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Sex- and Age-Related Differences in Ribosomal Proteins L17 and L37, as well as Androgen Receptor Protein, in the Song Control System of Zebra Finches

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Abstract

The zebra finch song system is sexually dimorphic – only males sing, and the morphology of forebrain regions controlling the learning and production of this song is greatly enhanced in males compared to females. Masculinization appears to involve effects of steroid hormones as well as other factors, perhaps including the expression of sex chromosome genes (males: ZZ, females: ZW). The present study investigated three proteins – two encoded by Z-linked genes, ribosomal proteins L17 and L37 (RPL 17 and RPL37), including their co-localization with androgen receptor (AR), from post-hatching day 25 to adulthood. Extensive co-expression of AR with the ribosomal proteins was detected in the three song nuclei investigated (HVC, RA, and Area X) across these ages. In general, more cells expressed each of these proteins in males compared to females, and the sex differences increased as animals matured. Specific patterns differed across regions and between RPL17 and RPL37, which suggest potential roles of one or both of these proteins in the incorporation and/or differentiation of song system cells.

Keywords

Songbird; sexual differentiation; steroid hormone; testosterone

1. INTRODUCTION

Sex differences in the morphology and function of neural circuits regulating reproduction exist in numerous vertebrates (e.g., Cooke et al., 1998, Balthazart and Adkins-Regan, 2002, Simerly, 2002). These dimorphisms are in many cases organized by testosterone or its metabolite estradiol during a critical period of development. While the role of gonadal steroids in masculinization has long been established for a number of model systems, information about interactions between these hormones and other molecules important for sexual differentiation are largely unknown (Forger, 2009, McCarthy, 2009, McCarthy et al., 2009).

Zebra finches offer an excellent opportunity to elucidate mechanisms regulating sexual differentiation. Males sing to court females, using vocalizations they learn from their fathers

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during development. While females produce some simpler calls, they do not sing. Parallel to these behavioral differences, the song circuit is highly sexually dimorphic. Forebrain regions involved in learning and production of song, including the robust nucleus of the arcopallium (RA) and HVC (used as a proper name; Reiner et al., 2004), are larger in volume and contain more and larger cells in males compared to females. Another region, Area X of the striatum, is not visible in females (Wade, 2001, Wade and Arnold, 2004).

These sexual dimorphisms in both brain and behavior are organized in the first few weeks after hatching. Initial results were consistent with the idea that mechanisms parallel those in rodents – testosterone from the testes is metabolized in the brain to estradiol (via the aromatase enzyme), and this hormone is directly responsible for masculinization (De Vries and Simerly, 2002). For example, treating female zebra finches with estradiol shortly after hatching masculinizes the morphology of the brain regions, including increasing the volumes of HVC and RA, and inducing a visible Area X, and the manipulation allows females to sing in adulthood (Gurney and Konishi, 1980, Gurney, 1981, Adkins-Regan and Ascenzi, 1987, Konishi and Akutagawa, 1988, Simpson and Vicario, 1991, Grisham and Arnold, 1995, Arnold, 1997). Other data, however, have raised questions about the role of naturally occurring estradiol in males, and are consistent with the idea that sex chromosome genes may be involved in masculinization (Mathews et al., 1988, Mathews and Arnold, 1990, 1991, Wade and Arnold, 1994, Balthazart et al., 1995, Wade and Arnold, 1996, Wade et al., 1996, Springer and Wade, 1997, Wade et al., 1999, Agate et al., 2003). Male birds are homogametic (ZZ) and females are heterogametic (ZW). As dosage compensation in birds is limited (Itoh et al., 2007), the potential for expression of Z-linked genes to facilitate masculinization is high.

Using a variety of procedures, including cDNA microarray analyses, real time quantitative (q)PCR, and *in situ* hybridization, we identified two ribosomal proteins as potential contributors to the masculinization process (Wade et al., 2005), both of which are encoded on the Z-chromosome. The mRNAs for both ribosomal proteins L17 and L37 (RPL17 and RPL37) are increased in males compared to females at post-hatching day 25 in RA and Area X. This age is early in the period during which males learn their songs and is a time at which morphological differentiation is occurring at a rapid pace (Bottjer et al., 1985, Kirn and DeVoogd, 1989, Nordeen and Nordeen, 1997). Thus, it is reasonable to hypothesize that RPL17 and RPL37 are involved in development of song system structure and/or function. This idea is supported by the facts that sex differences in the expression of these mRNAs is greatly reduced or absent in adulthood (Tang and Wade, 2006) and the proteins encoded by these two genes are expressed in most cells born beginning on post-hatching day 6 that are incorporated into these brain regions (Tang and Wade, 2009).

In addition, sexually dimorphic expression of RPL17 and RPL37 occur in regions with abundant androgen receptors (AR). The expression of AR is greater in males than females in HVC and Area X by post-hatching days 9–1 (Kim et al., 2004). Similar to morphological features, exogenous estradiol in females increases the expression of AR in HVC and Area X, as well as in the lateral magnocellular nucleus of the anterior nidopallium (IMAN; Nordeen et al., 1986, Kim et al., 2004). While treatment of females with androgen alone has only modest and somewhat inconsistent effects on masculinization of the forebrain song circuit (reviewed in Wade and Arnold, 2004), at least one study (Grisham et al., 2002) suggests that an estrogen-induced increase in AR is a critical step in morphological differentiation. In addition, the development of stereotyped song appears to require androgens (Bottjer and Johnson, 1997).

A primary focus of the present set of studies was to examine developmental changes in the numbers of cells expressing RPL17 and RPL37 in the song circuit of males and females. We

also wanted to address the potential for interactions of AR with RPL17 and RPL37 by determining whether they are co-expressed within cells. We used species specific polyclonal antibodies to the two ribosomal proteins to investigate protein expression rather than mRNA, and investigated birds at four key developmental stages. The youngest were 25 days old, to parallel our previous work on mRNA expression. We also investigated 35, 45 and 65 days of age to span the period of sexual differentiation of song system morphology and sensorimotor integration of the song. Fully mature adults were evaluated for comparison. If substantial co-localization exists between AR and either of the ribosomal proteins, the steroid receptor and ribosomal protein(s) could be involved in either the same or different pathways facilitating masculinization within a cell. In addition, if the pattern of co-expression differs in males and females particularly at one or more key developmental stages, it would provide additional evidence that the interaction between the RPLs and AR could be important for specific aspects of the masculinization process.

2. EXPERIMENTAL PROCEDURES

2.1 Animals

Male and female zebra finches were raised in mixed sex group aviaries in our colony at Michigan State University. They were housed on a 12:12 light:dark cycle, and seed and water were available *ad libitum*. The birds were also fed with bread mixed with hard-boiled chicken eggs, spinach and orange juice once a week. Nests were checked daily, and the day a hatchling was found was considered post-hatchling day 1. All procedures were conducted in accordance with NIH guidelines and approved by the Michigan State University IACUC.

2.2 Tissue Collection

Brains of male and female zebra finches from each age group (n=6/sex/age) were collected following rapid decapitation, snap frozen in cold methyl-butane, and stored at -80°C . Coronal sections (20 μm) through the entire telencephalon were cut on a cryostat, thaw-mounted onto 6 alternate sets of slides (SuperFrost Plus slides; Fisher Scientific, Hampton, NH), and stored with desiccant at -80°C until further processing.

2.3 Stereological Analyses of Double-Label Immunohistochemistry for RPL17 or RPL37 plus AR

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 3 times in PBS (5 min each). Slides were treated with 0.9% H_2O_2 /methanol for 30 min and incubated for 30 minutes in 10% normal goat serum (NGS) in PBS with 0.3% Triton X-100. The tissue was then incubated overnight at 4°C in PBS containing a RPL17 polyclonal antibody raised in rabbit against zebra finch RPL17 amino acids 61-74 (RPYNGGVGRCAQAKQ; 1 $\mu\text{g}/\text{ml}$; produced for us by the Antibody on Demand program of Affinity Bioreagents, Golden, CO), along with 0.3% Triton X-100, and 10% NGS. A biotin-conjugated goat anti-rabbit secondary antibody (1 $\mu\text{g}/2\text{ ml}$; Vector Labs, Burlingame, CA) was then used for 2 hours at room temperature, followed by treatment with *Elite* ABC reagents and diaminobenzidine (DAB) with 0.0024% hydrogen peroxide to produce a brown reaction product. Slides were then rinsed in PBS to be sure the reaction was terminated.

They were then incubated in 10% NGS for 30 min and exposed to the polyclonal AR primary antibody (1 $\mu\text{g}/3\text{ ml}$; sc-816; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C . Tissue was then exposed to a biotin-conjugated goat anti-rabbit IgG secondary antibody (1 $\mu\text{g}/2\text{ ml}$; Vector) for 2 hr at room temperature. The protein was visualized with *Elite* ABC reagents and the Vector SG substrate (Vector) per manufacturer's instructions to produce a blue-gray reaction product.

A second set of slides was processed exactly as above, 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. The labeling techniques were chosen to provide stable markers appropriate for stereological estimates of total numbers of cells expressing them (see below).

Detailed validation of the antibodies for the two ribosomal proteins is presented in Tang and Wade (2009), including production of a single band of the expected size in Western analyses and lack of labeling when the primary antibodies were omitted. Omission of the primary AR antibody also eliminated labeling in zebra finch brain (Wu et al., 2010). Additional tests were conducted for this set of studies. Preadsorption of the AR antibody with 30-fold excess of the immunizing peptide resulted in a complete absence of labeling, and Western analysis using zebra finch brain tissue indicated a band of the expected size (data not shown). Reversing the order of the reactions (AR before RPL) produced similar patterns of labeling as those used for the present analyses. Finally, to confirm that the peroxidase from the first reaction (DAB; RPL) was completely inactivated and did not contribute to labeling in the second reaction (SG; AR), we eliminated the ABC from the second reaction in one set of tissue for RPL17 and one for RPL37. In both cases, no blue-gray reaction product was detected (Figure 1).

Song nuclei (Area X, HVC and RA) from each animal were analyzed under brightfield illumination using Stereo Investigator (Microbrightfield Inc., Williston, VT) by an individual blind to sex and age of the animals. The border of each song nucleus was defined by tracing its edge throughout its rostrocaudal extent (sections were 120 µm apart) on one side of the brain, randomly chosen. All cells exhibiting neuronal morphology and clear reaction product for AR, RPL17 or RPL37, and those double-labeled for RPL17+AR or RPL37+AR, (Figure 1D,F) were counted in sections using the Optical Fractionator function (following Beck et al., 2008). The software also provided an estimate of the volume for each brain region.

2.4 Statistical Analyses

Within RA and HVC, effects of sex and age were analyzed by two-way ANOVA. These analyses were conducted for the estimates of total numbers of cells expressing each of the proteins, as well as those expressing both RPL17+AR or RPL37+AR. Percentages of double-labeled cells and brain region volumes were also analyzed in this manner. When significant interactions were detected, effects of age were investigated within each sex by one-way ANOVA. Pairwise comparisons among ages were done when appropriate by Tukey HSD. Area X cannot be detected with a Nissl stain in female zebra finches, and also could not be distinguished with these markers used in the present study (Figure 2). Therefore, effects of age were analyzed only in males, using one-way ANOVA followed as appropriate by Tukey HSD *post-hoc* analyses. Actual sample sizes varied slightly due to tissue quality, and a few animals were deleted from the analysis of RPL37-labeling in Area X because they were statistical outliers (one each at post-hatching day 25, 45 and 65; Dixon's test; Sokal and Rohlf, 1981). Final sizes of each group are indicated in the figures.

3. RESULTS

3.1 RPL17 + AR

3.1.1 RA—The estimated total number of cells expressing RPL17 was greater in males than females ($F=75.16$, $p=0.0001$), and a sex x age interaction was detected ($F=4.35$, $p=0.005$) while a main effect of age was not ($F=0.26$, $p=0.904$; Figure 2 left column). The number of RPL17+ cells did not differ across ages in males ($F=1.12$, $p=0.374$). Females, however, did

exhibit an effect of age ($F=12.8$, $p<0.0001$), with 25-day-olds having more RPL17+ cells than those at days 35-adult, and adults having fewer than day 45 (Tukey, $p<0.05$). The number of cells expressing AR was greater in males compared to females ($F=67.48$, $p<0.0001$; Figure 2 right column). A main effect of age was not detected, but sex and age interacted ($F=3.62$, $p=0.013$). AR+ cells did not change significantly in males as they matured ($F=1.97$, $p=0.136$), but did decline in females ($F=7.32$, $p=0.001$). The total number of double-labeled cells followed the same pattern due to extensive co-expression (see below). A main effect of sex ($F=74.30$, $p<0.0001$), but not age ($F=0.24$, $p=0.914$), was detected with a significant interaction between the two variables ($F=4.30$, $p=0.005$). Males did not differ across ages ($F=1.09$, $p=0.387$), but the total number of double-labeled cells declined in females as they matured ($F=12.80$, $p<0.0001$), parallel to total RPL17- labeled cells.

In both sexes, 100% of the RPL17+ cells co-expressed AR at all juvenile stages. This complete co-localization was also detected in adult females, and in adult males the percentage was 99.6 ± 0.38 . In contrast, about one-third of the AR+ cells co-expressed RPL17 (20–40%, depending on sex and age). Neither main effects of sex nor age, nor an interaction between the variables, was detected for either percentage (all $F\leq1.79$, $p\geq0.149$). The volume of RA was substantially larger in males than females ($F=476.64$, $p<0.0001$), and it differed across ages ($F=11.51$, $p<0.0001$). A significant interaction between sex and age was detected ($F=34.84$, $p<0.0001$). The volume increased in males ($F=24.63$, $p<0.0001$) and decreased in females ($F=21.21$, $p=0.001$) as they matured (data not shown).

3.1.2 HVC—RPL17-labeled cells exhibited a trend for an increase across ages ($F=2.62$, $p=0.051$), as well as a significant main effect of sex ($F=5.11$, $p<0.0001$) and a sex x age interaction ($F=5.11$, $p=0.002$; Figure 2 left). The number of RPL17+ cells increased with age in males ($F=4.46$, $p=0.010$); this value was greater in adults than d25- and 35-day old birds (Tukey, $p<0.05$). The opposite pattern was detected in females ($F=6.02$, $p=0.003$); more HVC cells expressed RPL17 at day 25 than at all other ages (Tukey, $p<0.05$). While no main effect of age was detected ($F=1.80$, $p=0.149$), males had more AR-positive cells than females ($F=143.93$, $p<0.0001$; right), and a sex x age interaction existed ($F=3.82$, $p=0.011$). The number of cells increased as males aged ($F=3.26$, $p=0.033$), although no pairs of groups differed significantly from each other (Tukey $p>0.05$). Within females, the number of AR-expressing cells decreased ($F=8.70$, $p=0.001$), such that 25 day-old birds had more than each of the other age groups (Tukey $p<0.05$). The estimated total number of cells expressing both RPL17 and AR was greater in males than females ($F=158.39$, $p<0.0001$) and appeared to differ across ages ($F=2.62$, $p=0.050$). A significant interaction was also detected ($F=5.12$, $p=0.002$). These cells increased in males across ages ($F=4.47$, $p=0.010$), with adults having significantly more than the two youngest ages analyzed (Tukey, $p<0.05$). In contrast, they declined in females ($F=6.10$, $p=0.003$), such that the value at 25 days of age was greater than all of the others (Tukey, $p<0.05$).

No significant effects were detected on the proportion of RPL17+ cells co-expressing AR (all $F<1.23$, $p>0.274$). As in RA, almost no variability was detected on this measure – group means ranged from 99.04–100.00%. The percentage of AR+ cells co-expressing RPL17 was greater in males than females ($F=6.71$, $p=0.014$), but a main effect of age ($F=0.46$, $p=0.761$) and interaction between the two variables ($F=0.958$, $p=0.442$) were not detected. On average, this percentage was 46.69 ± 1.12 for males and 39.35 ± 2.23 for females. Similar to RA, the volume of HVC was larger in males than females ($F=139.15$, $p<0.0001$) and tended to differ across ages ($F=2.51$, $p<0.058$). A sex x age interaction was also detected ($F=5.28$, $p=0.002$). The size of HVC increased across ages in males ($F=4.59$, $p=0.009$) and decreased in females ($F=6.67$, $p=0.002$; data not shown).

3.1.3 Area X—RPL17 single- and double-labeled cells increased across ages (both $F=4.7$, $p=0.006$); adults differed from 25-, 35-, and 45-day-old birds ($p<0.05$; Figure 2 left). The total number of cells labeled for AR also increased in males as they aged ($F=3.83$, $p=0.015$; Figure 2 right), with adults having significantly more than birds at 35- and 45- days of age (Tukey, $p<0.05$). The vast majority (99-100%) of cells expressing RPL17 also expressed AR; this value obviously did not differ across ages ($F=1.00$, $p=0.426$). The percentage of AR+ cells that co-expressed RPL17 increased across ages ($F=4.95$, $p=0.004$), and ranged from 51.7 ± 0.87 to $61.2\pm0.41\%$. Finally, the volume of Area X increased as animals mature ($F=7.61$, $p<0.0001$); the region was larger in adults than in males at post-hatching day 45 and younger (Tukey, $p<0.05$; data not shown).

3.2 RPL37 + AR

3.2.1 RA—More cells expressed RPL37 in males than females ($F=48.46$, $p<0.0001$). This variable did not differ across ages ($F=1.75$, $p=0.157$), but an interaction did exist ($F=3.60$, $p=0.013$; Figure 3 left). The interaction was due to a gradual increase across ages in males ($F=3.08$, $p=0.038$; no two pairs of ages differed significantly) but not females ($F=0.933$, $p=0.465$). The estimated total number of cells expressing AR was greater in males compared to females ($F=183.80$, $p<0.0001$; Figure 3 middle). While an effect of age was not detected ($F=1.59$, $p=0.196$), a sex x age interaction was present ($F=4.02$, $p=0.008$). Here, the number of cells declined across ages in females ($F=4.90$, $p=0.006$; day 25 > adult), but not males ($F=2.40$, $p=0.083$).

The number of double-labeled cells was greater in males than females ($F=29.68$, $p<0.0001$; Figure 3 right). Effects of age ($F=1.03$, $p=0.402$) and a sex x age interaction ($F=2.54$, $p=0.054$) were not detected. While the interaction suggested a trend, significant effects of age were not detected in either males or females (both $F<1.98$, $p>0.135$). On average $58\pm2\%$ of the RPL37-positive cells co-expressed AR. A smaller percentage of AR-positive cells co-expressed RPL37 ($17\pm2\%$). Neither percentage was affected by sex or age, and these variables did not interact (all $F<3.42$, $p>0.071$). The results for effects of sex and age on RA volume were statistically identical in this set of tissue as those labeled for RPL17+AR (see above).

3.2.2 HVC—Effects of sex (male > female), age, and an interaction between the variables were detected for the number of RPL37+ cells (sex: $F=151.13$, $p<0.0001$; age: $F=3.09$, $p=0.026$; interaction: $F=4.50$, $p=0.004$; Figure 3). Parallel results were obtained for the total number of cells immunolabeled for AR and AR+RPL37-labeled cells (all $F\geq 3.41$, all $p\leq 0.017$). In all cases, the numbers of labeled cells increased across ages in males ($F>4.38$, $p<0.010$) and decreased in females ($F>3.62$, $p<0.026$; see Figure 3 for details of pairwise comparisons). The percentage of RPL37-positive cells that co-expressed AR was greater in males than females ($F=12.64$, $p=0.001$) and declined from post-hatching day 35 through adulthood ($F=4.58$, $p=0.004$; Table 1). No interaction between sex and age was detected ($F=1.66$, $p=0.177$). In contrast, no significant effects were detected for the percentage of AR-positive cells co-expressing RPL37 ($24\pm1\%$; all $F<1.81$, $p>0.187$). Parallel to the results collected from the tissue labeled for RPL17+AR, the volume of HVC was greater in males than females, and increased significantly across ages in males while decreasing in females.

3.2.3 Area X—Trends for increases in the number of cells labeled for RPL37 alone and RPL37+AR were detected (both $F = 2.4$, $p=0.08$). In both cases, *post hoc* comparisons indicated that fewer cells were present in 25-day-old males compared to adults (Tukey $p<0.05$; Figure 3). The estimated total number of cells expressing AR increased as males matured ($F=6.55$, $p=0.001$). Significant effects of age were not detected on the percentage of RPL37-positive cells that co-expressed AR ($60\pm1\%$; $F=1.05$, $p=0.404$) or the reverse, AR-

positive cells that co-expressed RPL37 ($15 \pm 1\%$; $F=0.88$, $p=0.491$). As indicated for the RPL17+AR-labeled tissue (above), the volume of Area X increased as males aged.

4. DISCUSSION

The present data document a number of effects of both sex and age on the expression of RPL17 and RPL37 in the song system of zebra finches. In both RA and HVC, males have more cells expressing these proteins than do females, and the sex differences increase as the birds mature. The numbers of these cells generally increase in the Area X of maturing males, as well. In all cases, substantial co-expression with AR exists. However, specific patterns differ, both across the regions and between the two ribosomal proteins. These relationships allow us to generate some hypotheses regarding potential functions within each of these brain regions.

4.1 RA

Sexual dimorphisms in the size of RA mainly develop due to the loss of neurons in females from about post-hatching days 20 to 60 (Kirn and DeVoogd, 1989). The numbers of cells expressing RPL17 (both the total estimate and the subset that co-expresses AR) match this pattern well. They decline in females between 25 days after hatching and adulthood and do not increase significantly with age in males. Thus, it is quite possible that the sex difference in RPL17 we detected reflects a general loss of cells during period. An analysis of mRNA based on the density of silver grains from a radiolabeled probe covering fairly large cross-sectional areas within RA detected substantially less expression of RPL17 in adults compared to 25-day-old birds, as well as a smaller sex difference in the older birds (Tang and Wade, 2006). Thus, while males synthesize more RPL17 per cell than females, especially as juveniles, the rates of translation and/or protein degradation may change as animals age.

The pattern of RPL37 mRNA expression from the analysis of silver grains is similar to that for RPL17, although for RPL37 mRNA a sex difference existed at post-hatching day 25 and not in adults (Tang and Wade, 2006). In contrast, the present study suggests that the total number of cells containing RPL37 does not decline with age in females, and increases gradually in males. These results are consistent with a sex specific increase in cells that express RPL37 in males, one that does not result from a general change in cell number in this brain region as the birds age (Kirn and DeVoogd, 1989). As the density of Nissl-stained cells is greater in *females* (at least in adults; see Figure 1 of Nottebohm and Arnold, 1976), it seems reasonable to infer that the sex difference in RPL37 is due both to increased synthesis per cell and a greater number of cells expressing the protein in males compared to females.

While the function of RPL37 is unknown, one possibility is that enhanced expression promotes incorporation of new cells into RA, either by increasing their proliferation or survival in males. This idea is consistent with results from Tang and Wade (2009), which showed that the majority of song system cells generated beginning on post-hatching day 6 and surviving until at least day 25 expressed one or both of these ribosomal proteins. While more of the neurons born during this period were destined for Area X than RA or HVC, the high percentage of RPL37 (as well as RPL17) expression in them was consistent across these regions.

4.2 HVC

While as in RA some cells do die in the HVC of females, the major factor contributing the sex difference in HVC is the addition of cells in males (reviewed in Wade and Arnold, 2004). Our initial investigation of two individuals of each sex at post-hatching day 25 did

not indicate increased expression of either RPL17 or RPL37 mRNA in HVC compared to surrounding tissue (Tang and Wade, 2006). However, a confocal immunohistochemical analysis with the species specific antibodies used in the present study (Tang and Wade, 2009) clearly revealed the presence of these proteins in HVC, consistent with the present study. Here, we document male-biased sexual dimorphisms in the numbers of cells expressing each of the two ribosomal proteins. These sex differences become greater with age.

For RPL17, cells increase in males from day 35 to adulthood, and decrease in females between post-hatching days 25 and 35. The loss of these cells in females coincides with a peak in the density of dying cells in females from 20–3 days of age (Kirn and DeVoogd, 1989), and may therefore be relatively non-specific. However, in males, the increase in the number of RPL17-positive cells occurs later than the primary increase in overall cell number between days 10 and 30 (Kirn and DeVoogd, 1989). Thus, the results are consistent with this ribosomal protein having a specific function in HVC.

At this stage we can only speculate about potential functions. A role in cell incorporation is possible. Neurons are added to this region throughout life, and survival appears to increase as incorporation of new cells in adults decreases, creating a stable overall number of neurons in the adult HVC (Pytte et al., 2007). This pattern fits with the increase we detected as animals mature. Another intriguing possibility is that RPL17 is involved in the differentiation of newly incorporated cells. It is up-regulated during early retinoic acid-induced differentiation of cultured NTERA2 cells into neurons (Bévort and Leffers, 2000). The retinoic acid synthesizing enzyme, retinaldehyde dehydrogenase, is expressed in HVC as early as 4–6 days after hatching and continues into adulthood (Kim and Arnold, 2005). While the rate decreases as adult animals age, mature zebra finches do incorporate new neurons into HVC (Wang et al., 2002, Pytte et al., 2007), thus neuronal differentiation occurs throughout the lifespan to at least some extent.

The number of cells expressing RPL37 is greater in the HVC of males than females, and the sex difference increases as the birds mature. As in RA, the specific pattern differs between the two ribosomal proteins. The increase in RPL37+ cells in males begins at 25, rather than 35, days after hatching. The decrease in females is gradual such that no two pairs of ages differ significantly, despite a main effect of age in this sex. These results are consistent with changes in cell numbers overall in this brain region (Kirn and DeVoogd, 1989), and therefore may follow, rather than promote, them.

4.3 Area X

Changes in estimated numbers of total cells could be evaluated across ages in males, but not in females. As in Nissl-stained sections, the region was not morphologically distinct from the rest of the medial striatum in females in the present study. In males, cells expressing RPL17 increased between post-hatching day 45 and adulthood. A modest trend ($p=0.08$) existed for an increase in RPL37-labeled cells throughout the developmental period examined. Results from double-labeled cells were the same as those based on the total numbers of cells expressing each of the ribosomal proteins.

Like HVC, differentiation of Area X primarily involves the addition of cells in males; rates of cell death are very low (Nordeen and Nordeen, 1988, Kirn and DeVoogd, 1989, Wade and Arnold, 2004). To our knowledge, detailed information on cell addition to Area X throughout development is not available. However, the patterns of relatively late (RPL17) and limited (RPL37) addition of cells expressing these ribosomal proteins in a region with very little cell death overall, are consistent with one or both playing a role in the survival of existing cells. Earlier research from our lab (Tang and Wade, 2009) also supports a role for

either or both RPL17 and RPL37 in the post-hatching proliferation of neurons that are incorporated in area X. Additional research is needed to distinguish, for each of the three brain regions, what specific mechanisms might be modulated by these ribosomal proteins.

4.4 Potential Interactions of Ribosomal Proteins and AR

In addition to providing novel information on RPL17 and RPL37, the present data offer some new insights regarding AR expression. Previous work on these receptors during song system development has focused on mRNA, and investigated a smaller range of mostly younger ages (Gahr and Metzdorf, 1999, Kim et al., 2004). Here, we document a series of sex-specific changes in numbers of AR+ cells in the developing song system. Differences in staining characteristics between the tissue labeled for RPL17 and RPL37 make it difficult to draw conclusions about absolute numbers of cells expressing the AR protein (see below). However, consistent patterns indicate that males have more AR immunoreactive cells than females in RA and HVC. In RA, the number of these cells in males remains stable from post-hatching day 25 through adulthood, but declines during this period in females. In HVC, AR+ cells also decrease in females as they mature, but in males they increase. They also increase in males during this period in Area X. These patterns generally parallel overall changes in cell numbers (see above), so it is hard to hypothesize about developmental mechanisms specifically associated with AR.

The relationship between AR and the two ribosomal proteins is intriguing, however. Large numbers of cells in RA, HVC and Area X express AR (see Figures 2 and 3), a subset express RPL17 and/or RPL37, and many (if not all) ribosomal-protein containing cells are AR+. These results suggest the potential both for effects of androgen that are separate from those of these two ribosomal proteins and effects of RPL17 and RPL37 that directly involve AR. One possibility is that androgen, acting at AR, influences the expression of the ribosomal proteins. While results from studies in which androgen levels were manipulated during development have produced equivocal results on structural change, a key role for AR in the masculinization of song system morphology was suggested in one study involving co-administration of estradiol and the androgen receptor blocker Flutamide (see Introduction; Grisham et al., 2002). In parallel, the development of stereotyped song appears to require androgen (Bottjer and Johnson, 1997). An intact HVC appears critical for estradiol-induced masculinization of the song circuit (Herrmann and Arnold, 1991), and this region expresses estrogen receptor alpha mRNA during development (Jacobs et al., 1999). At this point, we can only speculate about how the factors might work together to facilitate masculinization. However, one possibility is that the process could require the action of estradiol in HVC along with the interaction of androgen and Z-linked RPL17 and RPL37 across the song circuit. Relationships among androgen, estrogen and the two ribosomal proteins must be further evaluated.

Cellular localization of immunoreactivity might provide clues as to potential roles of these proteins. In fact, we suspect that the difference in AR labeling between the sets of tissue stained for RPL17 and RPL37 may reflect differential function. The absolute numbers of AR+ cells, as well as the numbers and percentages of double-labeled cells were lower in tissue stained for RPL37 compared to RPL17. As the tissue came from the same individuals, it is clear that the former under-represents AR expression. We believe this is due to the following.

The color reactions were stopped to optimize detection of each of the ribosomal proteins, which were the focus of this set of experiments. RPL17 and RPL37 exhibited somewhat different staining characteristics, which likely resulted in diminished quantification of AR in the nuclei of RPL37+ cells. Specifically, while labeling RPL17 was more cytoplasmic, RPL37 appeared more nuclear (Figure 1, compare panels C and E). AR labeling was often

distinct (Figure 1), but in other cases it may have been difficult to see in conjunction with the nuclear staining with the RPL37 antibody. As indicated above, the procedures were chosen to facilitate analysis of changes in total numbers of cells expressing the ribosomal proteins as males and females mature. A secondary question was whether there was potential for direct interaction with androgen via AR in specific song control nuclei. A follow-up study, for example using fluorescent, confocal analysis of regions within RA, HVC and Area X could provide more accurate estimates of co-expression. However, the present work clearly provides a positive answer to the question of whether the ribosomal proteins and androgen might interact in the same cells. The next step is to use selective inhibition of each to evaluate specific roles, and the distribution of the proteins may provide some clues.

Labeling of AR in both the nucleus and cytoplasm is not surprising; it probably reflects the presence of both bound and unbound receptors. While one might not expect ribosomal proteins to exist in the nucleus, precedents do exist. Ribosomal protein L7 (RPL7) appears to serve two functions, one associated with translation at the ribosome, and another as a nuclear receptor co-regulator (including as a co-activator of estrogen receptor alpha; Duncan et al., 2009). Ribosomal protein S3 (RPS3) acts as a DNA binding subunit of nuclear factor kappa B (NF- κ B), and regulates its transcription (reviewed in Wan and Lenardo, 2010). Little is known about the functions of RPL17 or RPL37 in any model system. However, the patterns of their expression suggest potential roles in both facilitating the translation of proteins and enhancing the activity of steroid hormone receptors. These ideas will need to be specifically addressed in future studies.

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ABBREVIATIONS

AR	Androgen receptor
DAB	diaminobenzidine
IMAN	Lateral magnocellular nucleus of the anterior nidopallium
PBS	Phosphate buffered saline
RA	Robust nucleus of the arcopallium
RPL17	Ribosomal protein 17
RPL37	Ribosomal protein 37

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Figure 1.

Photographs indicating labeling characteristics of the antibodies – all from male HVC. (A) Blue-gray staining with the AR primary antibody was both nuclear and cytoplasmic. While some light labeling was detected surrounding AR+ neurons within song control nuclei, tissue just outside of these regions was almost completely blank. The image in (B) is immediately ventral to the HVC depicted (A), and similar to what was detected in the preadsorption control. In (C) and (E), the ABC reagent was eliminated for the second immunolabeling reaction (AR), and resulted in detection of only the ribosomal proteins (brown). RPL17 (C) is primarily cytoplasmic, whereas RPL37 (E) is more nuclear. (D) and (F) show double-labeling of AR and RPL17 and RPL37, respectively. Scale bar (in B) = 20 μ m for all images.

Figure 2.

Expression of RPL17 and AR in three song control nuclei of zebra finches across post-hatching (P) development (mean + SE). As nearly 100% of the RPL17+ cells co-expressed AR, results for the double-labeled cells are the same as those indicated for RPL17. Sample sizes for animals in this experiment are represented on the X-axis of the graphs in the left-hand column. For all graphs, different lower-case letters represent significant differences between ages within males or females from pairwise comparisons (Tukey HSD $p < 0.05$). Main effects and interactions are indicated in the text. Photographs of a representative male and female at 45 days of age are provided for each brain region. All scale bars = 100 μ m.

Figure 3.

Estimates of total numbers of cells expressing RPL37 or AR, as well as the number of double-labeled cells, in three song control nuclei of zebra finches across post-hatching (P) development (Mean + SE). Sample sizes for animals in this experiment are represented on the X-axis of the graphs in the left-hand column. For all graphs, different lower-case letters represent significant differences between ages within males or females based on pairwise comparisons (Tukey HSD $p < 0.05$). Main effects and interactions are indicated in the text.

Table 1

Percentages of cells for each sex and age of RPL37+ cells that are also labeled for AR in the HVC of zebra finches.

Post-hatching day	Male		Female	
	Mean	Std. Error	Mean	Std. Error
25	65.53	3.7	60.14	5.5
35	71.25	1.7	65.89	0.9
45	69.02	2.5	58.00	4.7
65	65.98	1.8	66.67	5.3
Adult	63.61	1.4	50.19	1.5