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Effects of Estradiol on Incorporation of New Cells in the Developing Zebra Finch Song System: Potential Relationship to Expression of Ribosomal Proteins L17 and L37

Yu Ping Tang and Juli Wade

Department of Psychology & Zoology, Neuroscience Program, Michigan State University, East Lansing, Michigan 48824

Abstract

Mechanisms regulating masculinization of the zebra finch song system are unclear; both estradiol and sex-specific genes may be important. This study was designed to investigate relationships between estrogen and ribosomal proteins (RPL17 and RPL37; sex-linked genes) that exhibit greater expression in song control nuclei in juvenile males than females. Four studies on zebra finches were conducted using bromodeoxyuridine (BrdU) injections on posthatching days 6–10 with immunohistochemistry for the ribosomal proteins and the neuronal marker HuC/D at day 25. Volumes of brain regions were also assessed in Nissl-stained tissue. Most BrdU+ cells expressed RPL17 and RPL37. The density and percentage of cells co-expressing BrdU and HuC/D was greatest in Area X. The density of BrdU+ cells in Area X (or its equivalent) and the percentage of these cells that were neurons were greater in males than females. In RA and HVC, total BrdU+ cells were increased in males. A variety of effects of estradiol were also detected, including inducing an Area X in females with a masculine total number of BrdU+ cells, and increasing the volume and percentage of new neurons in the HVC of females. The same manipulation in males decreased the density of BrdU+ cells in Area X, total number of BrdU+ cells in RA, and density of new neurons in HVC and RA. These data are consistent with the ideas that RPL17, RPL37, and estradiol might all influence sexual differentiation, perhaps with the hormone and proteins interacting, such that an appropriate balance is required for normal development.

Keywords

sex difference; estrogen; RPL17; RPL37; brain development

INTRODUCTION

Sexually dimorphic neural systems provide exceptional models for elucidating factors crucial to development and maintenance of structure and function of the brain. The zebra finch song system is particularly useful for understanding ontogenetic mechanisms, as both anatomical and behavioral differences between males and females are dramatic and permanently established prior to sexual maturity. Under normal circumstances, only males sing, and a variety of sex differences in morphology of song control regions exist. For example, forebrain areas including HVC (used as a proper name) and the robust nucleus of the arcopallium (RA) are larger in volume in males, because of an increase in the number and size of neurons. Area

X, which is substantial in size in males, cannot be detected in females with Nissl stains (all reviewed in Arnold, 1997; Wade, 2001; Wade and Arnold, 2004).

It is clear that steroid hormones can impact differentiation of both structure and function. In particular, estradiol administered to posthatching females enhances the morphology of song control regions and can organize the capacity for adult song (reviewed in Wade, 2001; Wade and Arnold, 2004). Endogenous estradiol produced in the brains of juvenile males is likely responsible for the growth of the projection from HVC to RA (Holloway and Clayton, 2001). However, it has been difficult to draw conclusions about other morphological effects of estradiol (for example on neuron survival or soma size) because, while estradiol treatment masculinizes females, inhibiting secretion or action of the hormone has generally not inhibited the process in males (Arnold, 1975; Mathews et al., 1988; Adkins-Regan and Ascenzi, 1990; Mathews and Arnold, 1990, 1991; Balthazart et al., 1995; Wade and Arnold, 1994, 1996; Wade et al., 1999).

Genetic differences between the sexes are responsible for some aspects of sexual differentiation. The most direct demonstration comes from a spontaneously occurring gynandromorphic zebra finch, which had plumage typical of females and an ovary on the left paired with masculine plumage and a testis on the right (Agate et al., 2003). Although analysis of the brain of this bird indicated that some diffusible signal was also likely involved, the influence of the male complement of genes on the right side of the body (and/or female on the left) clearly influenced differentiation of the song system, HVC in particular.

Specific genetic mechanisms regulating sexual dimorphisms in the song system are unknown. Recent studies have demonstrated increased expression of particular genes in song nuclei of developing males compared to females (Kim et al., 2004; Chen et al., 2005; Duncan and Carruth, 2007; Tang et al., 2007). We identified greater expression of the mRNA encoding ribosomal proteins L17 and L37 (RPL17 and RPL37) in the Area X and RA of 25-day-old males compared to females, sex differences that are greatly reduced or eliminated in adults (Wade et al., 2005; Tang and Wade, 2006). This pattern was also seen in the ventral portion of the ventricular zone (VVZ), a region likely to generate neurons destined for Area X (DeWulf and Bottjer, 2002, 2005; Scott and Lois, 2007), suggesting that RPL17 and/or RPL37 might facilitate masculinization by increasing the survival of and/or recruitment of neurons into Area X in particular.

Although many details remain to be worked out, collectively the data suggest that both genes and steroid hormones are involved in sexual differentiation of the zebra finch song system. One particularly intriguing idea is that expression one or more genes on the sex chromosomes interact in some way with estradiol to effect complete masculinization. BLASTn comparisons of the sequences of RPL17 and RPL37 to the map of the zebra finch genome released in 2008 (NCBI database) suggest that both are located on the Z chromosome (males are homogametic, ZZ, where as females are heterogametic, ZW). Thus, they seem good candidates for this scenario.

The focus of the present set of experiments was to begin to investigate potential interactions of RPL17 and RPL37 and estradiol in the masculinization of Area X. Questions to be addressed included whether: (1) cells incorporated into the portion of the medial striatum containing Area X that were born prior to the obvious signs of sexual dimorphism that exist at approximately posthatching day 11 (e.g., Kim et al., 2004) express either of the RPL17 or RPL37; (2) estradiol mediates the presence of these cells; (3) the new cells become neurons. Although focusing on mechanisms influencing sexual differentiation of Area X, RA was also evaluated due to the sexually dimorphic expression of RPL17 and RPL37 mRNAs during development (see earlier). HVC was used as a control, as the mRNAs for these ribosomal proteins are not specifically

expressed in that region (that is, to no greater extent than in surrounding tissue; Wade et al., 2005), and it projects to the both of the other two areas.

Four experiments were conducted on alternate series of sections from the same set of tissue. Bromodeoxyuridine (BrdU) was injected on posthatching days 6-10 in birds administered estradiol or blank implants on day 3, and they survived until they were juveniles (posthatching day 25). Tissue was collected then because sex differences in morphology and expression of RPL17 and RPL37 are known to exist in Area X at that age, and changes in the brain regions are unlikely to be affected by song-related activities. It is before males form a template of their fathers' songs or begin to vocalize (Nordeen and Nordeen, 1997); thus the experiences of males and females should not have differed substantially up to this point. Experiment 1 involved immunohistochemistry with fluorescent tags for BrdU plus RPL17 or RPL37 proteins followed by the analysis using confocal microscopy to determine the densities of single and double-labeled cells. The same procedures were used in Experiment 2 in tissue labeled with antibodies to BrdU and the neuronal marker HuC/D. In Experiment 3, nonfluorescent chromagens were used to label BrdU and HuC/D to allow estimates of total BrdU+ and double-labeled cells to be generated with stereology in brightfield. Finally, the volumes of Area X, RA, and HVC were quantified in Nissl-stained sections from these birds in Experiment 4 because data previously collected on zebra finches treated with estradiol during development involved primarily females, and in most cases the brains were evaluated when the birds were far older than posthatching day 25, as was used in the present experiment (Gurney, 1981; Simpson and Vicario, 1991; Adkins-Regan et al., 1994; Grisham and Arnold, 1995).

METHODS

Animals

Zebra finches were raised in colonies containing approximately 5-7 breeding pairs and their offspring. They were housed on a 12:12 light:dark cycle and seed and water were available *ad libitum*. Each week, the birds also received either spinach or oranges and bread mixed with hard-boiled chicken eggs. Nests were checked daily; when a hatchling was found was considered posthatching day 1.

Manipulations and Tissue Collection for all Experiments

On posthatching day 3, males and females each received a subcutaneous implant of either estradiol or a vehicle control ($n = 6$ per sex per treatment). Hormone pellets were produced using a 1:5 uniform mixture of 17β -estradiol (Steraloids, Welton, NH) and silicone sealant (Type A, Dow Corning, Midland, MI) that was extruded through a 3-mL syringe in a straight line onto wax paper and dried overnight. The mixture was cut into 1 mm lengths, which were then quartered. Each of these treatments contained approximately 100 μ g estradiol. Control, blank pellets were synthesized in the same way without the addition of the hormone.

Birds received a daily injection of BrdU dissolved in 0.75% saline beginning on posthatching day 6 (50 μ g/g body weight; Sigma #B5002, St. Louis, MO) for five consecutive days. They were euthanized at 25 days of age by rapid decapitation. Gonads were examined at this time to determine the sex of each individual. Brains were frozen in cold methyl-butane and stored at -80°C , then sectioned (20 μ m) on a cryostat, and thaw-mounted onto SuperFrost Plus slides (Fisher Scientific, Hampton, NH). Six sets of slides containing coronal sections of each brain throughout the telencephalon were stored with desiccant at -80°C until further processing.

Experiment 1: Double-Label Immunofluorescence for BrdU Plus RPL17 or RPL37

One set of slides was warmed to room temperature, rinsed in 0.1M phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 15 min, and washed three times in PBS (5 min each).

Slides were treated with 0.1N HCl for 30 min at 37°C, followed by two rinses in 0.1M sodium borate buffer pH 8.5 (5 min each). They were then washed three times for 5 min in PBS, exposed to 0.9% H₂O₂/methanol for 30 min and incubated for 30 min in 3% normal donkey serum in PBS with 0.3% Triton X-100. The tissue was then incubated overnight at 4°C in PBS containing a BrdU mouse monoclonal antibody (1 µg/mL; Roche Diagnostics #11170376001, Indianapolis, IN) and a RPL17 polyclonal antibody raised in rabbit against zebra finch RPL17 amino acids 61-74 (RPYNGGVGRCAQAKQ; 1 µg/mL; produced for us by the Antibody on Demand program of Affinity Bioreagents, Golden, CO), along with 0.3% Triton X-100, 3% NDS, and 30% glycerol. A mixture of Cy2-conjugated donkey anti-mouse (for BrdU) and TRITC-conjugated donkey anti-rabbit (for RPL17) secondary antibodies (1 µg/mL each; Jackson ImmunoResearch, West Grove, PA) was then used for 2 h at room temperature. Slides were quickly dehydrated in ethanol and coverslipped with DPX (Fluka, St. Louis, MO). A second set of slides was processed exactly as mentioned earlier, except that a zebra finch RPL37 polyclonal antibody raised in rabbit (against amino acids 25-39; KAYHLQKSTCGKCGY; 1 µg/mL; Antibody on Demand) was used rather than the RPL17 antibody. Prior to use on brain sections as indicated earlier, both antibodies were tested on Western blots. Each produced single, clean band of the predicted size (not shown).

Experiment 2: Double-Label Immunofluorescence for BrdU Plus HuC/D

A third set of slides was processed using the same procedure as mentioned earlier except that the HuC/D primary antibody (raised in mouse; Molecular Probes #A21271, Eugene, OR) was substituted for those against the ribosomal proteins. Following exposure to the primary antibodies, slides were sequentially incubated at room temperature in Cy2-conjugated goat anti-mouse IgG subclass 1 (2 h; Jackson ImmunoResearch, West Grove, PA) and Rhodamine Red-X-conjugated goat anti-mouse IgG subclass 2b (1 h; Jackson ImmunoResearch, West Grove, PA) secondary antibodies for BrdU and HuC/D, respectively.

Control runs were done in which each of the four primary antibodies (against BrdU, HuC/D, RPL17, and RPL37) were omitted. Because antibodies to BrdU (IgG₁) and HuC/D (IgG_{2b}) were both raised in mouse, tests were also for cross-reaction between the secondary antibodies to these IgG subclasses and each of the primaries. These were done using the chromogens in Experiment 3 (see later). In all cases, labeling was completely absent.

Experiments 1 and 2: Confocal Analysis

Tissue was analyzed by an individual blind to experimental group. Sections were examined with a scanning confocal microscope (Olympus FV1000) using a UPlan FluoriteN 40×/1.3 NA (Area X, RA, and HVC) or PlanApoN 60×/1.42 NA (VVZ) oil objective. All confocal images were captured using sequential line scanning with Argon-488 nm and HeNe-543 nm lasers at emissions of 505-525 nm (for Cy2) and 560-620 nm (for TRITC and Rhodamine). Within each song nucleus, 4-8 nonoverlapping images were collected through the Z-plane from each side of the most middle two sections in which the brain region appeared. For the VVZ, images were taken from the same sections in which Area X were analyzed. The image size for Area X, RA, and HVC was 318 × 318 µm² (in the X-Y plane) which covers more than 50% of the cross-sectional area for each of the regions. As the VVZ is narrower than the confocal images captured, they were imported into Scion (NIH) Image software, and the border was traced to obtain a cross-sectional area that approximated those used in Tang and Wade (2006). These individual values for the VVZ were normalized to 100 × 100 µm². In all cases, densities were averaged across sections to obtain one value for each brain region per individual.

Experiment 3: Stereological Analysis of Double-Label Immunohistochemistry for BrdU plus HuC/D

A fourth set of slides was processed for BrdU plus HuC/D using a procedure similar to Experiment 2. However, the tissues were sequentially incubated with the mouse IgG₁ monoclonal BrdU antibody overnight at 4°C followed by biotin-SP-conjugated goat anti-mouse IgG₁ secondary antibody (1 µg/mL; 1 h; Jackson ImmunoResearch, West Grove, PA). The protein was visualized with *Elite* ABC reagents (Vector Laboratory, Burlingame, CA) and the Vector SG substrate according to manufacturer's instructions to produce a blue-gray reaction product in the nucleus. The tissues were then rinsed with PBS and incubated with the mouse IgG_{2b} monoclonal HuC/D antibody overnight at 4°C followed by biotin-SP-conjugated goat anti-mouse IgG_{2b} secondary antibody (1 µg/mL; 1 h; Jackson ImmunoResearch, West Grove, PA), followed by treatment with *Elite* ABC reagents and diaminobenzidine (DAB) with 0.0024% hydrogen peroxide to produce a brown reaction product.

Song nuclei (Area X, HVC, and RA) from each animal were analyzed under brightfield illumination using Stereo Investigator (Microbrightfield, Williston, VT) by an individual blind to experimental manipulation. The border of each song nucleus was defined by tracing its edge throughout its rostrocaudal extent in sections 120 µm apart. All cells positive for BrdU and those double-labeled for HuC/D were counted in sections using the Optical Fractionator function (as in Beck and Wade, 2008). Volumes and estimates of total numbers of these cells were averaged for the two sides of the brain prior to use in statistical analyses. In two cases, values could only be obtained for one side due to tissue damage.

Experiment 4: Brain Region Volumes

A fifth set of the same tissue was stained with cresyl violet, and the cross-sectional areas of Area X, HVC, and RA were traced using Scion (NIH) Image in each section in which they appeared, on both the left and right sides of the brain. This process was conducted by an individual without knowledge of experimental group. Volumes were separately calculated from each side by summing the areas and multiplying by the sampling interval (0.12 mm). The values from the left and right sides were averaged prior to statistical analysis.

Statistics

In Experiment 1, the densities of immunohistochemically labeled cells were evaluated by mixed-model ANOVAs. Effects of sex and estradiol treatment were analyzed between individuals separately for each of the song nuclei (Area X, RA, and HVC), as well as the VVZ. Because alternate sets of tissue from the same animals were used for the two ribosomal proteins in this study, and we wanted to determine whether they responded in parallel to the hormone manipulation, differences across the tissue sets (RPL17 vs. RPL37) were analyzed within individuals.

In Experiment 2, the percentage of BrdU-labeled cells that were positive for HuC/D, as well as the density of these double-labeled cells, were analyzed by mixed model ANOVAs. Effects of sex and treatment were considered between individuals. However, in this case, because we were interested in relative effects across the three song nuclei evaluated (HVC, RA, and Area X), differences among these brain regions were also assessed analyzed within individuals. Effects of sex and estradiol manipulation were separately evaluated for the VVZ, as it is not a song nucleus and, as expected, BrdU-labeling was far greater than in the song control regions (see later). Finally, based on results from Experiment 1 and an initial examination of the tissue from this experiment (see later), we computed three-way mixed-model ANOVAs for each brain region to evaluate the density of BrdU labeling alone across all three sets of series of tissue sections with fluorescent markers (RPL17, RPL37, and HuC/D). The effects of sex and treatment were analyzed between individuals, and tissue series within individuals.

In Experiment 3, estimates of the total number of BrdU+ cells, the number of these that are neurons (co-expressing BrdU and HuC/D), as well as the percentage of the new cells that were HuC/D+ were analyzed by ANOVA. In RA and HVC, two-way analyses (sex $\times$ treatment) were computed as mentioned earlier. However, it was impossible to do this for Area X. As this region cannot be identified in control females (see Introduction), there is no border that one can trace, which is required for an estimate of the total number of cells within a region to be calculated. Thus, the variables were analyzed for Area X by one-way ANOVA across the three groups in which the quantification could be conducted (estradiol-treated females and males, and control males).

In Experiment 4, effects of sex and treatment on the volumes of Area X, HVC, and RA were individually evaluated by two-way ANOVA; relative sizes across the brain regions held no particular relevance.

RESULTS

Experiment 1: Co-expression of RPL17 or RPL37 with BrdU--Density Analyses

All BrdU-labeled cells detected in each of the analyzed brain regions also expressed RPL17. Similarly, across the treatment groups, 93-98% of the BrdU-positive cells in Area X (see Fig. 1), RA, and HVC, and more than 99% in the VVZ, expressed RPL37. Not surprisingly, therefore, the effects of sex and estradiol on the densities of BrdU-labeled cells were equivalent, regardless of whether one considers quantification of BrdU-positive nuclei alone or in conjunction with each of the ribosomal proteins.

In Area X, estradiol had opposite effects in males and females. In this region, the hormone increased the density of BrdU-positive cells in females, and decreased it in males (sex $\times$ treatment interactions: BrdU alone $F = 4.48$, $p = 0.048$; BrdU double labeled with RPL17 or RPL37 $F = 4.53$; $p = 0.047$; Fig. 2 left four panels). This interaction between sex and estradiol manipulation was not detected in any of the other regions in which labeling was quantified [HVC, RA, or the VVZ; all $F < 2.55$, $p > 0.126$; Fig. 3(A)]. Main effects of sex and treatment were not detected, and none of the other possible interactions among the three variables were statistically significant in any of these areas (all $F < 1.51$, $p > 0.233$).

In a few cases, effects of tissue set were detected, with more BrdU labeling, and thus more double-labeling, occurring in tissue that had been counter-stained for RPL17 compared to RPL37 (Area X: both $F > 12.14$, $p < 0.003$; HVC double-label analysis $F = 4.51$, $p = 0.046$, BrdU only $F = 3.85$, $p = 0.064$; data not shown). These effects can be explained by an unfortunate situation in which the quality of the water from our filtration system deteriorated after the first of the three immunohistochemistry runs for BrdU+RPL17, resulting in high background. Although we had intended to use a single lot of the BrdU primary antibody for the entire study, the last of it was depleted in resolving this issue. Thus, two runs of BrdU+RPL17 and all of the BrdU+RPL37 were reacted with another lot of antibody which produced a slightly lower level of labeling. As two animals from each of the groups were in all cases reacted simultaneously, the greater labeling in the first run was evenly distributed across experimental manipulations and thus should not affect any of the results, particularly as the question of interest involved whether an interaction between the ribosomal proteins and estradiol exists.

Experiment 2: Co-expression of BrdU with HuC/D--Density Analyses

A relatively small percentage (15-17%) of the BrdU+ cells in the VVZ co-expressed the neuronal protein HuC/D. More double-labeled neurons appeared in the song control regions (see Fig. 4). In the analysis of the percentage of BrdU-positive cells that expressed HuC/D (Table 1A), significant effects of sex (male $>$ female; $F = 5.05$, $p = 0.036$) and brain region

($F = 180.53, p < 0.0001$) were detected. The percentages differed significantly among all three areas (Tukey/Kramer, all pairwise comparisons $p < 0.05$), with Area X showing the greatest percentage of neurons, HVC an intermediate value, and RA the least. The interaction between sex and treatment did not quite reach statistical significance ($F = 3.48, p = 0.077$). However, the interactions between brain region and sex ($F = 8.25, p = 0.001$), as well as between brain region and hormone manipulation ($F = 3.74, p = 0.032$) were both statistically significant.

When these effects were evaluated in more detail, it became clear that the results differed rather substantially across the three song control nuclei (Table 1A). Within Area X, males had a greater percentage of the double-labeled cells than females ($F = 11.77, p = 0.003$; Fig. 4), but the effect of treatment ($F = 1.63, p = 0.216$) and interaction between the sex and hormone manipulation ($F = 0.01, p = 0.910$) were not statistically significant. In HVC, estradiol produced an increase in the percentage of these cells ($F = 4.64, p = 0.044$; Fig. 5), but the effect was clearly due to females (sex $\times$ treatment interaction: $F = 12.06, p = 0.002$). A main effect of sex was not detected ($F = 1.26, p = 0.274$) in this region, and no significant effects were detected in RA (all $F < 2.46, p > 0.132$).

If one considers the density rather than percentage of double-labeled neurons (BrdU+/Hu+), the results are similar, but not identical (Table 2). In the overall ANOVA, a significant main effect of sex was detected ($F = 16.83, p = 0.0006$), with the value greater in males compared to females. A main effect of treatment was not detected ($F = 2.72, p = 0.115$), but a sex $\times$ treatment interaction was ($F = 9.79, p = 0.005$). An effect of brain region (Tukey/Kramer $p < 0.05$, Area X greater than both RA and HVC, which did not differ from each other) and sex $\times$ brain region interaction were also revealed (both $F > 24.31, p < 0.0001$). Within Area X (or the region of the medial striatum containing it), males exhibited an increased density of these double-labeled neurons compared to females ($F = 27.29, p < 0.0001$) and an interaction existed ($F = 5.05, p = 0.036$). The pattern of estradiol was opposite in the two sexes (an estradiol-induced increase in females and decrease in males), but pairwise comparisons within each sex indicated that neither was statistically significant (both $t < 1.96, p > 0.080$). The same interaction was detected in HVC ($F = 9.74, p = 0.005$) without main effects of sex or treatment (both $F < 2.77, p > 0.111$). In this case, the interaction stemmed from an estradiol-induced decrease in males ($t = 3.12, p = 0.011$) but not females ($t = 1.51, p = 0.162$). Finally, in RA, an effect of treatment ($F = 6.10, p = 0.023$) and marginal interaction between sex and treatment ($F = 4.28, p = 0.052$) were revealed; estradiol induced a 38% decrease in the density of BrdU+/Hu+ cells in the RA of males only.

Finally, our initial question involved the incorporation of cells generated beginning on post-hatching day 6 into Area X in particular, including the extent to which they express RPL17 and RPL37. Those issues were addressed by Experiment 1. However, because of the unforeseen technical issue with the immunohistochemistry involving those proteins (see earlier), and because it became clear upon initial examination of the tissue from this second experiment that BrdU labeling was greater than in the previous study, we decided to add the quantification of these cells in this tissue to the previous analysis to generate a single, global assessment for each brain region, allowing us to draw conclusions based on convergence of available evidence. As indicated in the Methods section, a three-way ANOVA was used for each of the three song control nuclei. As in Experiment 1, the effects of sex and treatment were of primary interest, and these two factors could now be assessed with additional power between subjects. In addition, the values for BrdU-immunoreactivity obtained in the three alternate sets of tissue with fluorescent labels were analyzed as a third variable within individuals. For all three brain regions, HVC, RA, and Area X, the density of BrdU+ cells in the tissue co-labeled for HuC/D was roughly three times greater than in the tissue exposed to the RPL antibodies (all $F > 73.39, p < 0.0001$; Figs. 2 and 3). The increased sensitivity was presumably due to the secondary antibodies used for BrdU necessitated by the hosts in which the primaries were raised (see

Methods). Although the signal for BrdU in Experiment 2 was enhanced compared to Experiment 1, the patterns of labeling among the groups were the same across all three instances (double-labeling with RPL17, RPL37, and HuC/D). In addition to the differences in labeling sensitivity/intensity, a variety of other effects were detected, in Area X only. In this region, the density of BrdU+ cells was greater in males than females ($F = 7.68, p = 0.012$), and a sex $\times$ treatment interaction was detected ($F = 11.22, p = 0.003$). Across the three tissue sets, E2 induced a significant decrease in males ($t = 2.66, p = 0.026$). The direction of the effect of hormone in females was the opposite, although the increase did not reach statistical significance ($t = 1.95, p = 0.080$). A significant interaction between tissue set and treatment was also detected ($F = 5.49, p = 0.008$), which appeared to be due to a greater effect of estradiol in the brain sections double-labeled for HuC/D (likely due to increased sensitivity of the assay).

Experiment 3. Co-expression of BrdU with HuC/D--Stereological Estimates of Total Number of Labeled Cells

In Area X, no differences were detected among the three groups evaluated in the number of BrdU+ cells (see Fig. 6), or the percentage or total number of these new cells that were HuC/D+ ($F < 0.73, p > 0.497$; Fig. 7; Tables 1B and 3), which means that E2 induced a change in females in each of these features. As expected, Area X was visible in E2-treated females and its values were equivalent to both groups of males. In HVC, males had more BrdU+ cells than females ($F = 7.13, p = 0.0156$), but effects of treatment ($F = 0.02, p = 0.885$) and the sex $\times$ treatment interaction ($F = 3.45, p = 0.080$) were not statistically significant, although the interaction data are consistent with a modest E2-induced increase in females and decrease in males (see Fig. 6). The estimated total number of cells double-labeled for BrdU and HuC/D was also unaffected by treatment ($F = 0.10, p = 0.761$; Table 3). Effects of sex ($F = 3.22, p = 0.090$) and its interaction with treatment ($F = 3.29, p = 0.087$) were not statistically significant either. No significant effects were detected on the percentage of BrdU+ cells that also expressed Hu (all $F < 0.98, p > 0.337$). In RA, more BrdU+ cells were detected in males than females ($F = 6.41, p = 0.014$). Estradiol treatment had no effect ($F = 2.23, p = 0.151$), but an interaction between sex and treatment existed ($F = 7.39, p < 0.014$; Fig. 6). Planned comparisons indicate that this is due to an estradiol-induced decrease in males ($t = 2.39, p = 0.041$) but not females ($t = 1.178, p = 0.266$). When one considers the total number and percentage of cells co-expressing BrdU and HuC/D no significant effects were detected (all $F < 1.81, p > 0.195$; Table 3).

The percentages in the SG/DAB-labeled tissue (Experiment 3) are uniformly lower than those in the fluorescent tissue (Experiment 2; see Table 1). A variety of factors differed between these studies. In addition to issues related to the sensitivity of reagents used for detection, the ability to clearly identify whether the two labels truly existed in the same cell was a concern. It was far easier to be certain in the fluorescent confocal images from Experiment 2 than in brightfield in Experiment 3. To be conservative, if there was any doubt, a cell was not counted as double-labeled. Despite differences in statistical outcomes for HVC, the pattern of results on percentages of BrdU-labeled neurons for this brain region was similar in Experiments 2 and 3. In fact, if one considers these data together with a mixed model ANOVA, in addition to the effect of procedural differences ($F = 159.44, p = 0.0001$), the same interaction was detected as in the analysis of fluorescent tissue alone ($F = 13.69, p = 0.002$; Table 1). Estradiol increased the percentage of these cells in females ($t = 3.02, p = 0.015$) and caused a marginal decrease in males ($t = 2.16, p = 0.059$). The results in Experiments 2 and 3 were the same for RA, and the data cannot be analyzed collectively for Area X due to the inability to obtain data from the DAB/SG labeled tissue in control females (Experiment 3).

Experiment 4: Brain Region Volume

In Area X, significant main effects of sex and treatment, as well as a significant interaction between the two variables, were detected (all $F > 127.62$, $p < 0.0001$; Fig. 8). The brain region was larger in males than in females, and estradiol increased the size. The interaction was due to the fact that Area X was induced in estradiol-treated female and equivalent in size to control males ($t = 1.41$, $p = 0.188$), while the estradiol-induced increase in males was only 10% ($t = 2.29$, $p = 0.045$). HVC was also larger in males than females ($F = 55.55$, $p < 0.0001$), and a significant interaction between sex and steroid treatment was detected ($F = 6.37$, $p = 0.020$). Estradiol increased HVC volume in females ($t = 2.49$, $p = 0.032$), but not males ($t = 0.274$). Like the other two song control nuclei, RA was larger in males than in females ($F = 5.25$, $p = 0.035$). However, the effects of treatment and the interaction between sex and treatment were not statistically significant (both $F < 0.80$, $p > 0.385$). Main effects and interactions between sex and treatment on volume assessed via Microbrightfield in the alternate set of tissue labeled for BrdU and Hu via SG/DAB were statistically identical.

DISCUSSION

Phenotype of Song System Cells Born from Posthatching Days 6-25

Almost all of the BrdU-positive cells also expressed RPL17 and RPL37. Among the three song control nuclei investigated, Area X had the greatest density and percentage of neurons born beginning on post-hatching day 6 (cells double-labeled for BrdU and HuC/D). Close to half of these cells were neurons, which is consistent with recently reported data. Rochefort et al. (2007) demonstrated that in 25-dayold zebra finches injected with BrdU on posthatching day 4, approximately equal proportions of the labeled cells in Area X were positive and negative for HuC/D. Collectively, the results are consistent with the idea that RPL17 and RPL37 can influence the development of sexual dimorphisms in the song circuit, and that of the cells born during this period, Area X may be particularly sensitive to those effects. The potential ways in which this might happen are discussed later.

A number of sex differences and effects of estradiol were also detected in this study. They demonstrate consistency with earlier research on the development of song control nuclei in zebra finches and provide novel information that facilitates our understanding of the mechanisms regulating sexual differentiation of the brain. For simplicity, interpretations derived from specific, localized effects are provided for the individual brain regions below, followed by more general conclusions based on integration of results.

Area X

Sexual differentiation of Area X largely involves the addition of cells in males. They are added during the first 2 to 3 months after hatching, and include neurons born throughout at least the first month of life (Nordeen and Nordeen, 1988; Kirn and DeVoogd, 1989; Rochefort et al., 2007). In addition, cell death in Area X is increased in females compared to males during posthatching days 15-25 (Kirn and DeVoogd, 1989), which likely contributes to the sexual dimorphism. This region is detectable in males but not females by posthatching day 11. The portion of the medial striatum where Area X exists in males expresses more androgen receptors in males than females by that age, an effect that is likely facilitated by endogenous estradiol (Kim et al., 2004).

The greater volume of Area X detected in males compared to females in this study, along with increased density of cells, including those co-expressing HuC/D, born beginning on posthatching day 6 all suggest that more neurons and glia are incorporated between days 6 and 25 to the portion of the medial striatum containing Area X in males than females. At this point it is not clear whether the mechanisms involve increased neurogenesis, migration, or survival.

Similarly, the equivalent estimates of total counts of BrdU+ cells in estradiol-treated females and both groups of males suggest that estradiol increases the incorporation of cells born beginning on day 6 to create an identifiable Area X in females; apparently this applies both to neurons and other cell types. Thus, the hormone appears to masculinize the addition or survival of these cells, apparently without a substantial effect on their density (which is greater in males than females). This effect contributes to the increase in volume in estradiol-treated females and is consistent with previous research suggesting that estradiol masculinizes the Area X of females by increasing the incorporation or survival of neurons (Nordeen and Nordeen, 1989). Conversely, the decreased density of cells born to estradiol-treated males beginning on posthatching day 6 probably contributed to the increase in the volume of their Area Xs. As the decrease in density in males was detected in BrdU+ cells, but not those that co-expressed HuC/D, it appears that it occurred primarily in nonneuronal cells. These data are consistent with the relatively small (10%) difference between estradiol- and blank-treated males.

The male-biased sex difference in Area X volume we report is consistent with previous work, as is the induction of an Area X in estradiol-treated females (reviewed in Wade, 2001). In this study, a statistical interaction between sex and treatment was also detected, because of the enhancing effect of estradiol being far greater in females than males. Such an interaction was previously reported in juvenile animals collected at the same age (Mathews and Arnold, 1991). In that study, however, estradiol *decreased* Area X volume in males in addition to inducing a detectable brain area to develop in females. Reasons for the difference between the two experiments are not clear, but may involve methodology. For example, animals in the earlier study were injected daily for 25 days after hatching, whereas our birds were given implants on posthatching day 3, which might be less stressful. Interestingly, these authors did not see the negative effects of estradiol in other male brains collected at posthatching day 60, weeks after treatment ended. They speculated that normal testicular function which resumed after the treatment was completed allowed the animals to compensate so that the brain regions eventually developed normally. Although a reasonable idea at the time, it has since become clear that testicular tissue is most likely not the source of masculinizing agents (Wade and Arnold, 1994, 1996; Wade et al., 1999).

RA

The larger volume detected in males compared to females at posthatching day 25 in this study stems at least in part from a general increase in the estimated total number of cells born beginning on day 6 after hatching. Estradiol treatment of males caused a decrease in the number of new cells in this brain region, and appeared to do the same for the density of new neurons specifically. The sex difference in RA volume largely arises due to increased cell death in females (reviewed in Wade and Arnold, 2004). Thus, estradiol administration to males appears to increase the death of cells, or in other words, feminizes their rate of survival. As this happens, the neurons move further apart. Thus, it seems reasonable to infer that the estradiol increases the death of non-neuronal cells.

HVC

The equivalent density of cells born beginning on posthatching day 6 in males and females with a greater number of new cells overall contributed to the male-biased sex difference in volume we detected. Estradiol appears not to affect either of these characteristics. However, it did increase the volume of this brain region in females and decrease the density of new neurons specifically in males (without producing a substantial change in the estimated total number of neurons in either sex). One might have expected the volume to decrease in males with estradiol treatment in parallel, but as neurons represent less than a third of these new cells, perhaps that is not sufficient to create the larger scale morphological change. Consistent with this idea, the mean values from both the Nissl- and SG/DAB-labeled tissue were smaller in estradiol-treated

compared to control males. Interestingly, Mathews and Arnold (1991) saw such a decrease in the volume of HVC in estradiol treated males, but as for Area X, the effect no longer existed at 60 days of age.

The estradiol-induced increase in HVC volume in females is more difficult to interpret based on the present data, as the only other measure of this area the hormone appeared to affect in this study was the percentage of new neurons. Perhaps this increase contributes to the volume, if new neurons are larger than the majority of the gial cells born beginning on day 6. Previous work suggests that estradiol can masculinize neuron number in HVC by influencing post-mitotic events other than survival, such as phenotypic determination (Burek et al., 1997). Estradiol treatment of females also increases soma size (reviewed in Wade, 2001), so that could have contributed to the increased volume we detected. In addition, the timing of BrdU treatment in this study may be important. As in Area X, cells appear to be incorporated into HVC roughly equally from those born throughout the first month after hatching (Kirn and DeVoogd, 1989). If the estradiol we administered facilitated the survival of cells that would normally die (see Burek et al., 1995), but they were born before day 6, we would not be able to detect the effect in our BrdU sample.

Integration Among Brain Regions: Potential Sites of Estradiol Action and Roles for RPL17 and RPL37

At 25 days of age, substantial mRNA for estrogen receptor α has been detected in HVC, while little was detected in RA and none in Area X (Jacobs et al., 1999). The sample size in that study was too small to evaluate whether sex differences exist. Other experiments investigating juvenile zebra finches reported: (1) no binding of 3H-estradiol in song nuclei of 20-day-old birds (Nordeen et al., 1987), and (2) mRNA in HVC on posthatching days 15 and 30 (comparable expression in males and females; Gahr, 1996). While not completely consistent, on balance these data support the idea that direct action of estradiol via estrogen receptor α is plausible in HVC, and less likely in RA and Area X.

As available data suggest that the expression of these receptors is similar in the two sexes (see earlier), and no consistent evidence is available to indicate that developing males and females have divergent levels of estrogen availability (Hutchison et al., 1984; Adkins-Regan et al., 1990; Vockel et al., 1990; Schlinger and Arnold, 1992; Wade et al., 1995), it is plausible that differential action of estradiol results from influences of genes, perhaps RPL17, RPL37, or others. It is of course also possible that estrogen receptors other than α are important. However, the one report of estrogen receptor β in the songbird brain (Bernard et al., 1999) indicates that these receptors are not expressed in song control regions. Other possibilities include membrane estrogen receptors that have relatively recently been identified (Revankar et al., 2005; Thomas et al., 2005; Toran-Allerand, 2005). These, however, have not been considered in the context of the developing brain (McCarthy, 2008).

The effects of estradiol, localization of estrogen receptors α , and what is known about projections among the three song nuclei, allow us to generate some hypotheses regarding mechanisms of action. In males, HVC projections grow into RA at about post-hatching day 30, whereas HVC to Area X projections are already present by day 20 (Konishi and Akutagawa, 1985; Mooney and Rao, 1994). Thus in this study, in which birds were euthanized at 25 days of age, estradiol might have acted at estrogen receptors in HVC both to directly influence the survival of cells there and to mediate downstream changes in Area X. This scenario would be less likely to affect RA using the paradigm in this study, and in fact we did not see estradiol-induced masculinization in this brain region in females. In parallel, the sex difference in HVC cell number is likely not influenced by a sexually dimorphic RA (Burek et al., 1994). The idea of trans-synaptic masculinization from HVC to Area X is supported by an experiment in which HVC was lesioned in 20-day-old females. This procedure prevented estradiol's ability to

masculinize both Area X and, presumably later, RA (Herrmann and Arnold, 1991). One important question, however, is whether a projection from HVC to Area X exists in females, and/or whether it is induced in estradiol-treated females in a manner similar to what normally exists in males. This idea certainly seems plausible, but to our knowledge an answer is not presently available.

Expression of RPL17 and RPL37 mRNAs are enhanced in Area X and RA at 25 days of age and are greater in males than females at this time. These sex differences are greatly reduced or eliminated in adults (Tang and Wade, 2006). However, in HVC, expression is equivalent to surrounding tissue in juveniles of both sexes (Wade et al., 2005). These genes appear to be on the Z chromosome (see Introduction), and as limited dosage compensation exists in birds (Itoh et al., 2007), increased expression may well be involved in masculinization. However, if so, the influence is selective. For example, the increased density and percentage of BrdU-labeled neurons in males compared to females in Area X is consistent with the possibility that the ribosomal proteins support proliferation of cells destined for Area X or perhaps differentiation of the cells into neurons in this region. Parallel effects may not occur in RA because sexual dimorphism in that region is due more to increased cell death in females than addition of cells in males (reviewed in Wade and Arnold, 2004). Increased incorporation of cells plays a large role in sexual differentiation of HVC, like Area X (reviewed in Wade and Arnold, 1994). However, as RPL17 and RPL37 mRNAs are not specifically expressed in HVC (see earlier), it seems unlikely that they would mediate morphological or functional change.

Little information is available on the functions of RPL17 and RPL37. However, existing data suggest that ribosomal mRNAs are generally influenced by rates of growth and proliferation of cells, and there is some indication that RPL17 might be involved with neuronal differentiation, at least in cell culture (see Tang and Wade, 2006 and references therein). Although information is not presently available, it is also conceivable that the ribosomal proteins might influence the survival of cells in particularly in RA, as the estimated total number of BrdU+ cells was greater in males than females, parallel to the expression of RPL17 and RPL37. Future work is required to address all of these issues, as well as potential interaction between these proteins and estradiol. One tantalizing idea is that what is required for normal masculinization is an appropriate balance between the activities of estradiol and these (or other) sex-linked genes.

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Figure 1.

Example of co-expression of BrdU and the two ribosomal proteins (RPL17 and RPL37) in Area X. Nuclear BrdU labeling is depicted in the left panel (green) and the more diffuse ribosomal proteins in the center (red). Merged images are on the right. Scale bar = 5 μ m for all images.

Figure 2.

Photomicrographs of BrdU-positive cells in Area X of 25-day-old zebra finches from each of the four experimental groups. The four panels on the left are from tissue that was also labeled for RPL17 (Experiment 1), and those on the right were from tissue used to detect co-expression of HuC/D (Experiment 2). These additional labels are not shown. Scale bar = 10 μ m.

Figure 3.

Mean number of BrdU-positive cells (plus standard errors) counted in areas of known sizes in three song control regions and the ventral portion of the ventricular zone (VVZ). Panel A depicts data from tissue that had been double-labeled for RPL17 (Experiment 1); B shows results from tissue that was also used to detect HuC/D (Experiment 2).

Figure 4. Photomicrographs of BrdU (green) and HuC/D (red) immunoreactive cells in Area X and the ventral ventricular zone (VVZ) of 25-day-old zebra finches that received control implants. White arrowheads in the top left panel indicate double-labeled cells. Scale bar = 10 μ m. LV = lateral ventricle.

Figure 5. Photomicrographs of cells labeled for BrdU (green) and HuC/D (red) in the HVC of 25-day-old zebra finches. White arrowheads indicate double-labeling. Scale bar = 10 μ m.

Figure 6. Estimates of total numbers of BrdU+ cells (mean + standard error) determined in three song control nuclei by stereology. NM, not measured.

Figure 7. Co-expression of BrdU (blue/gray) and HuC/D (brown) in Area X (Experiment 3). The photomicrograph in panel A is from an estradiol-treated female, B is from a control male, and C represents an estradiol-treated male. Scale bar 20 μ m.

Figure 8.

Volumes of three song control nuclei (means plus standard errors) in 25-day-old males and females treated with estradiol or blank implants. Note the different axes, as Area X is much larger than the other two brain regions. Sample sizes were in 6 all cases except estradiol-treated males ($n = 5$) and females ($n = 4$) due to tissue damage.

Percentage of BrdU+ Cells in Song Control Nuclei that Co-express HuC/D Calculated from Densities Quantified Using Confocal Microscopy in Fluorescently Labeled Tissue (A: Left Columns) and from Estimates of Total Numbers of Labeled Cells Using Stereological Methods in Brightfield (B: Right Columns)

	A. Experiment 2				B. Experiment 3			
	Female		Male		Female		Male	
	Blank	Estradiol	Blank	Estradiol	Blank	Estradiol	Blank	Estradiol
Area X ^a	39.2 (2.6)	41.6 (2.0)	46.1 (1.6)	49.0 (2.0)	NM	32.9 (0.7)	33.4 (1.4)	31.5 (1.9)
HVC ^b	28.0 (1.2)	34.8 (1.6)	33.6 (1.2)	32.0 (0.6)	17.2 (1.6)	17.6 (1.9)	18.8 (1.1)	16.2 (1.1)
RA	25.0 (1.8)	24.9 (1.7)	26.1 (1.1)	21.6 (1.1)	13.4 (2.1)	11.7 (0.8)	11.5 (3.3)	14.5 (2.6)

n = 5-6 per cell; numbers represent means with standard error in parentheses.
^aEffect of sex (male > female) in A only.
^bEffect of treatment (estradiol > blank) in A, and sex × treatment interaction in A, as well as A and B when the data are analyzed together.

Table 2Density of Cells (Number per $318 \mu\text{m} \times 318 \mu\text{m}$) in Song Control Nuclei Co-expressing BrdU and HuC/D

	Female		Male	
	Blank	Estradiol	Blank	Estradiol
Area X ^{a,b}	30.7 (2.9)	35.6 (3.3)	61.4 (5.3)	47.8 (4.5)
HVC ^b	17.4 (1.5)	21.8 (2.5)	26.3 (2.2)	19.1 (0.7)
RA ^{b,c}	16.3 (1.6)	15.7 (2.0)	19.4 (1.6)	11.9 (1.0)

n = 6 per cell; numbers represent means with standard error in parentheses.

^aEffect of sex (male > female).

^bInteraction between sex and treatment (marginal for RA, in which the effect is clearly due to the decrease in males exposed to estradiol).

^cEffect of treatment (estradiol < blank).

Table 3

Total Number of Cells in Song Control Nuclei Co-expressing BrdU and HuC/D

	Female		Male	
	Blank	Estradiol	Blank	Estradiol
AreaX	NM	10,750 (1,292)	14,750 (2,353)	14,175 (4,271)
HVC	426 (56)	730 (227)	1,155 (266)	726 (120)
RA	410 (63)	420 (53)	620 (178)	510 (99)

n = 5-6 per cell; numbers represent means with standard error in parentheses.