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Sexually Dimorphic SCAMP1 Expression in the Forebrain Motor Pathway for Song Production of Juvenile Zebra Finches

Yu Ping Tang^{1,3}, Camilla Peabody^{1,3}, Michelle L. Tomaszynski^{1,3}, and Juli Wade^{1,2,3,*}

¹Department of Psychology, Michigan State University, East Lansing, Michigan 48824-1101

²Department of Zoology, Michigan State University, East Lansing, Michigan 48824-1101

³Neuroscience Program, Michigan State University, East Lansing, Michigan 48824-1101

Abstract

Mechanisms regulating sexual differentiation of the zebra finch song system are not well understood. The present study was designed to more fully characterize secretory carrier membrane protein 1 (SCAMP1), which was identified in a cDNA microarray screen as showing increased expression in the forebrains of developing male compared with female zebra finches. We completed the sequence of the open reading frame and used *in situ* hybridization to compare mRNA in song control regions of juvenile (25-day-old) individuals. Expression was significantly greater in the HVC (used as a proper name) and robust nucleus of the arcopallium (RA) in males than in females. Immunohistochemistry revealed that SCAMP1 protein is also expressed in these two brain regions, and qualitatively appears greater in males. Western analysis confirmed that the protein is increased in the telencephalon of males when compared with females at 25 days of age. These results are consistent with the idea that SCAMP1 is involved in masculinization of these brain areas, perhaps facilitating the survival of cells within them.

Keywords

sex difference; brain development; songbird; HVC; RA

INTRODUCTION

Sexually dimorphic neural systems in diverse vertebrate groups have provided exceptional models for elucidating the factors regulating the development of brain and behavior. In most cases, gonadal steroids are critical for masculinization (and/or defeminization) (Cooke et al., 1998; De Vries and Simerly, 2002). However, relatively little is known about molecular mechanisms associated with these processes. Zebra finches offer a unique opportunity for these types of investigations. The sex differences in structure and function are large. Males sing whereas females do not, and morphology of brain regions controlling song learning and production are substantially enhanced in males compared with females (Nottebohm and Arnold, 1976). These areas include the HVC (used as a proper name; Reiner et al., 2004) and the robust nucleus of the arcopallium (RA), which regulate motor aspects of song production, as well as Area X and the lateral magnocellular nucleus of the anterior nidopallium (IMAN), which are important for song learning during development (Bottjer and Johnson, 1997).

We created a cDNA microarray to screen for factors involved in sexual differentiation of the zebra finch song system (Wade et al., 2004). Among others, a gene tentatively identified as secretory carrier membrane protein 1 (SCAMP1) showed increased expression in the forebrain of hatchling and 25-day-old males in the microarray analysis, as well as in separate individuals using real-time quantitative PCR (qPCR) (Wade et al., 2005). These procedures utilized cDNA generated from the entire telencephalon. *In situ* hybridization on a small number of juvenile males and females then indicated that the mRNA was present only in males in the song control regions HVC, RA, IMAN and Area X, although expression in several other telencephalic regions was detected in both sexes (Wade et al., 2005).

SCAMP1, along with four related genes (SCAMPs 2–5), encode proteins involved in vesicle trafficking (Fernández-Chacón and Südhof, 2000). They are found in membranes and appear to function as vesicular carriers in the cell surface recycling system (Wu and Castle, 1998). Most are widely expressed in diverse tissue types at least in mammals, but SCAMP5 seems specific to brain cells, perhaps mature neurons. The distributions of SCAMPs 1 and 5 are similar in brain and their expression is associated with synaptic vesicles (Fernández-Chacón and Südhof, 2000).

In an effort to begin elucidating potential roles for SCAMP1 in masculinization of the song system, goals of the present study included (1) completing the sequence of the open reading frame for the zebra finch, (2) conducting a more detailed analysis of the mRNA in the song control regions of 25-day-old males and females, and (3) determining whether the expression of SCAMP1 protein is parallel. As before, this age was selected because it is during the time when song is memorized and morphological differentiation of the song control regions is enhanced (reviewed in Doupe et al., 2004; Wade and Arnold, 2004).

METHODS

Sequencing

The sequence of the entire open reading frame was obtained for zebra finch SCAMP1 using a combination of 5' RACE and primers designed from sequences of other species. Every base was obtained at least twice, once in each direction. Initially, the GeneRacer kit (Invitrogen, Carlsbad, CA) was used per manufacturer's instructions to generate cDNA from RNA that had been extracted with Trizol (Invitrogen) from the telencephalon of a 25-day-old male. Platinum Taq High Fidelity DNA polymerase (Invitrogen) was then used in the first stage PCR with the GeneRacer kit's 5' primer and a 3' zebra finch specific primer obtained from our recently published partial sequence (Wade et al., 2005; Genbank accession number AY833080), corresponding to bases 3623–3651 in the compiled sequence (updated in Genbank under the same accession number; see Results section). The subsequent nested PCR using the product from the first reaction involved a primer designed from bases 3576–3605, along with the GeneRacer 5' nested primer. This product was gel-purified using the SV Gel and PCR Cleanup System (Promega, Madison, WI) per manufacturer's instructions. It was cloned into pGEM T-Easy vector (Promega) and transformed into One Shot Top-10 competent cells (Invitrogen). The plasmid was isolated with the Wizard Plus kit (Promega), and initially sequenced using M13 forward on a 3130 DNA sequencer (Applied Biosystems, Foster City, CA) with Big Dye Terminator Kit 3.1 (Applied Biosystems). This procedure revealed bases 2717–3609 in the compiled sequence (see Results section), which overlapped with the 3' sequence we had determined previously (Wade et al., 2005).

Obtaining the rest of the 5' sequence involved the use of primers designed from Genbank sequences from human, mouse, rat, pig, and chicken. Three clones were produced including bases 1–772, 305–1190, and 733–2900 of the compiled sequence (see Results section). Template cDNA was prepared from 25-day-old males with the High Capacity cDNA Archive

Kit (Applied Biosystems; Wade et al., 2005), and used with 0.6 μ M 5' primer, 0.4 μ M 3' primer, 2 mM MgSO₄, 0.2 mM dNTPs, and 2.5 units Platinum Taq High Fidelity DNA polymerase (Invitrogen). Products were ligated, transformed, purified, and sequenced as above. However, in one case the PCR product was directly ligated into the vector, in another, it was extracted from 0.75% Seaplaque GTG agarose (Cambrex, Rockland, ME), and in the third, it was purified from a 1% agarose gel with the GenElute column system (Sigma, St. Louis, MO), depending on the size of the amplicon(s) and whether one or more were detected. Sequence information was first obtained from these three plasmids using vector primers (T7, SP6, and M13 forward) plus an internal one corresponding to bases 2235–2258 in the final sequence. Once this information was compiled, three additional primers using bases 1445–1469, 1975–1999, and 2804–2824 were employed to confirm sequence information in the opposite direction as necessary.

Tissue Collection for Histology

Post-hatching day 25 (day 1 = the day of hatching) zebra finches were rapidly decapitated (n = 6 per sex). Brains were frozen in methyl-butane and stored at -80°C . Later, coronal 20 μ m sections were thaw-mounted in six series onto SuperFrost Plus slides (Fisher Scientific, Hampton, NH). They were stored with desiccant at -80°C until further processing.

In Situ Hybridization

Two adjacent sets of tissue sections (for antisense and sense probes) were used for *in situ* hybridization, as described in Wade et al. (2005). All slides were hybridized simultaneously. The tissue sections were allowed to come to room temperature, rinsed in phosphate buffered saline (PBS), fixed in 4% paraformaldehyde, and washed in 0.1% diethylpyrocarbonate-treated water. Following another PBS rinse, they were incubated in 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min, dehydrated, and air dried. The tissue was prehybridized in a solution containing 50% formamide (buffer recipe in Wade et al., 2005) and then hybridized overnight at 55°C with hybridization buffer, 10% dextran-sulfate, 50% formamide, and 5×10^6 cpm ^{33}P -UTP-labeled RNA probe. Probes were prepared using the MAXIscript In Vitro Transcription Kit with SP6/T7 RNA polymerases (Ambion, Austin, TX). After hybridization, slides were washed in SSC, treated with RNase A, washed again, dehydrated, and air dried. Slides were exposed to Hyperfilm MP (Amersham Biosciences, Piscataway, NJ) with an intensifying screen (BioMax Transcreen LE; Eastman Kodak, Rochester, NY) to confirm sufficient labeling, and then were dipped in emulsion (NTB-2; Eastman Kodak). The slides were developed 2 weeks later and lightly counter-stained with cresyl violet.

Silver grain labeling representing mRNA expression was quantified in song control areas in which we had qualitative evidence that SCAMP1 was increased in males compared to females at 25 days of age. These regions included IMAN, Area X, HVC, and RA (Wade et al., 2005). When Area X was not visible (as it typically is not in females), a comparable region of the MST was assessed. As a control, silver grains were also quantified in a portion of the lateral arcopallium (A) outside of RA, which is not involved in song learning, production, or perception. All analyses were done without knowledge of the sex of the individuals.

Within each brain region, images from both sides of each coronal section containing the area of interest (bilateral measurements in 3–6 sections per animal) were captured using Scion (NIH) Image with darkfield microscopy. The captured area was $264 \times 198 \mu\text{m}^2$, which covered at least 50% of the cross-sectional area for each of the song control regions. As in Veney and Wade (2004, 2005), the density of labeling (percent area covered by silver grains) was quantified using the “density slice” function. For each brain region, labeling in adjacent sense-treated sections was subtracted from the antisense values, and an average for each animal was calculated from the multiple measurements for use in statistical analyses.

Effects of sex (between individuals) and brain region (within individuals) were analyzed by two-way ANOVA. Because of a significant interaction (see below), one-way ANOVAs within sex were also performed with Tukey/Kramer *post hoc* comparisons among brain regions. Planned pairwise comparisons were used to further probe sexual dimorphisms within each of the brain areas, using Bonferroni corrections (adjusted $\alpha = 0.01$ for five regions). All statistics were computed using Statview (SAS Institute, Cary, NC).

Immunohistochemistry

As SCAMP1 is nearly ubiquitously expressed (Singleton et al., 1997) and immunohistochemistry is not particularly conducive to quantifying levels of expression within cells, our main goal with this tissue was simply to evaluate whether the mRNA was translated in the regions in which it appeared sexually dimorphic. Therefore, brains from additional 25-day-old zebra finches ($n = 2$ per sex) were obtained and sectioned as above for evaluation of SCAMP1 protein. Slides were warmed to room temperature and fixed in 4% paraformaldehyde in 0.1 M PBS for 15 min, followed by a 30 min incubation in 0.6% H₂O₂/methanol to remove endogenous peroxidase and a 30 min incubation in 3% normal donkey serum in 0.1 M PBS with 0.3% Triton X-100. The tissue was then incubated in a SCAMP1 goat polyclonal antibody (sc-13614; 5 μ g/ml; Santa Cruz Biotechnology, Santa Cruz, CA) in 0.1 M PBS containing 0.3% Triton-X100, 3% NDS, 30% glycerol for 48 h at 48°C. A biotinylated donkey anti-goat secondary antibody was then used for 2 h at room temperature (1:500; Jackson ImmunoResearch, West Grove, PA) along with *Elite* ABC peroxidase reagents (Vector Laboratory, Burlingame, CA), and diaminobenzidine. Slides were dehydrated and coverslipped with DPX mounting medium (Fluka, St. Louis, MO). As a negative control, the primary antibody was preadsorbed for 72 h at 4°C with 10-fold excess of the peptide used to generate it (sc-13614P; Santa Cruz Biotechnology).

Western Analysis

Telencephalons from day 25 male and female zebra finches ($n = 6$ per sex) were collected by rapid decapitation, frozen on dry ice, and stored at -80°C . Individual telencephalons were sonicated at 4°C in RIPA Lysis buffer, according to manufacture instructions for protein extraction (Santa Cruz Biotechnology). Protein concentrations were quantified with a Bradford assay. From each telencephalon, 50 μ g was electrophoresed on a 10% Tris-HCl gel and transferred to a PVDF membrane at 4°C. The membrane was incubated in 5% nonfat milk for 30 min at room temperature to block nonspecific binding. It was then exposed to the same primary antibody used for immunohistochemistry (1 μ g/ml) overnight at 48°C. The membrane was treated with horseradish peroxidase-conjugated donkey anti-goat antibody (1:10,000) at room temperature for 1 h (Santa Cruz Biotechnology). Immunoreactivity was detected by chemiluminescence (ECL, Amersham Pharmacia; Piscataway, NJ) followed by exposure to X-OMAT film (Eastman Kodak, Rochester, NY). The mean optical density of the SCAMP1 band in each lane was quantified using Scion (NIH) Image. The membrane was then stripped in buffer containing 50 mM Tris-HCl, 0.2% SDS, and 0.8% 2-mercaptoethanol at 60°C for 30 min, washed in 1 $\times$ PBS-Tween 20, and reprobed using an antibody against the immediate early gene *ZENK* (Egr-1; sc-189, 1:1000; Santa Cruz Biotechnology) overnight at 4°C. It was then incubated in a horseradish peroxidase-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch) for 1 h at room temperature. Optical densities were determined for the *ZENK* bands as above, and a ratio of SCAMP1 to *ZENK* labeling was calculated for each animal. These values were compared between males and females by two-tailed *t* test. Protein from mouse brain (hippocampus; 50 μ g) was used as a positive control. As a negative control, the primary antibody was preabsorbed with 5-fold excess of the peptide (sc-13614P; Santa Cruz Biotechnology) for 2 h at room temperature before exposure to the membrane.

RESULTS

Sequence

The compiled zebra finch sequence consists of 4092 nucleotides: 3 bases of 5' UTR, 1017 in the protein coding region (339 amino acids; labeled as bases 4–1020 in Genbank, accession number AY833080), and 3072 bases in the 3' UTR including the stop codon. The open reading frame is very similar to other species. For example, it shares 81–85% identity with mammalian species including human (variant 1), mouse, and pig, as well as cow and dog. The chicken sequence is incomplete, but based on the information available, it is 93% identical to the zebra finch (Fig. 1). The avian sequence has one more predicted amino acid than the mammalian versions, a glycine inserted at position 116, which corresponds to nucleotides 349–351 of the zebra finch sequence.

mRNA Expression

Main effects of sex ($F = 10.96$, $p = 0.008$), brain region ($F = 3.93$, $p = 0.009$), and an interaction between the two variables ($F = 6.29$, $p = 0.0005$) were detected (Figs. 2 and 3). Planned comparisons ($\alpha = 0.01$ for five brain regions) revealed enhanced expression in males compared with females in HVC ($t = 4.40$, $p = 0.0013$) and RA ($t = 4.26$, $p = 0.002$), but not in IMAN, Area X (or the equivalent portion of the medial striatum in females), or the control region A (all $t < 1.85$, $p > 0.094$). One-way ANOVAs revealed that mRNA levels differed across brain regions within males ($F = 6.33$, $p = 0.002$) but not females ($F = 1.52$, $p = 0.233$). Among males, expression in HVC was increased compared with all brain regions (Tukey/Kramer all $p < 0.05$) other than RA.

Protein Expression

As expected from the literature (Fernández-Chacón and Südhof, 2000), immunohistochemistry detected SCAMP1 in numerous cells throughout the brain. Substantial labeling appeared in the HVC and RA of both males and females (Fig. 4), and it was completely eliminated in tissue exposed to antibody that had been preadsorbed with the peptide against which it was raised. Qualitatively, it appeared that the staining was darker in HVC and RA compared with the surrounding tissue in males than in females (Fig. 4).

As expected, Western analysis showed a band of ~37 kDa in each zebra finch brain as in the mouse, and no labeling was seen when the primary antibody was preadsorbed with the peptide against which it was raised (data not shown). Relative expression of the SCAMP1 protein was increased in the forebrain of males compared with females ($t = 3.73$, $p = 0.004$; Fig. 5).

DISCUSSION

Results from *in situ* hybridization documented enhanced expression of SCAMP1 mRNA in the HVC and RA of 25-day-old male zebra finches. Western analysis indicated increased SCAMP1 protein in homogenates of the telencephalons males compared with females at this age, and immunohistochemistry revealed that the protein is widely expressed in the brain, including in HVC and RA. As indicated in the Introduction section, sexually dimorphic SCAMP1 gene expression was initially identified in zebra finches via a microarray screen. We tested RNA extracted from the whole telencephalon in males and females at post-hatching day 25, as in the present study, and on the day of hatching. The microarray results were verified by qPCR. Both procedures revealed expression in males 1.3–1.4 times that of females. Preliminary analyses by *in situ* hybridization suggested that SCAMP1 mRNA was visually detectable in several areas in both sexes at 25 days of age, but in the song control nuclei IMAN, RA, HVC, and Area X, it was seen only in males (Wade et al., 2005). In the present study, silver grain density was quantified in each of these regions. It was numerically greater in males

than females in all of them, with on average a 1.2-fold difference in IMAN, 1.4 in Area X, 1.7 in RA, and 2.5 in HVC, compared with no difference between the sexes (ratio of 1.0) in the control region, A. The values only reached statistical significance for RA and HVC, but clearly they mirror the previously collected data. Quantification of relative protein levels in the telencephalon as a whole using Western analysis on still another set of individuals showed a 1.7-fold increase in males compared with females. Thus a similar sex difference in protein levels exists. Collectively, the results are consistent with the idea that SCAMP1 is involved in the masculinization of forebrain song control regions, and specifically in differentiation of the forebrain components critical to motor output (HVC and RA) rather than those more involved in song learning (IMAN and Area X).

At this stage, the function of SCAMP1 in song system development is not clear. SCAMPs were initially discovered as secretory vesicle components in exocrine glands, but their distribution is very widespread. Five isoforms have been identified. While SCAMPs 2–4 are mainly produced elsewhere, SCAMP5 appears to be specific to the brain, and SCAMP1 is concentrated in synaptic vesicles. The proteins are composed of four transmembrane segments with N- and C-terminal cytoplasmic extensions. SCAMPs have been implicated in both exocytosis and endocytosis, and could also play a role in the maintenance of organellar pH and volume homeostasis (Singleton et al., 1997; Fernández-Chacón and Südhof, 2000; Lin et al., 2005). Perhaps surprisingly, SCAMP1 knock-out mice exhibit minimal deficits. No major aberrations in the brain were detected, although some change in the efficiency of exocytosis in mast cells was noted (Fernández-Chacón et al., 1999). However, given the similarity in distribution with SCAMP5, it is possible that there is some functional redundancy. Alternatively, it may be that SCAMP1's role in the brain during development and/or adulthood is more pronounced in systems other than in the mouse model tested or that further characterization of the knock-out mice would provide additional deficits.

To our knowledge, neither the function of SCAMP proteins in birds nor potential sex differences in expression in any model system have been investigated. However, based on what is known about the development of the song control regions in which SCAMP1 mRNA is increased in juvenile males, some hypotheses can be generated. For example, beginning about 30 days after hatching, females show increased cell death in RA compared with males. In contrast, in HVC more neurons are added in males than females, such that cell number becomes sexually dimorphic there by day 20 (Kirn and DeVoogd, 1989). Although the sex difference in cell number in HVC is largely due to this increased addition of neurons in males (Nordeen and Nordeen, 1988), females exhibit significantly more pyknotic cells in the region during the relatively restricted time period of post-hatching days 20–30 (Kirn and DeVoogd, 1989). These data suggest that some neuronal death also is involved in creating the sexual dimorphism in this brain area.

In RA, the increased cell death in females occurs largely after the sex difference in SCAMP1 expression was detected. As we only investigated post-hatching day 25, we do not yet know the time-course of its expression. Obviously, it will be important to learn more, but the available information suggests that the diminished SCAMP1 mRNA in the RA of females is not due to prior loss of SCAMP1-expressing cells. Rather, the data are consistent with the idea that either an equivalent number of individual cells express SCAMP1 in the two sexes with less per neuron in females, or a smaller proportion of neurons express the gene in females compared with males. Either way, it is possible that fewer cells survive in the female RA because they express SCAMP1 insufficiently. The immunohistochemical procedure we used to identify the protein is not amenable to quantification of level per cell. However, based on the timing, the fact that most if not all brain cells seem to express SCAMP1, and the somewhat lighter labeling in cells in RA in females compared with males, the former seems a more likely possibility.

The increased pyknotic cells in the female HVC at 20–30 days of age (Kirn and DeVoogd, 1989) coincide with the decreased expression of SCAMP1 detected in the present study, and is thus also consistent with the idea that SCAMP1 influences neuronal survival. However, we cannot be sure that the decreased expression is not due to prior cell loss, as we did not quantify SCAMP1 mRNA before day 25. Still, our analysis is in the heart of the relatively restricted period when females show some enhanced cell death, so it is tempting to consider the idea that SCAMP1 availability influences that process. Similarly, as males have an increased number of HVC cells by day 20, we do not know whether SCAMP1 influences this characteristic or more passively reflects the addition of SCAMP1-containing cells. If it is actively involved in masculinization of HVC, this gene could be involved in the migration or perhaps differentiation of neurons. However, considering the results from RA and HVC together, along with what is known about the mechanisms creating the sex difference in cell number in these areas, the collective picture suggests that SCAMP1 could function to promote cell survival.

How might that occur? Although it would not be prudent to speculate much at this stage, it is at least worth considering the possibility that SCAMP1 may be involved in a pathway through which brain-derived neurotrophic factor (BDNF) masculinizes the song system. High affinity receptors for this protein (tyrosine kinase B; trkB) are required for neuron survival, as well as normal axon growth, synaptogenesis, and the functional maturation of presynaptic machinery, including development of the appropriate number of synaptic vesicles and/or normal endo- and exo-cytotic cycles (Chao and Hempstead, 1995; Martinez et al., 1998). Similarly, exercise modulates levels of the synaptic vesicle-associated proteins synapsin I and synaptophysin in the adult rodent hippocampus via BDNF (Vaynman et al., 2006). Thus, some precedent exists for an association between trkB/BDNF and synaptic vesicle function. TrkB is on the zebra finch Z sex chromosome (males are homozygous, ZZ, whereas females are heterozygous, ZW; Chen et al., 2005). It is expressed in the developing forebrain and, at least at some ages, exhibits increased expression in the HVC and RA of males compared with females (Dittrich et al., 1999; Wade, 2000). BDNF is also expressed in the HVC of developing males, including about a third of the neurons that project to RA (Dittrich et al., 1999). Estradiol, which can masculinize the song system, increases BDNF expression in the juvenile HVC (Dittrich et al., 1999). Similarly, the effects of testosterone on facilitating the survival of newly generated neurons in the adult canary HVC are mediated through BDNF (Rasika et al., 1999). While the effect of trkB on SCAMP1 is presently unknown, SCAMP1 is phosphorylated on tyrosine residues by the epidermal growth factor receptor (Wu and Castle, 1998). Thus, it might be advisable to determine whether trkB has a similar effect on SCAMP1. If so, it would be consistent with the idea that BDNF facilitates neuronal survival at least in part via this mechanism. It is also conceivable that a different association might occur, with SCAMP1 increasing neuron survival through BDNF by increasing its transport from HVC to RA. However, this seems less likely as differentiation of RA does not require input from its afferent structures, IMAN and HVC (Burek et al., 1995).

Although clearly many details regarding timing and process need to be investigated, the available evidence promotes the hypothesis that SCAMP1 is involved in masculinization of motor portions of the forebrain song control circuit. Increasing our understanding of the specific ways in which it is involved will facilitate the understanding of the molecular mechanisms regulating sexual differentiation of the nervous system.

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Zebra finch	MSDFDSNPFA	DPDLNNPFKD	PSVTQVTRNV	PPGLDEY.NP	FSDSRTPPPG
Mouse	MSDFDSNPFA	DPDLNNPFKD	PSVTQVTRNV	PPGLDEY.NP	FSDSRTPPPG
Pig	MSDFDSNPFA	DPDLNNPFKD	PSVTQVTRNV	PPGLDEY.NP	FSDSRTPPPG
Human	MSDFDSNPFA	DPDLNNPFKD	PSVTQVTRNV	PPGLDEY.NP	FSDSRTPPPG
Chicken	~~~~~	~~~~~	~~~~~m	PPGivslpsv	laavvraPPG
Zebra finch	51				100
Mouse	NVKMPNVpST	QPAIMKPTEE	PSSYTQIAQE	HALAQAEllK	RQEELERKAA
Pig	NVKMPNVpST	QPAIMKPTEE	hpayTQItke	HALAQAEllK	RQEELERKAA
Human	NVKMPNVpST	QPAIMKPTEE	hpayTQIAke	HALAQAEllK	RQEELERKAA
Chicken	NVKMPNVpST	QPAIMKPTEE	hpayTQIAke	HALAQAEllK	RQEELERKAA
Zebra finch	101				150
Mouse	ELDRREREMQ	SLNQGGGRKN	NWpPLpENFP	VGpCFYQDFS	VDIPVEFQKT
Pig	ELDRREREMQ	nLsQh.GRKN	NWpPLpENFP	VGpCFYQDFS	VDIPVEFQKT
Human	ELDRREREMQ	nLsQh.GRKN	NWpPLpENFP	VGpCFYQDFS	VDIPVEFQKT
Chicken	ELDRREREMQ	SLNQGG....			
Zebra finch	151				200
Mouse	VKIMYYLWMF	HTVTLFLNIF	GCLAWFYVDA	TRGVDFGLSI	LWFLFLTPCS
Pig	VKIMYYLWMF	HaVTLFLNIF	GCLAWFcVDs	sRaVDFGLSI	LWFLFLTPCS
Human	VKIMYYLWMF	HaVTLFLNIF	GCLAWFcVdp	sRGVDFGLSI	LWFLFLTPCS
Chicken	v	HTVTLFLNIF	GCLAWFYVDA	TRGVDFGLSI	LWFLFLTPCS
Zebra finch	201				250
Mouse	FVCWYRPLYG	AFRSdSSFRF	FVFFpFYICQ	FAVHVLQAAG	FQRWNGCGWI
Pig	FVCWYRPLYG	AFRSdSSFRF	FVFFpFYICQ	FAVHVLQAAG	FhnWNGCGWI
Human	FVCWYRPLYG	AFRSdSSFRF	FVFFpFYICQ	FAVHVLQAAG	FhnWGiGWI
Chicken	FVCWYRPLYG	AFRSdSSFRF	FVFFpFYICQ	FAVHVLQAAG	FQRWNGCGWI
Zebra finch	251				300
Mouse	SSLTGLNKSI	PVGIMMVIIA	ALFTASAVIS	LVMFKKVHGL	YRTTGASFEK
Pig	SSLTGLNKSI	PVGIMMIIIA	ALFTASAVIS	LVMFKKVHGL	YRTTGASFEK
Human	SSLTGLNqSI	PVGIMMIIIA	ALFTASAVIS	LVMFKKVHGL	YRTTGASFEK
Chicken	SSLTGLNKSI	PVGIMMIIIA	ALFTASAVIS	LVMFKKVHGL	YRTTGASFEK
Zebra finch	301				340
Mouse	AQQEFATGVM	SNKTVQTAAA	NAASTAATSA	AQNAFKGNRM	
Pig	AQQEFATGVM	SNKTVQTAAA	NAASTAATSA	AQNAFKGNqM	
Human	AQQEFATGVM	SNKTVQTAAA	NAASTAAsSA	AQNAFKGNqi	
Chicken	AQQEFATGVM	SNKTVQTAAA	NAASTAATSA	AQNAFKGNRM	

Figure 1.

Comparison of predicted amino acids in three mammalian species and chicken to the zebra finch. Those that differ from the zebra finch are indicated with lower case letters. The available sequence for the chicken does not cover the entire protein coding region.

Figure 2.

SCAMP1 mRNA in the forebrain of 25-day-old zebra finches. Analyses of the percent area covered by silver grains were conducted for regions in which expression had been previously detected (see text for details), including the lateral magnocellular nucleus of the anterior nidopallium (IMAN), the portion of the medial striatum that in males contains Area X, HVC (used as a proper name), and the robust nucleus of the arcopallium (RA). As a control, silver grains were also assessed in a region of the arcopallium (A) outside of RA. Overall, expression was increased in males compared with females, and differed among brain regions only in males.

* indicates significant difference between the sexes within brain area.

Figure 3. SCAMP1 mRNA in the song control nuclei HVC and RA. Darkfield images from each brain region and some surrounding tissue are depicted from representative males and females. In addition to the tissue labeled with the antisense probe, adjacent tissue sections incubated with the sense probe are shown to indicate the level of background. Scale bars: 500 μ m for each brain region.

Figure 4. SCAMP1 protein expression in HVC and RA, as detected with immunohistochemistry. Arrows in each of the low magnification images indicate the borders of the song nucleus. The small box includes a high magnification image of a cell from the male's HVC. Scale bars: 100 μ m for the lower magnification photographs of HVC and RA, and 5 μ m for the insert.

Figure 5. Quantification of protein expression in the forebrain of males and females from a Western blot. The optical densities of bands representing SCAMP1 and ZENK were obtained for each individual and a ratio was calculated. Values represent the mean + standard error of six birds of each sex; * $p = 0.004$. Representative bands for each protein are shown below the histogram.