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Sexually Dimorphic and Developmentally Regulated Expression of Tubulin Specific Chaperone Protein A in the LMAN of Zebra Finches

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Abstract

Sex differences in brain and behavior exist across vertebrates, but the molecular factors regulating their development are largely unknown. Songbirds exhibit substantial sexual dimorphisms. In zebra finches, only males sing, and the brain areas regulating song learning and production are much larger in males. Recent data suggest that sex chromosome genes (males ZZ; females ZW) may play roles in sexual differentiation. The present studies tested the hypothesis that a Z-gene, tubulin specific chaperone protein A (TBCA), contributes to sexual differentiation of the song system. This taxonomically conserved gene is integral to microtubule synthesis, and within the song system, its mRNA is specifically increased in males compared to females in the lateral magnocellular nucleus of the anterior nidopallium (LMAN), a region critical for song learning and plasticity. Using *in situ* hybridization, Western blot analysis, and immunohistochemistry, we observed effects of both age and sex on TBCA mRNA and protein expression. The transcript is increased in males compared to females at three juvenile ages, but not in adults. TBCA protein, both the number of immunoreactive cells and relative concentration in LMAN, is diminished in adults compared to juveniles. The latter was also increased in males compared to females at post-hatching day 25. With double-label immunofluorescence and retrograde tract tracing, we also document that the majority of TBCA+ cells in LMAN are neurons, and that they include RA-projecting cells. These results indicate that TBCA is both temporally and spatially primed to facilitate the development of a sexually dimorphic neural pathway critical for song.

Keywords

sex difference; song system; neural development; sex chromosome

Sex differences in brain structure and function exist across vertebrates. At the most basic level, sex chromosomes are responsible. For example, in rodents a Y chromosome gene controls testis formation. Secretion of testosterone, following metabolism into estradiol, then masculinizes reproductive behavior and brain morphology. Recent work has also implicated

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more direct effects of sex chromosomes on neural development (Lenz et al., 2012). However, information about the roles of specific genes, both those acting downstream of steroid hormones and those with potentially independent masculinizing actions on brain and behavior, are largely unknown.

Zebra finches provide a terrific model for elucidating these factors. Only males sing (Zann and Bamford, 1996). Area $\times$ and the lateral magnocellular nucleus of the anterior nidopallium (LMAN) are critical for song learning (Brenowitz et al., 1997). HVC and the robust nucleus of the arcopallium (RA) are required for its production. These two regions contain more and larger cells in males; Area $\times$ is undetectable in females (Wade and Arnold, 2004). LMAN is similar in size between the sexes, but its projection to RA is sexually dimorphic (Nordeen et al., 1992).

Microarray screens and follow-up studies identified sex chromosome genes (males = ZZ, females = ZW) that may contribute to song system differentiation (Wade et al., 2004, Wade et al., 2005, Tang and Wade, 2006, Tang et al., 2007, Tang and Wade, 2009, Tomaszycski et al., 2009, Tang and Wade, 2010, Wu et al., 2010, Tang and Wade, 2012). Tubulin specific chaperone protein A (TBCA) is one such Z-gene. This molecule is a chaperone critical for microtubule formation (Tian et al., 1996), structures essential for a variety of cellular functions, including transport and axon growth (Conde and Caceres, 2009). While present in a variety of brain regions, in the song circuit increased expression in males compared to females is detected specifically in LMAN at post-hatching day 25 (D25; Qi et al., 2012). Transport of neurotrophins from LMAN to RA regulates RA cell survival (Johnson et al., 1997). Thus, increased TBCA may facilitate masculinization within LMAN and also RA, in part by supporting the projection between these areas.

The present study quantified TBCA mRNA and protein in LMAN at four ages to elucidate potential roles in developmental events. LMAN neurons project to RA by D15 and likely D12 (Mooney and Rao, 1994); analysis at D12 was designed to capture this feature. D25 replicates our earlier study (Qi et al., 2012), and is early in the period when males form memories of a tutor song. Additionally, between D25 and adulthood, females lose a significant number of RA-projecting LMAN neurons while males do not (Nordeen et al., 1992). D45 is during sensorimotor integration, when males match their song to the stored template. Around this time, LMAN soma size increases in males and decreases in females (Nixdorf-Bergweiler, 1998). By D100, song is stable and sexual differentiation of the neural song system is complete. To facilitate interpretations, double-label immunofluorescence was employed to determine whether TBCA+ cells in LMAN are neurons, and retrograde tracing assessed whether they project to RA.

2. Experimental Procedures

2.1 Animals and Tissue Collection

Male and female zebra finches were reared in walk-in colony cages, each of which contained approximately 5-7 male and female pairs with their offspring. Animals were exposed to a 12:12 light: dark cycle, and provided free access to drinking water, seed (Kaytee Finch Feed; Chilton, WI), gravel and cuttlebone. Their diets were supplemented weekly with spinach, oranges and hard-boiled chicken eggs mixed with bread.

Evaluation of mRNA was done by *in situ* hybridization. Individuals of both sexes were collected during development, at D12, D25, D45, and in adulthood (over 100 days of age). Based on the results from that analysis (see below), protein was investigated in males and females at D25 and in adulthood by immunohistochemistry and Western blot analysis. All birds were rapidly decapitated, and whole brains were frozen in methyl-butane. Samples

were stored at -80°C until processing. Adults were sexed using plumage characteristics; males have black and white stripes on their necks and orange cheek patches that females do not possess. The sex of younger birds was determined by examining the gonads under a dissecting microscope at the time of euthanasia. All procedures were approved by The Institutional Animal Care and Use Committee (IACUC) of Michigan State University.

2.2 *In Situ* Hybridization

Brains ($n=4$ per age per sex) were coronally sectioned at $20\mu\text{m}$ and thaw-mounted in six series onto SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA). Tissue was stored at -80°C with desiccant until processing. Two adjacent sets of tissue sections (one for antisense and one for control, sense probes) were used. Due to the large number of slides, tissue was processed in two runs, each of which contained the same number of animals from each group and followed an identical protocol.

The probes and procedures were the same as in Qi et al., (2012). Briefly, the tissue was rinsed in phosphate buffered saline (PBS), fixed in 4% paraformaldehyde, and dehydrated in a series of ethanols. The slides were then pre-hybridized for 2 hours and exposed to ^{33}P -UTP-labeled RNA probes overnight. The tissue was washed in saline-sodium citrate (SSC) buffers to remove excess probe and dehydrated in a series of ethanols. Slides from one randomly selected animal per group were initially exposed to autoradiography film (HyBlot CL, Denville Scientific Inc, Metuchen NJ) for four days for qualitative assessment of the pattern and level of signal. Slides containing tissue from all animals were then dipped in NTB emulsion (Eastman Kodak, Rochester, NY) and stored in the dark at 4°C for 5 weeks. They were developed using Kodak Professional D-19 Developer and Fixer (Eastman Kodak, Rochester, NY) and lightly counter-stained with cresyl violet to facilitate identification of the anatomy.

All analyses from emulsion coated slides were completed without knowledge of each animal's sex or age. LMAN was first located using brightfield microscopy and then captured in darkfield using Image J (National Institutes of Health). The threshold function (default settings) was used on these images to manually define the silver grains within a 0.052mm^2 box placed in the center of LMAN. The software calculated the area covered by this labeling, and a percentage of the analyzed region was calculated. This procedure was used in each section containing LMAN on both the left and right sides of the brain. Background labeling from neighboring sense-treated tissue was subtracted from values obtained from sections exposed to the antisense probe, and the resulting numbers were averaged within individuals (as in Qi et al., 2012).

A 2-way ANOVA was used (SPSS 19.0, IBM, Armonk, NY) to analyze the effects of sex and age on the percent area covered by silver grains in LMAN. Following a significant interaction between these variables (see Results), 1-way ANOVAs tested effects of age within sex, and Tukey's HSD was used to analyze differences among the ages as appropriate. Finally, planned comparisons with Bonferroni corrections were used to test for sex differences within each of the four developmental stages ($\alpha = 0.0125$).

2.3 Western Blot Analysis

Brains ($n=6$ per age per sex) were coronally sectioned at $300\mu\text{m}$, thaw-mounted onto SuperFrost Plus slides (Fisher Scientific, Hampton, NH), and stored at -80°C until collection of LMAN. Sections were kept cold on a metal platform surrounded by dry ice under a dissecting microscope. LMAN was removed using a 17-gauge micropunch (Stoelting, Wood Dale, IL). For each individual, 3 punches were obtained from the center of LMAN on each of the left and right sides. LMAN was readily visible in the frozen sections

based on its opaqueness compared to surrounding landmarks. The 6 punches maximized the tissue collected within the borders of LMAN and included little, if any, from outside (Figure 1). They covered the rostrocaudal extent of the nucleus and were placed consistently across individuals. The punches were immediately put into cold RIPA lysis buffer (Santa-Cruz Biotechnology, Santa Cruz, CA) and homogenized using a sonic dismembrator (Fisher Scientific, Pittsburgh, PA). They were centrifuged at $10,000 \times g$ for 10 minutes at 4°C . The protein supernatants were collected, and an aliquot of each sample was quantified using a Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). The remainder of the samples was stored at -20°C until processing.

Samples were divided among three gels, each of which contained the same number from each group and a Kaleidoscope pre-stained standard (Bio-Rad Laboratories, Hercules, CA). For each gel, total protein ($30\mu\text{g}$ per sample) was loaded into 4-20% mini-protean TGX gels (Bio-Rad Laboratories, Hercules, CA). Separated samples were then electrophoretically transferred to PVDF membranes (Millipore, Billerica, MA) at 4°C . Membranes were cut in half so that TBCA (13kDa) and the loading control, actin (43kDa), could simultaneously be probed.

Each membrane was incubated in 5% non-fat milk for 1 hour at room temperature to block non-specific binding. TBCA was detected with a rabbit polyclonal primary antibody ($2\mu\text{g}/\text{ml}$, #SAB1100935 Sigma-Aldrich, St. Louis, MO) at 4°C for 24 hours. This antibody was designed for human TBCA, but the sequence is 93% identical to zebra finch. Membranes were then exposed to a goat anti-rabbit HRP-linked secondary antibody (1:5000, #7074 Cell Signaling Technology Inc., Danvers, MA) at room temperature for 1 hour. Actin was detected by the same procedures with a goat polyclonal primary antibody ($1\mu\text{g}/\text{ml}$, #SC-1615 Santa-Cruz Biotechnology, Santa-Cruz, CA) and a donkey anti-goat HRP-linked secondary ($1\mu\text{g}/15\text{ml}$, #SC-2020 Santa-Cruz Biotechnology, Santa Cruz, CA). After washes in PBS, blots were processed for enzyme-linked chemiluminescent detection (ECL Plus, GE Healthcare, Pittsburgh, PA), followed by exposure to autoradiography film (HyBlot CL, Denville Scientific Inc, Metuchen NJ). Film was developed in Kodak Professional D-19 Developer and Fixer (Eastman Kodak, Rochester, NY).

Validation of the TBCA antibody was accomplished by comparison to mammalian tissue and omission of the primary antibody. While it would be ideal to also preadsorb the antibody with the peptide against which it was raised, that peptide is not commercially available. Labeling of the intended protein is strongly suggested by the facts that (1) one distinct band of 13kD was detected as expected from each zebra finch sample used in the present study, (2) simultaneous comparison of whole female rat telencephalon and two D25 male zebra finch LMAN samples produced identical results, and (3) labeling was eliminated with omission of the antibody (Figure 2).

For each experimental animal, labeling for TBCA and actin was analyzed using Image J under constant lighting conditions (as in Tang et al., 2007, Tang and Wade, 2012). A box was sized to fit the center of the smallest band for each blot and used across each of the samples for determination of optical density. Background was quantified by placing the same box immediately beneath each corresponding band; this value was subtracted from each sample before analysis. A ratio of TBCA to actin labeling was calculated for each animal, and a 2-way ANOVA (SPSS 19.0 IBM Armonk, NY) determined the effects of sex and age on this relative value. Bonferroni corrected planned comparisons were used as appropriate for pairwise analyses within age and sex ($\alpha = 0.0125$).

2.4 Immunohistochemistry

Tissue sections from six individuals of each sex at D25 and in adulthood (100+ days) were used. All tissues except those from two animals came from alternate sections of the animals used for *in situ* hybridization. Brains of these two additional animals were removed and sectioned in the exact same manner as the others. All tissue was run in two sets under identical conditions; each contained three animals from each group.

Slides were warmed to room temperature, rinsed in 0.1 M PBS, fixed in 4% paraformaldehyde for 15 min, and washed in PBS. They were treated with 0.9% hydrogen peroxide/methanol for 30 min and incubated for 30 min in 5% normal goat serum in PBS with 0.3% Triton X-100. The tissue was then incubated in the same TBCA primary antibody and in the same concentration as that used for the Western blots in 0.1 M PBS containing 0.3% Triton X-100 and 5% NGS for 24 hours at 4°C. The next day, after rinses in PBS, the tissue was incubated in a biotin-conjugated goat anti-rabbit secondary antibody (1µg/2ml, #BA-1000 Vector Laboratories, Burlingame, CA, USA) in PBS with 0.3% Triton X-100 for 2 hours at room temperature. The protein was visualized with Elite ABC reagents (Vector Laboratories, Burlingame, CA, USA) per manufacturer's instructions, including diaminobenzidine (Sigma-Aldrich, St. Louis, MO) with 0.0024% hydrogen peroxide. Slides were then rinsed in PBS, dehydrated in a series of ethanols and coverslipped with DPX (Sigma-Aldrich, St. Louis, MO). Sections exposed to the same protocol, but with the primary antibody omitted, exhibited no labeling.

LMAN was analyzed under bright field illumination using Stereo Investigator (Microbrightfield, Inc., Williston, VT, USA) without knowledge of the animal's sex or age. The optical fractionator function was used to estimate the total number of TBCA+ cells in and the volume of LMAN (as in Beck et al., 2008, Tang and Wade, 2009, Wu et al., 2010). TBCA positive cells were defined as those with a distinct neuronal morphology exhibiting a brown cytoplasmic reaction product. The borders of LMAN on one side of the brain (randomly selected) were defined by tracing its edge, and cells were manually counted in sampling sites determined by the software. The density of TBCA labeled cells was calculated by dividing the estimate of the total number by the volume of LMAN. Parameters for acceptable coefficient of errors were based on Slomianka and West (2005).

A 2-way ANOVA was used (SPSS 19.0 IBM Armonk, NY) to analyze the effects of sex and age on the estimated number and density of labeled cells. Where appropriate, planned comparisons were used with Bonferroni corrections to test for differences within age and sex ($\alpha = 0.0125$).

Specificity of effects (see Results) was determined by qualitatively assessing TBCA-labeled cell bodies in HVC and RA. An observer blind to sex and age rated this immunohistochemical signal on a three point scale (0 = no detectable cells; 1 = a few somata without a distinct border of the brain region being visible with this marker; 2 = numerous somata that cause the brain region to stand out from surrounding tissue).

2.5 Double-Label Studies

Two techniques were used to determine whether (1) TBCA+ cells in LMAN are neurons, and (2) they project to RA. First, immunofluorescence for TBCA with HuC/D was performed on D12 and D45 animals (2 per sex per age). Tissue was processed in the same manner as for the TBCA immunohistochemistry described above up to the incubation with the primary antibody. Here, the TBCA antibody concentration was increased to 4µg/ml and was applied to the tissue with the mouse HuC/D primary antibody (1µg/ml, #A21271 Molecular Probes, Eugene, OR) in 0.1 M PBS with 0.3% Triton X-100. The solution also

contained 3% NGS and 0.15% sterile glycerol. Following overnight incubation in primary antibodies, slides were sequentially incubated at room temperature in DyLight 488 goat anti-rabbit (1 μ g/ml, #DI-1488 Vector Laboratories, Burlingame, CA,) and Rhodamine Red-X-conjugated goat anti-mouse (1 μ g/5ml, #115-295-207 Jackson ImmunoResearch, West Grove, PA), secondary antibodies for TBCA and HuC/D, respectively.

Second, four D25 males received bilateral injections of the retrograde neuronal tracer Fluorogold (Fluorochrome, LLC, Denver, CO) into RA (as in Kirn and Nottebohm, 1993). Birds were deeply anesthetized with isoflurane (Abbot Laboratories, Abbot Park, IL), and a Hamilton syringe was lowered into RA at a 9° angle from vertical. In each hemisphere, 1 μ l of Fluorogold was injected over a five-minute period, at a rate 0.2 μ l per minute. Birds were allowed a 3-day survival, after which they were rapidly decapitated, and brains were collected. Tissue was coronally sectioned at 20 μ m and thaw-mounted in six series onto SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA). One set of slides was processed for fluorescent TBCA immunohistochemistry as above, with the exception that the secondary antibody was Rhodamine TRITC donkey anti-rabbit (1 μ g/ml, #711-025-152 Jackson ImmunoResearch, West Grove, PA) because it was easier to visualize in combination with the Fluorogold. To maintain consistency, TBCA is depicted in green for both this and the double-label immunofluorescence described immediately above.

Tissues from both the tract tracing and double-label immunohistochemistry were examined using a scanning confocal microscope (Olympus FluoView1000 LSM). For TBCA plus HuC/D immunofluorescence, all confocal images were captured using sequential line scanning with Argon-488 nm and HeNe-543 nm lasers at emissions of 520 nm (for TBCA) and 591 (for Rhodamine Red-X). A Z-stack was generated from the center of LMAN of one randomly selected section on one side of each brain using a 40 $\times$ oil objective. A grid of 64 squares (40 $\times$ 40 μ m each) was placed over each image, and both single and double-labeled cells were counted using stereological procedures. That is, in addition to those fully within each square, only the cells touching the top and right edges were counted, while those touching the left and bottom sides were not. The percentage of TBCA+ cells that are neurons was determined by dividing the number of double-labeled cells by the value for TBCA for each individual and multiplying by 100.

For Fluorogold plus TBCA immunofluorescence, all confocal images were captured using sequential line scanning with 405 nm diode and HeNe-543 nm lasers at emissions of 461 nm (for Fluorogold) and 591 nm (for TBCA-TRITC). A Z-stack was generated from the center of LMAN of one randomly selected section on one side of each brain using a 60 $\times$ oil objective, and images were qualitatively examined for co-localization.

3. Results

3.1 *In situ* Hybridization

Qualitative assessment of the films indicated results parallel to our earlier work which only considered expression in the song system (Qi et al., 2012). Tissue exposed to the sense probe had a homogeneous and very low level of signal across all regions. Slides exposed to the antisense probe showed increased labeling compared to the sense-treated tissue across much of the brain, excluding the striatum. Among the song nuclei, the only area that stood out from surrounding tissue was LMAN. In this region, the levels appeared far greater in sections of male compared to female tissue in juveniles.

Quantitative analysis of silver grains on emulsion-coated slides indicated that TBCA mRNA was greater in the LMAN of males than females (main effect of sex: $F_{1,24} = 55.98$, $p < 0.001$; Figure 3). A main effect of age was also detected ($F_{3,24} = 10.91$, $p < 0.001$);

expression was lower in adults compared to all other ages (all Tukey HSD, $p < 0.050$). An interaction between sex and age also existed ($F_{3,24} = 5.09$, $p = 0.007$), as TCBA mRNA differed across ages in males ($F_{3,12} = 13.00$, $p < 0.001$) but not females ($F_{3,12} = 1.05$, $p = 0.408$; Figure 4). Among males, expression in adults was significantly less than that at each of the other ages (all Tukey HSD, $p < 0.050$). TBCA mRNA was increased in males compared to females throughout development (all $t_6 = 4.31$, $p = 0.005$), but not in adulthood ($t_6 = 0.73$, $p = 0.492$).

3.2 Western blot analysis

Relative TBCA protein levels were significantly increased in males compared to females (main effect of sex: $F_{1,20} = 14.37$, $p = 0.001$). A main effect of age also existed ($F_{1,20} = 4.42$, $p = 0.048$); protein concentrations were lower in adults than juveniles. An interaction between sex and age was also detected ($F_{1,20} = 8.11$, $p = 0.010$), which reflected a sex difference that existed in juveniles ($t_{10} = 3.99$, $p = 0.003$) but not adults ($t_{10} = 0.85$, $p = 0.416$; Figure 5). Within males, expression in juveniles was 2.2 times that in adults, although this did not reach statistical significance using the conservative Bonferroni correction for multiple comparisons ($t_{10} = 2.77$, $p = 0.020$). Total protein in LMAN was equivalent between ages in females ($t_{10} = 0.83$, $p = 0.424$).

3.3 TBCA Immunohistochemistry

Within LMAN, main effects of age were detected for both the estimated total number and the density of TBCA labeled cells, (number: $F_{1,20} = 17.82$, $p < 0.001$; density: $F_{1,20} = 12.11$, $p = 0.002$; Figure 6). Both values were lower in adults compared to juveniles. Although main effects of sex were not detected (number: $F_{1,20} = 0.51$, $p = 0.483$; density: $F_{1,20} = 0.04$, $p = 0.840$), interactions between sex and age existed (number: $F_{1,20} = 6.35$, $p = 0.020$; density: $F_{1,20} = 4.82$, $p = 0.040$). Labeling in LMAN was decreased in adult compared to juvenile males (number: $t_{10} = 3.58$, $p = 0.005$; density: $t_{10} = 3.32$, $p = 0.008$; Figure 7). In females, cell number ($t_{10} = 2.52$, $p = 0.030$) and density did not differ between the two ages ($t_{10} = 1.24$, $p = 0.245$).

Qualitative analysis of two other song nuclei indicates specificity of the protein expression and the effect of age. No labeled cell bodies were detected in RA in any animal. In HVC, nine of the 24 animals had a few detectable cells. However, in none was the border of the brain region clearly distinct in the immunohistochemically labeled tissue. These animals were spread across the four groups as follows: 2 adult females, 1 D25 female, 3 adult males, and 3 D25 males. No individual received a rating of 2 for HVC, which would have indicated labeling that is substantial and distinct from surrounding tissue.

3.4 Fluorescence

The majority of TBCA+ cells also expressed Hu C/D. Values were similar across the sexes and ages, and ranged from 81% to 90% co-expression (Figure 8). The pattern of labeling in each of the four D25 males injected with Fluorogold in RA was very similar; TBCA was detected in the majority of the back-filled LMAN cells (Figure 9).

4. Discussion

4.1 Summary

The present study identified sexually dimorphic and developmentally regulated expression of TBCA in the LMAN of zebra finches. TBCA mRNA was selectively increased in males compared to females at D12, D25, and D45. This sexual dimorphism was no longer detected in adult birds, when expression in males was reduced to the level in females. Relative TBCA protein concentration in LMAN was also greater in males than females at D25 and lower in

adults than juveniles. The estimated number and density of TBCA immunoreactive cells were decreased in adults as well, although they did not exhibit a significant sex difference at D25.

The discrepancy in results from juveniles using the two methods of detecting protein in the present study suggests that similar numbers of cells may express TBCA in the male and female LMAN at D25, but the amount per cell is increased in males. It is also possible that the number of cells expressing TBCA protein is functionally increasing in males at this developmental stage. On average, the number of TBCA+ cells is more than 50% greater in juvenile males compared to females. However, a fair amount of variability existed in this sample of males, possibly due to the developmental trajectories of these particular birds. While the number of animals is not sufficient to analyze quantitatively, the confocal images from the TBCA plus HuC/D double-labeling are consistent with both of these ideas (Figure 8).

We also considered the possibility that the difference reflects the types of cells included in analyses by immunohistochemistry and Western blot. That is, in the former we targeted TBCA-labeled cells exhibiting a neuronal morphology whereas in the latter, protein from all cells was included in the LMAN punches. Our analysis of mRNA using silver grains showed increased TBCA in developing males, and also did not consider cell type. Thus, the sex differences we detected at D25 could reflect increased expression in glial cells, which contain microtubules (Richter-Landsberg, 2008, Kreft et al., 2009). This scenario, however, seems relatively unlikely as consistently more than 80% of the TBCA containing cells in LMAN were neurons (see Results), many of which appear to project to RA.

4.2 Functional Implications

The TBCA gene is highly conserved in vertebrates and is one of five tubulin chaperone proteins (TBCA-TBCE) required for proper folding of β -tubulin and α -tubulin, the basic structural units for microtubules. TBCA is unique to the β -tubulin pathway (Melki et al., 1996, Lewis et al., 1997, Lopez-Fanarraga et al., 2001). Perturbations in this family of proteins are associated with a variety of brain abnormalities in mammalian brain regions including the cortex and basal ganglia (Jaglin et al., 2009). TBCA, in particular, is vital for cytoskeletal integrity and cell viability (Nolasco et al., 2005).

Enhanced TBCA in the LMAN of developing males may facilitate sensorimotor integration in song development by providing cytoskeletal support for the projection to RA. Both males and females have projections from LMAN to RA early in development (Mooney and Rao, 1994), but females lose a portion of these between D25 and adulthood (Nordeen et al., 1992). It is possible that this loss of LMAN input is due at least in part to decreased TBCA expression in females, and results in limited communication between the anterior forebrain pathway critical to learning and the motor pathway required for normal production of song. While some evidence exists for females forming memories of tutor song (Miller, 1979), they do not go through this period of practice or develop this type of vocalization.

The integrity of the LMAN to RA projection is important for vocal plasticity during song learning. During sensorimotor integration, variable song production begins around D40 and becomes more consistent by D60 (Slater et al., 1988). Lesions or inactivation of LMAN in this period reduce song plasticity so that the vocalizations become stable prematurely, resulting in a repetitive and simplified form (Bottjer et al., 1984, Scharff and Nottebohm, 1991, Olveczky et al., 2005). Lesions of LMAN in juvenile males also alter synaptic connectivity and excitatory neuronal transmission within RA in a manner consistent with the idea that plasticity is reduced and adult song characteristics are achieved earlier than normal (Kittelberger and Mooney, 1999). Thus, LMAN input to RA may play an important role in

modulating the variability of vocalizations, thereby influencing the learning process (Sizemore and Perkel, 2008).

The male-biased expression of TBCA in LMAN may also support sex specific maturation within RA. Here, differential neuronal death contributes to the development of sex differences. Most RA neurons are born before hatching, and females, but not males, lose many of these during development (Konishi and Akutagawa, 1985, Nordeen and Nordeen, 1988, Kirn and DeVogd, 1989, Konishi and Akutagawa, 1990). The projection from LMAN to RA appears to be critical for the survival of RA neurons in developing males (Johnson and Bottjer, 1994, Johnson et al., 1997). If LMAN is lesioned in 20-day old male birds, RA loses over 40% of its neurons within six days. Infusions of brain-derived neurotrophic factor (BDNF) or neurotrophin-3 (NT-3) into RA, however, can prevent this cell death (Johnson et al., 1997). Neurotrophin transport requires an intact microtubule system (Chowdary et al., 2012). TBCA's integral role in microtubule biosynthesis could enhance transport of these trophic factors from LMAN to RA, facilitating the survival of RA cells in males. While further investigation is necessary before we can draw such a conclusion, the present data support this idea as TBCA is present in many of the LMAN neurons that project to RA, at least in males around 25 days of age.

The consistent sex difference in TBCA mRNA expression within LMAN through D45 and decline to female levels in adult males may reflect a diminished need for trophic support within RA after D45. Western blot data also suggest this possibility. While they are limited in females, HVC projections enter RA in males at about one month of age (Konishi and Akutagawa, 1985). In parallel, lesions of LMAN at D40 no longer induce RA cell death in males (Johnson and Bottjer, 1994). Thus, it is possible that the decline in TBCA transcript between D45 and adulthood reflects a reduced need of RA neurons for trophic support from LMAN, due at least in part to the presence of an intact projection from HVC.

Finally, increased TBCA expression in juvenile males might support maturation within LMAN itself, an idea not mutually exclusive from those discussed above. The frequency of spines on LMAN neurons declines in males between 35 days of age and adulthood, overlapping with the period of sensorimotor integration (Nixdorf-Bergweiler et al., 1995). As microtubules modify spine morphology (Jaworski et al., 2009), it is possible that the decline in TBCA in adults compared to juveniles facilitates this process.

4.3 Future Direct

The present results suggest the potential for specific roles of TBCA in development of neural structure and function, including maturation of song, morphology of RA and LMAN, and the projection between them. Future work should directly test these ideas by inhibiting expression of TBCA protein in males during critical phases of song system differentiation. This type of influence of a sex chromosome gene is particularly attractive to consider in birds, as dosage compensation is limited in this vertebrate group (Itoh et al., 2007). It will also be important to isolate factors directly influencing and influenced by TBCA expression. The loss of sexual dimorphism in adults detected in the present study indicates that the male-biased expression in juveniles does not depend solely on Z-gene dosage, as we would expect that to be consistent across ages. Steroid hormones should be considered. For example, it is possible that an increase in androgen availability in sexually mature males facilitates the decline of TBCA expression in their LMANs. In development, estradiol may be important. As in rodents (see Introduction), this steroid hormone can masculinize morphology of the song circuit and create the potential for singing behavior in female zebra finches. However, estradiol alone is not sufficient to fully masculinize (Wade and Arnold, 2004). One exciting hypothesis is that the hormone and sex chromosome genes work in concert to facilitate normal development of brain and behavior.

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Highlights

Tubulin Specific Chaperone Protein A is detected in LMAN of the song system.

mRNA is increased in males compared to females during development but not adulthood.

The protein is also increased in juveniles compared to adults.

This Z-gene may be involved with sexual differentiation of the song system.

Figure 1.

Example of a LMAN microdissection taken from a thionin stained coronal hemi-section of an adult male zebra finch brain. LaM = lamina mesopallialis; LPS = lamina pallio-subpallialis; LV = lateral ventricle. Scale bar = 1mm.

Figure 2.

Replicate Western blots from the whole telencephalon of an adult female rat (left lane) and LMN micropunches from two 25 day old male zebra finches (middle and right lanes). The two images on the left show the single 13 kDa band produced by the TBCA primary antibody and the 43 kDa band produced on the same blot with the actin primary antibody. The two images on the right show the lack of labeling with omission of the TBCA antibody (top) with consistent labeling of actin.

Figure 3. TBCA mRNA levels in LMAN from post-hatching day 12 through adulthood. Values represent means + 1 standard error. Main effects of sex and age, as well as an interaction between the two variables, were detected. Asterisks indicate significant effects of sex within age.

Figure 4. Darkfield images depicting representative TBCA mRNA labeling from the center of LMAN. Males exhibited greater expression than females at post-hatching days 12, 25, and 45. The magnitude of the sex difference was similar across these ages, so only post-hatching day 25 is depicted. Scale bar = 20 μ m for all photographs.

Figure 5.

Relative TBCA protein levels in LMAN. The histogram indicates mean + 1 standard error for values, corrected for actin. Main effects of sex and age, as well as an interaction between the variables, were detected. The asterisk indicates a significant difference between the sexes at post-hatching day 25, based on pair-wise comparisons. Representative images from Western blots above the bars for juvenile and adult birds of each sex indicate relative levels of TBCA and actin labeling.

Figure 6.

Quantification of TBCA immunohistochemical labeling. (A) Estimated total number of TBCA+ cells in LMAN (mean + 1 standard error). (B) Density of TBCA+ cells (mean + 1 standard error). The black asterisks above the bars indicate significant main effects of age. White asterisks indicate significant effects of age within males.

Figure 7. TBCA immunohistochemistry in LMAN. Images depict a representative post-hatching day 25 male and an adult male. Arrows indicate the lateral borders of the brain region. Inserts show higher magnification images from the center of LMAN in each photograph. Scale bar for the lower power images = 500 μ m, higher power =

Figure 8.

TBCA+ and Hu C/D labeling near the center of LMAN in a 45-day-old male (A) and female of the same age (B). These confocal images indicate substantial co-expression, with TBCA in green and Hu C/D in red; more than 80% of the TBCA+ cells are neurons in both sexes. Scale bar = 10 μ m.

Figure 9. Confocal image of LMAN cells expressing TBCA protein (green) and sending projections to RA (magenta; retrograde tracing using Fluorogold). Injections were made into the RA of four 25-day-old males, which were euthanized three days later. The pattern of labeling was very similar across all animals. Scale bar = 10 μ m.