern of data is not explained by differences in the efficacy of centrifugation because neither the weights nor protein content of formed pellets was influenced by sex or brain area (see Results). We therefore conclude that the observed differences in aromatase activity accurately reflect higher E-synthetic capability at male synapses relative to female synapses. This pattern of results may reflect a greater number of aromatase-expressing presynaptic boutons in the male telencephalon relative to that in the female. Indeed, EM investigations support this view, suggesting that telencephalic loci of the adult male zebra finch do contain more aromatase-expressing presynaptic boutons in the HP, NCM, and HVC (Peterson et al., 2005). The present results may also reflect a higher concentration of aromatase within individual synapses in the male relative to the female. Quantitative EM studies of aromatase expression within individual presynaptic boutons are necessary to evaluate this hypothesis.

Earlier reports localized immunoreactive aromatase to presynaptic boutons within HVC and HP of adult male zebra finches (Saldanha et al., 2000; Peterson et al., 2005). Although these studies were successful in describing greater numbers of aromatase-positive synaptic boutons in males relative to females, whether (or not) these boutons were capable of synthesizing E was unknown. In excellent agreement with previous data (Peterson et al., 2005), the present report suggests that synaptosomes of the adult male zebra finch contain higher aromatase activity than those from adult females (see Fig. 2). Taken together, these studies strongly implicate synapses, and not the somal or dendritic aromatase localized in microsomes, as a dimorphic source of E available to modulate E-dependent physiology in the zebra finch brain.

The posterior telencephalon including HP, NCM, and other areas around HVC and RA emerged as a strong contributor to sex-specific synaptosomal aromatase. These areas are critical modulators of singing behavior (Nottebohm and Arnold, 1976) and song

recognition (Mello et al., 1992; Chew et al., 1996; Bailey et al., 2002; Bailey and Wade, 2003). It is possible that synaptic aromatase may contribute to these processes in the zebra finch. Indeed, provision of E or its precursor testosterone has potent effects on singing behavior in several songbird species (Harding et al., 1983; Walters and Harding, 1988; Oberlander et al., 2004). Further work is needed to ascertain the specific role of synaptic E-provision in these behaviors.

Why package aromatase in synaptosomes? The present results add to the increasing body of evidence suggesting that hormonal provision at synaptic connections may represent an important mode of combining electrical and endocrine signaling in the vertebrate brain (Hojo et al., 2004; Peterson et al., 2005). Importantly, the preponderance of axonal and synaptic aromatase in males relative to females (Saldanha et al., 2000; Peterson et al., 2005; present results) strongly suggests a functional nexus between circuits responsible for singing behavior and the steroidal modulation of these circuits. Further work in our laboratories is currently focused on the developmental and seasonal regulation of this dimorphism in E-provision within the passerine brain.

We thank Noel Alday for technical assistance and Ryan Wynne and Brad Walters for helpful comments on a draft of this manuscript.

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