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Developmental Changes in the Sexually Dimorphic Expression of SCAMP1 Protein and Its Co-Localisation with Androgen Receptor Protein in the Zebra Finch Song System

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Abstract

The song system of zebra finches differs dramatically between the sexes in terms of both structure and function. Only males sing and the brain regions regulating the learning and production of this behaviour are far more developed in males than females. Mechanisms regulating sexual differentiation likely include both direct genetic and hormonal processes. Expression of both mRNA and the protein product for secretory carrier membrane protein 1 (SCAMP1), a sex chromosome gene, are increased in the brains of juvenile males compared to females. Here we investigated developmental changes in SCAMP1 containing cells in song nuclei and co-localisation with androgen receptor (AR) protein from post-hatching day 25 through adulthood. Almost all SCAMP1 cells co-expressed AR and about half of the AR cells expressed SCAMP1 in the HVC and RA of both sexes and in the Area X of males (which could not be clearly defined in females). In HVC and RA, more single and double-labeled cells were detected in males than females overall, and the sex differences increased as animals matured. The results suggest the potential for interaction of these two proteins in regulating development of brain and/or behaviour.

Keywords

Songbird; sexual differentiation; steroid hormone; testosterone

Introduction

Steroid hormones play critical roles in sexual differentiation of brain and behaviour in a number of organisms. For example, in rodents oestradiol exposure around birth is critical for masculinisation. The hormone creates permanent changes in the brain which enable testosterone and its metabolites to activate male specific sexual behaviour in adulthood (1, 2). Similar to mammalian systems, zebra finches exhibit prominent sex differences in the forebrain. For example, only males sing, and the regions controlling the learning and production of this behaviour, including Area X, the lateral magnocellular nucleus of the anterior nidopallium (IMAN), HVC (used as a proper name) and the robust nucleus in the arcopallium (RA), are significantly larger in males, due to an increase in both cell size and number (2).

The actions of steroid hormones are commonly mediated through binding to intracellular receptors. While oestrogen receptors are expressed in the developing song system to only a limited extent (3-5), androgen receptor (AR) mRNA is abundant in the song nuclei during

development as well as adulthood; expression is greater in males than females (6, 7). Androgens are not as effective as oestradiol in masculinising song system structure and function (8). However, blocking AR prevents oestrogen-induced masculinisation in female zebra finches (9). These results provide support for the idea that AR is critical to the sexual differentiation process.

Direct genetic effects are also likely important for masculinisation of the song system. Perhaps the best evidence comes from analysis of a gynandromorphic zebra finch (10). Morphology of the circuit in this spontaneously occurring bird, which was genetically male (two Z chromosomes) on the left and female on the right (ZW), suggested the influence of both neural gene expression and diffusible factors such as steroid hormones. Dosage compensation in birds is limited (11), providing a mechanism for sex chromosome gene expression to readily contribute to differentiation of brain and behaviour. Patterns of mRNA for several Z-chromosome genes in the developing song system are consistent with this idea (12-16).

One candidate is secretory carrier membrane protein 1 (SCAMP1). cDNA microarray and qPCR analyses showed enhanced expression in the forebrain of hatchling and juvenile males compared to females (17). Western blot analyses on proteins extracted from the whole telecephalon also revealed that juvenile males had increased SCAMP1 compared to females, and *in situ* hybridization demonstrated significant and specific male-biased expression of SCAMP1 mRNA in HVC and RA of 25-day-old zebra finches (12). SCAMP1 protein and other members of this family, SCAMP2-5, are involved in vesicle trafficking. They function as carriers in the cell surface recycling system, and have been implicated in both exocytosis and endocytosis; SCAMP1 is widely expressed in brain (18-22). SCAMP1 specifically is involved in trafficking of secreted proteins. Its 1st transmembrane domain (TMD) is critical for targeting proteins from the trans-golgi network to the plasma membrane; TMDs 2 and 3 are essential for golgi export; TMD 4 is important for maintaining SCAMP1 structure (23). While interaction of SCAMP1 with steroid hormones has not been reported, SCAMP4 is decreased in the ventromedial hypothalamus of rats during proestrus compared to diestrus. In parallel, oestradiol plus progesterone treatment decreases SCAMP4 in the ventromedial hypothalamus(24). Thus, precedent exists for interactions between steroid hormones and the SCAMP family in a brain area with reproductive function (albeit one quite different from courtship song). Manipulations of SCAMP1 will eventually be required to determine whether and how it is specifically involved in sexual differentiation of the song system. However, before undertaking that effort, it is important to determine whether the naturally occurring pattern of expression is consistent with such a possibility.

Therefore, the main focus of this study was to examine developmental changes in the numbers of SCAMP1 protein expressing cells in the song control nuclei of male and female zebra finches. We investigated birds at post-hatching days 25-65. This age range was selected to capture the song learning period, as well as a time of rapid sexual differentiation of song system morphology. Adult zebra finches (> 100 days of age) were also examined for comparison. Co-expression with AR was also investigated to assess the potential for interaction between androgen and this Z-chromosome gene.

Materials and Methods

Animals

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 a mixture of bread and hard-boiled chicken eggs, as well as spinach and orange, once a week. Nests were checked daily; the day of

hatchling was found was considered post-hatching day 1. All procedures were conducted in accordance with NIH guidelines and approved by the Michigan State University IACUC.

Tissue Collection

Brains of juvenile (post-hatching day 25, 35, 45, and 65) and adult zebra finches (all $n=6$ /sex/age) were collected following rapid decapitation, snap frozen in cold methyl-butane, and stored at -80°C . Coronal sections ($20\text{ }\mu\text{m}$) through the entire telencephalon were cut on a cryostat, thaw-mounted into 6 alternate sets (SuperFrost Plus slides; Fisher Scientific, Hampton, NH), and stored with desiccant at -80°C until further processing. Two series of these brain sections were used in other studies to examine expression proteins encoded by other Z-chromosome genes (14).

Double-Label Immunohistochemistry for SCAMP1 and AR

One set of slides was warmed to room temperature, rinsed in 0.1 M 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 min in 10% normal horse serum in PBS with 0.3% Triton X-100. The tissue was then incubated in a SCAMP1 goat polyclonal antibody ($1\text{ }\mu\text{g/ml}$; sc-13614; Santa Cruz Biotechnology, Santa Cruz, CA) in 0.1 M PBS containing 0.3% Triton X-100, and 10% NHS for 48 hr at 4°C . This primary antibody was previously validated, using a preadsorption control and western blot analysis (12). A biotinconjugated horse anti-goat secondary antibody ($1\text{ }\mu\text{g/2 ml}$; Vector Labs, Burlingame, CA) was then used for 2 hrs 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.

The slides were then incubated in 10% normal goat serum for 30 min and exposed to the rabbit polyclonal AR primary antibody ($1\text{ }\mu\text{g/3 ml}$; sc-816; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C . The tissue was then incubated in a biotin-conjugated goat anti-rabbit IgG secondary antibody ($1\text{ }\mu\text{g/2 ml}$; Vector Labs) for 2 hrs at room temperature. The protein was visualized with *Elite* ABC reagents and the SG substrate (Vector Labs) per manufacturer's instructions to produce a blue-gray reaction product (Figure 1). Omission of the primary AR antibody (16) and preadsorbing it with 30-fold excess of the immunizing peptide (present study; SC-816P, Santa Cruz; data not shown) resulted in a complete absence of immunohistochemical labeling. Western analysis using zebra finch brain tissue indicated a single band of the expected size for this species (Ensembl Genome Browser), which was also eliminated by preadsorption (Figure 2).

Stereological Analyses

HVC and RA were analysed in both males and females. However, Area X cannot be detected with a Nissl stain in female zebra finches, and also could not be clearly distinguished with the markers used in the present study (Figure 3). Therefore, labeled cells were only quantified in males. Cells were not counted in IMAN because its size is not sexually dimorphic (25) and borders can be hard to identify. Regions of interest from each animal were analysed under brightfield illumination using a Stereo Investigator (Microbrightfield Inc., Williston, VT) by an individual blind to sex and age of the animals. The border of each song nucleus on one side of the brain (randomly selected) was defined by tracing its edge throughout its rostrocaudal extent (sections were $120\text{ }\mu\text{m}$ apart). All cells exhibiting neuronal morphology and clear reaction product for SCAMP1 or AR, and those double-labeled for SCAMP1-AR were counted in sections using the Optical Fractionator function (14).

Statistical Analyses

Within RA and HVC, effects of sex and age were analysed 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 SCAMP1 and AR (Figure 4). Percentages of double-labeled cells and brain region volumes were also analysed in this manner, as were densities of labeled cells. This value was calculated by dividing the estimated total counts by the volume of each brain region for each individual (determined by Stereo Investigator based on the tracings). When significant interactions were detected, effects of age were investigated within each sex by one-way ANOVA. When appropriate, pairwise comparisons among ages were assessed using Tukey HSD, and sex differences within each age group were analysed with unpaired t-tests. In Area X, effects of age were analysed only in males, using one-way ANOVA followed by Tukey HSD.

Results

SCAMP1 Immunohistochemistry

Estimated Total Numbers of Labeled Cells

HVC: More SCAMP1 immunopositive (SCAMP1+) cells were detected in males than females overall, and the sex difference increased with age. Specifically, main effects of sex ($F_{1,39}=2.76$, $p=0.041$) and age ($F_{4,39}=170.77$, $p<0.0001$) were detected, as well as an interaction between age and sex ($F_{4,39}=3.74$, $p=0.011$). Males showed a gradual increase ($F_{4,20}=3.13$, $p=0.038$), whereas females exhibited a decrease ($F_{4,19}=13.82$, $p<0.0001$) in the number of SCAMP1+ cells. For all analyses on total counts of labeled cells, significant differences between sexes and ages based on pairwise comparisons are noted in Figure 4.

RA: A pattern similar to HVC was detected in this brain region. More SCAMP1+ cells were seen in males than females ($F_{1,39}=93.78$, $p<0.0001$), and an interaction between age and sex ($F_{4,39}=8.27$, $p<0.0001$) existed. However, a significant effect of age was not detected ($F_{4,39}=0.92$, $p=0.467$). Overall, the total number of SCAMP1+ cells increased as males developed ($F_{4,19}=3.19$, $p=0.037$), but decreased in females ($F_{4,20}=11.10$, $p<0.0001$).

Area X: SCAMP1+ cells increased significantly as males matured ($F_{4,24}=5.79$, $p=0.002$).

Density of Labeled Cells

HVC: The density of SCAMP1+ cells was greater in females than males. Females had $211,444 \pm 9,190$ and males had $177,688 \pm 5,974$ cells/mm³ – a 19% difference ($F_{1,39}=10.77$, $p=0.002$). Effects of age ($F_{4,39}=1.84$, $p=0.141$) and a sex x age interaction ($F_{4,39}=1.53$, $p=0.213$) did not exist.

RA: As in HVC, the density of SCAMP1+ cells was increased in females compared to males. This value was $212,940 \pm 13,450$ cells/mm³ in females and $119,367 \pm 5,604$ in males – a 78% difference ($F_{1,39}=37.10$, $p<0.0001$). Effects of age ($F_{4,39}=1.02$, $p=0.407$) and a sex x age interaction ($F_{4,39}=0.93$, $p=0.457$) were not detected.

Area X: A significant effect of age was detected in males ($F_{4,24}=3.40$, $p=0.024$). The density gradually increased, with only the post-hatching day 25 and adults differing significantly (Tukey $p<0.05$). The density (cells/mm³) increased by 30% between post-hatching day 25 ($216,229 \pm 10,723$) and adulthood ($281,040 \pm 19,367$).

AR Immunohistochemistry

Estimated Total Numbers of Labeled Cells

HVC: A greater number of AR+ cells existed in males compared to females, and the difference increased across ages. That is, significant main effects of age ($F_{4,39}=2.79$, $p=0.039$) and sex ($F_{1,39}=158.74$, $p<0.0001$), as well as a significant interaction between age and sex ($F_{4,39}=4.53$, $p<0.005$), were detected. The cells increased in males ($F_{4,20}=3.58$, $p=0.023$) and decreased in females ($F_{4,19}=11.76$, $p<0.0001$) as they matured.

RA: Males had more AR+ cells than females in this brain region as well. A significant main effect of sex was detected ($F_{1,39}=110.93$, $p<0.0001$), and while there was no main effect of age ($F_{4,39}=0.67$, $p=0.615$), a significant interaction between age and sex ($F_{4,39}=7.41$, $p<0.0001$) did occur. Females exhibited a significant decrease of AR+ cells ($F_{4,20}=9.45$, $p<0.0001$) with age, whereas males displayed a trend for an increase ($F_{4,19}=2.75$, $p=0.059$).

Area X: The estimated total number of AR+ cells increased significantly in males during development ($F_{4,24}=4.26$, $p=0.010$).

Density of Labeled Cells

HVC: The density of AR+ cells was greater in females than males ($424,041 \pm 18,190$ cells/mm³ vs. $342,004 \pm 10,581$ – a 23% difference). This sex difference was statistically significant ($F_{1,39}=15.71$, $p<0.0001$). Effects of age ($F_{4,39}=1.46$, $p=0.232$) and a sex $\times$ age interaction ($F_{4,39}=0.62$, $p=0.653$) were not detected.

RA: A sexual dimorphism on the density of AR+ cells was also detected in this brain region. Females had $353,479 \pm 24,255$, and males had $212,890 \pm 8,787$ cells per mm³ – a 66% difference ($F_{1,39}=22.94$, $p<0.0001$). Neither an effect of age ($F_{4,39}=0.33$, $p=0.856$) nor a sex $\times$ age interaction ($F_{4,39}=0.78$, $p=0.545$) was detected.

Area X: The density of AR+ cells did not change as males matured ($F_{4,24}=1.58$, $p=0.211$). They had an average of $224,560 \pm 5,929$ cells/mm³.

SCAMP1 and AR Double-Labeled Cells

Estimated Total Numbers of Labeled Cells

HVC: A greater number of cells expressing both SCAMP1 and AR was detected in males compared to females. In fact, main effects of sex ($F_{1,39}=168.48$, $p<0.0001$) and age ($F_{4,39}=3.00$, $p=0.030$), as well as a significant interaction between the two variables ($F_{4,39}=3.93$, $p=0.009$), were all detected. The number increased in males as they matured ($F_{4,20}=3.39$, $p=0.028$). In contrast, females showed a decrease in the number of double-labeled cells ($F_{4,19}=12.42$, $p<0.0001$). Neither the percentage of SCAMP1+ cells that were also AR+ (average $99.2 \pm 0.5\%$) nor AR+ cells that were also SCAMP1+ (average $51.0 \pm 0.5\%$) showed effects of sex or age (all $F<1.90$, $p>0.131$).

RA: As in HVC, males had more double-labeled cells than females. This effect of sex was statistically significant ($F_{1,39}=78.99$, $p<0.0001$). In addition, while there was no main effect of age ($F_{4,39}=0.94$, $p=0.451$), a significant interaction between age and sex was detected ($F_{4,39}=7.42$, $p<0.0001$). In males, the number of double-labeled cells increased across the ages ($F_{4,19}=2.93$, $p=0.048$), whereas females displayed a significant decrease ($F_{4,20}=9.49$, $p<0.0001$).

The percentage of SCAMP1 cells that co-expressed AR was greater in females ($F_{1,39}=5.851$, $p=0.02$; 95.7 ± 0.9 vs. $91.2 \pm 1.4\%$). The percentage of AR+ cells that expressed SCAMP1

also revealed a significant main effect of sex ($F_{1,39}=8.804$, $p<0.005$). This proportion was greater in females ($57.9 \pm 1.4\%$) than males ($51.4 \pm 1.9\%$). Effects of age and sex by age interactions were not detected (all $F<1.53$, $p>0.214$).

Area X: A main effect of age was detected in the estimated total number of double-labeled cells, such that they increased as males matured ($F_{4,24}=4.84$, $p=0.005$). However, age had no effect on either the percentage of SCAMP1 cells co-expressing AR or the percentage of AR cells co-expressing SCAMP1 (averages: $58.4 \pm 7.5\%$ and $92.24 \pm 0.79\%$, respectively; both $F<1.97$, $p>0.131$).

Density of Labeled Cells

HVC: The density of cells expressing SCAMP1 plus AR was greater in females than males ($210,416 \pm 9,129$ cells/mm³ in females and $176,068 \pm 6,492$ in males – a 19% difference). This effect of sex was statistically significant ($F_{1,39}=10.60$, $p=0.002$). Effects of age ($F_{4,39}=1.99$, $p=0.115$) and a sex x age interaction ($F_{4,39}=1.50$, $p=0.220$) were not detected.

RA: As in HVC, females exhibited a greater density of double-labeled cells than males in RA ($F_{1,39}=39.59$, $p<0.0001$). Females had $203,901 \pm 13,190$ and males had $108,626 \pm 5,161$ cells/mm³ (88% difference). Effects of age ($F_{4,39}=0.83$, $p=0.514$) and a sex x age interaction ($F_{4,39}=0.95$, $p=0.446$) were not detected.

Area X: A trend for an increased density of cells positive for both SCAMP1 and AR was detected ($F_{4,24}=2.59$, $p=0.062$). Similar to the pattern for single-labeled SCAMP1+ cells, the density appeared to increase with age in males.

Discussion

This study documents several effects of sex and age on both SCAMP1 and AR protein in the song nuclei of zebra finches during development. Overall, males have more cells expressing either or both genes/proteins than do females in HVC and RA, and the sex differences increase as zebra finches mature. These patterns of expression are consistent with previous work in zebra finches on both SCAMP1 and AR (see 6, 12 and references therein). The antibody used here revealed both nuclear and cytoplasmic labeling of AR, which likely represents bound and unbound receptors, respectively. It also supports the comparatively recent detection of substantial AR mRNA in the Area X of zebra finches (6).

Interestingly, while significant sex differences in SCAMP1, AR and double-labeled cells were detected in the HVC of 25-day-old zebra finches, none was seen in RA until 35 days after hatching. As sex differences in cell numbers are present before day 25 in both brain regions (2), these results suggest that SCAMP1 and/or AR might specifically mediate sexual differentiation in RA but not HVC. However, consideration of the effects of age increases our ability to draw conclusions about potential mechanisms. For example, in HVC, single and double-labeled SCAMP1+ cells increased gradually but significantly in males while showing more dramatic decreases in females, and similar patterns were detected in both sexes in RA and males in Area X. In contrast, single-labeled AR+ cells in RA declined in females without significantly increasing in males, a pattern that generally follows changes in total numbers of cells in this brain region (26).

Thus, collectively the results suggest that: (1) AR alone may not specifically regulate differentiation of cell number in the song system; and (2) SCAMP1 could facilitate cell survival, particularly in RA as the primary mechanism regulating its sexual dimorphism is increased cell death in females (26). Data on density are generally consistent with this idea. That is, the fact that the densities of single- and double-labeled cells in HVC and RA do not

differ across ages, as the sex difference in volume increases (14), means that the number of cells expressing SCAMP1 and/or AR contributes to overall changes in the sizes of these brain regions. While this information does not imply anything about specific functions of these proteins in the development of the sexual dimorphism in volume, the fact that *both* the density and number of SCAMP1+ cells specifically increase in Area X as males age lends support to the idea that this protein may reflect increased cell survival. More details are provided below.

Across these three brain regions, more than half of the AR cells co-express SCAMP1 and almost all of the SCAMP1 cells express AR. Thus, ample opportunity exists for these two proteins to work together some way. While androgen and SCAMP1 need not necessarily interact directly, this pattern of co-expression suggests that any effects of SCAMP1 on song system differentiation might require or be strongly influenced by androgen. In contrast, androgen probably acts in additional ways that do not involve SCAMP1. Our data suggest the potential for differences between SCAMP1-AR interactions among the brain regions, as the percentages of double labeled cells are greater in females than males in RA but not HVC. However, the biological importance of these 4-7% differences is not obvious.

Our previous investigation of SCAMP1 indicated that 25-day-old males express more mRNA than females in HVC (12). Here, we report a male-biased dimorphism in the number of cells containing SCAMP1 protein, as well as those expressing AR and the two proteins together, occurring as early as day 25 and continuing throughout development. The sex difference in the present study became greater with age primarily as a result of females losing about half of their SCAMP1+ cells in HVC from 25 to 35 days-of-age, whereas the number in males increased only modestly across development and not at all during this period. Neither of these patterns parallels general changes in overall HVC cell number reported by Kirn and DeVogd (26). Overall cell loss in females at this stage is far less than the decrease in labeling we detected, and the gradual and consistent increase in overall cell number males experience through at least day 45 occurs without a substantial change in the number of SCAMP1 expressing cells. Thus, the changes in SCAMP1 expression cannot simply be the result of changes in total HVC cell number in either sex. We hypothesize that increased SCAMP1 expression in males facilitates survival of their HVC neurons compared to females.

In contrast to the development of HVC, the sex difference in RA mainly results from cell death in females, which that peaks at day 30 (2, 26). This pattern fits with emergence of a sex difference in SCAMP1 expressing cells between days 25 and 35, suggesting that the dimorphism is facilitated by the survival of cells expressing SCAMP1 (or increased SCAMP1). Significant male-biased sex differences of SCAMP1, AR, and double labeled cells numbers detected in the RA of 35-day-old zebra finches coincide with the timing of RA projecting HVC fibers entering RA (around 30 to 35 days of age) in males (27). Thus, it is possible that those RA-projecting HVC cells may play a role in supporting RA cells survival in males by mechanisms involving SCAMP1 and AR.

Sexual differentiation of Area X is largely due to the addition of cells in males. Increased cell death in females also appears to enhance the dimorphism (26). We found that the density and percentage of Area X neurons born beginning on post-hatching day 6 are both greater in male zebra finches than in the comparable area of females at day 25 (28). Data at 30 days of age suggest that males exhibit increased mitotic activity in particular regions of the ventricular zone that might contribute cells to Area X (29, 30). Thus, it seems likely that both increased cell proliferation and cell survival are involved in the masculinisation of Area X. Overall, the pattern of results from the present paper is consistent with a role of SCAMP1, perhaps in concert with AR, in facilitating masculine rates of cells survival.

However, additional work is necessary to confirm this idea and to determine whether other effects occur as well.

Based on the present data, it is interesting to consider potential mechanisms that might be associated with SCAMP1- and AR-mediated sexual differentiation. For example, brain derived neurotrophic factor (BDNF) and fibroblast growth factor 2 have both been implicated in masculinisation of morphology of the zebra finch song system (31-33). Insulin-like growth factor 2 is also expressed in the developing zebra finch HVC, with an apparent peak around day 70, an age at which song is crystallising in males. Most of these neurons appear to project to Area X (34). In adult canaries, singing appears to induce a BDNF-mediated increase in cell survival in HVC, an effect that could influence downstream portions of the motor pathway, as the expression primarily occurs in RA-projecting neurons (35). BDNF expression is detectable in the HVC of untreated male canaries, but not females, however it is increased in females with testosterone (36). Thus, the potential exists for androgen to increase the synthesis of trophic factors in the song system, and for SCAMP1 to increase their release (see Introduction). Future work should to evaluate the particular role(s) of SCAMP1 in the developing zebra finch song system, as well as how it may interact with androgens and trophic factors to potentially support masculinisation of structure and/or function.

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Figure 1. Photograph of cells double-labeled for SCAMP1 and AR (arrowheads) in the Area X of an adult male zebra finch. Cells single-labeled for androgen receptor are also indicated (arrows). Scale bar=10 μ m.

Figure 2.

Western blot analysis documenting specificity of the androgen receptor antibody. The image on the left indicates labeling with the primary antibody in protein extracted from the whole telencephalon of two different 25-day-old males. The image on the right shows the absence of labeling on a parallel blot with preadsorption of the primary antibody (20-fold excess with the peptide against which it was raised). Western techniques were as in reference 12.

Figure 3.

Representative images of the general patterns of SCAMP1+AR labeling in the three song control nuclei investigated at post-hatching days 25 (D25), 45 (D45) and in adults. Arrows indicate borders of the regions traced. Scale bars=200 μ m.

Figure 4.

Estimates of total numbers of SCAMP1-positive, androgen receptor-positive and double-labeled cells in HVC, RA and Area X (Mean $\pm$ SEM). Lower-case letters represent significant differences between ages within each sex. In HVC and RA, values were significantly greater in males than females at each age except those marked "ns".