SEXUAL DIFFERENTIATION OF THE ZEBRA FINCH NEURAL SONG CIRCUIT

By

Linda Qi Beach

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ABSTRACT

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The Australian zebra finch is an advantageous model for investigating mechanisms regulating neural structure and behavior. Males and females exhibit remarkable differences in brain morphology and in the song behavior that they subserve. Thus, we can exploit these sexually dimorphic traits to begin to understand the factors that underlie development of the nervous system. While early studies implicated a critical role of estradiol (E_2) in masculinizing both structure and function, Z-linked genes (males: ZZ; females: ZW) might also contribute.

In the experiments conducted for this dissertation, I investigated the role of one Z-gene, tubulin specific chaperone protein A (TBCA) and its potential interactions with E_2, in masculinizing the zebra finch song system. TBCA is one of several chaperone proteins involved in the formation of β-tubulin, and is critical for microtubule biosynthesis and integrity.

I show that TBCA exhibits male-biased expression in the lateral magnocellular nucleus of the anterior nidopallium (LMAN). I also find that TBCA transcript and its protein product are developmentally regulated, such that this expression is higher in juveniles compared to adults. Further, TBCA is expressed in neurons that project to an efferent target, the robust nucleus of the arcopallium (RA). While the morphology of LMAN is not particularly different between the sexes, the projection from LMAN to RA is more robust in males, and this might influence masculine

development of RA. Thus, TBCA is both temporally and spatially primed to influence sex-specific development.

TBCA expression does not appear to be modulated by E_2, as administration of this hormone did not influence TBCA mRNA quantity or stereological cell counts of TBCA+ cells in LMAN. However, treatment of males with the aromatase inhibitor, fadrozole, induced a hypermasculine phenotype in neural structures, including in the volume of LMAN, cell size in RA, and of the projection between these two regions.

Finally, TBCA knockdown in LMAN *in vivo* demasculinized these same morphological parameters in both males and females. Moreover, I did not detect an interaction between TBCA and E_2 in facilitating masculine development, nor did I observe an additive effect of the two factors. Collectively, the present body of work represents an initial effort in determining the role of a Z-gene in the development of brain and behavior. The results here serve as an important platform from which we can begin to explore the mechanisms regulating the observed effects, including those underlying cell survival and maintenance of neural projections.

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For Susie, Paul, Iris, and Drake, of course.

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KEY TO ABBREVIATIONS

Tubulin specific chaperone protein A	TBCA
Lateral magnocellular nucleus of the anterior nidopallium	LMAN
Robust nucleus of the arcopallium	RA
Estradiol	E_2
Testosterone	T
Androstenedione	AE
Dihydrotestosterone	DHT
Progesterone	P
Mitochondrial ribosomal protein S27	MRPS27
DNA excision repair protein	XPACCH
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH
Immunohistochemistry	IHC
Brain-derived neurotrophic factor	BDNF
Tyrosine kinase receptor B	Trk-B
Saline-sodium citrate	SSC
Neurotrophin-3	NT-3
1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate	DiI

INTRODUCTION

Men and women differ in numerous ways. Humans, like many other animals, exhibit sex differences in physiology, brain and behavior. In humans, the differences involving the nervous system are expressed in the susceptibility to and development of diseases, including neurodegenerative ones like Alzheimer's and Parkinson's and mental illness disorders as diverse as anorexia nervosa, schizophrenia, attention deficit hyperactivity disorder, autism spectrum disorders, and Tourette's syndrome (Becker, 2008; Arnold, 2010). This underlying risk factor for disease is gaining attention in the fields of health and medicine, and in 2001, the Institute of Medicine published a paramount report titled, "*Exploring the Biological Contributions to Human Health: Does Sex Matter*?"

Sex differences in the brain are mediated by various factors including genetics, hormones, and environment. The influence of environment is now becoming more relevant than ever before due to the increasing interest in the role of epigenetic changes on our genome. However, in order to begin to understand the purely biological factors that mediate cellular mechanisms underlying sex-specific development, it is beneficial to examine the root of these differences in animal models that exhibit sexual dimorphisms in brain and behavior.

The Zebra Finch Model of Sexual Differentiation

The song system of the zebra finch has become as an invaluable model for studying questions in behavioral neuroendocrinology. The neural circuitry that controls singing behavior, which is used in courtship and defense of nest sites (Zann and Bamford, 1996), is highly sexually dimorphic. Only males sing, and the brain

regions controlling this song are substantially more developed in males (Wade and Arnold, 2004).

General Characteristics of Song Development

Male zebra finches, like all songbirds, undergo distinct phases of song development (Marler, 1997). The first begins around post-hatching day 20 (D20) and involves forming a template of song from adult conspecifics, most often the father (Konishi, 1965; Immelmann, 1969; Marler, 1970; Marler and Peters, 1977). The next stage is the sensorimotor phase, starting around D35, at which time the juvenile attempts to match his vocal output to that of his memorized template. Initially, birds produce sub-song, a quiet, unstructured and variable version of the final song product. They then develop plastic song, which is louder and more structured, but still variable. Song is crystallized (acquires its final structure) by D90.

Neural Circuitry of Singing and Song Learning

Song is governed by a network of several discrete brain regions (Figure 0.1) (Nottebohm et al., 1976). This circuit includes Area X of the basal ganglia, the lateral magnocellular nucleus of the anterior nidopallium (LMAN), the robust nucleus of the arcopallium (RA), and HVC (proper name) (Reiner et al., 2004). The first two structures are critical for song learning (Nottebohm et al., 1976, 1982; Bottjer et al., 1984) and the others comprise the descending motor pathway that is required for song production. HVC projects to RA, which innervates the tracheosyringeal portion of the hypoglossal nucleus (nXIIts). This brainstem motor nucleus projects to the muscles of the vocal organ (syrinx) (Nottebohm et al., 1982). Damage to any of these

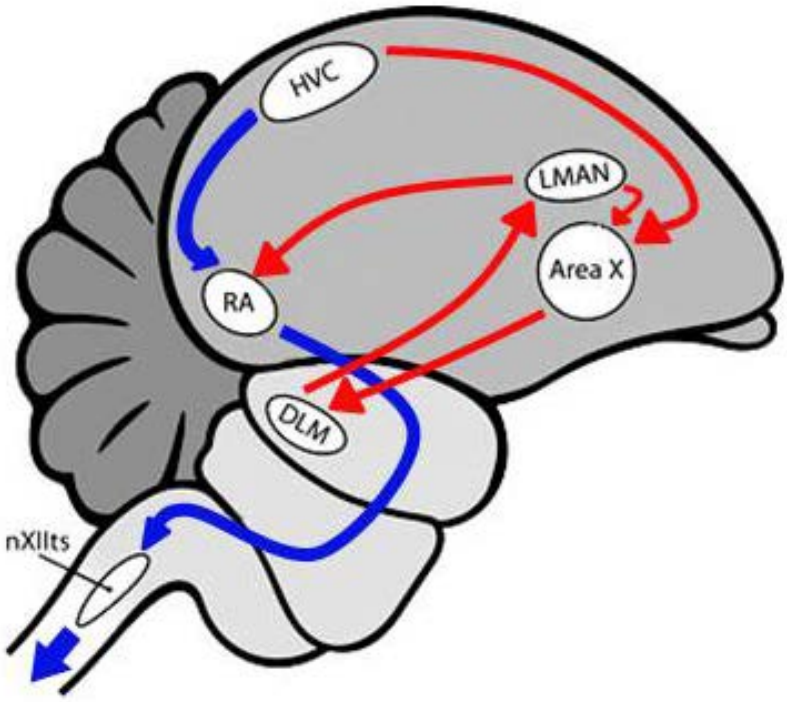

Figure 0.1 The zebra finch neural song circuit.

nuclei compromises song production (Nottebohm et al., 1976; Simpson and Vicario, 1990).

Sexual Dimorphisms in the Song Circuit

The sex difference in singing behavior is directly reflected in differences in morphology of these brain regions and their projections. HVC and RA are about five times larger in volume in males than in females, and the neuronal somata in these regions are substantially larger in males (Nottebohm and Arnold, 1976). Additionally, anatomical and electrophysiological evidence suggest that the projection from HVC to RA is greatly diminished in females (Konishi and Akutagawa, 1985; Williams, 1985).

Sex differences in both incorporation of new neurons and cell death contribute to the sex differences in the song system. In HVC, neurons are added in males to a greater degree between D20 and D55, with the number of HVC neurons in males becoming three times as many as in females by D55. Males also add a significant number of neurons to Area X during this period, and Area X does not appear as a distinct nucleus in females at all during development or adulthood (Nordeen and Nordeen, 1988). In contrast to HVC and Area X, sex differences in RA arise from differential cell loss between D20-D60 in females (Kirn and DeVoogd, 1989). Thus, sex differences in the structures of the zebra finch song system seem to result from a greater degree of neuron addition in males and a greater loss of neurons in females.

While LMAN is for the most part sexually monomorphic, sex differences do exist in its projections to RA, which synapse by D15 (Mooney and Rao, 1994). Both

males and females undergo cell death in LMAN around D20, but despite this neuron loss, the number of RA projecting neurons in males remains intact while a proportion are lost in females between D25 and adulthood (Nordeen et al., 1992). This loss of innervation from LMAN to RA in females overlaps with naturally occurring cell death in their RA (between D20 and D60) (Kirn and DeVoogd, 1989), an event that contributes extensively to the sexually dimorphic morphology of RA in adults.

Roles of Steroid Hormones in Sexual Differentiation of the Song System

Estrogen (E_2)

Early evidence suggested that the sexual differentiation of zebra finch neural structure and function is similar to that in mammals, with early, permanent influences of gonadal hormones on brain structures, followed by activation of these neural substrates later in life to mediate masculine behaviors (Wade and Arnold, 2004).

Treatment of female zebra finches with E_2 during the first few weeks after hatching masculinizes a variety of song system features, including larger volumes of RA, HVC, and Area X (Gurney and Konishi, 1980; Gurney, 1981; Simpson and Vicario, 1991; Adkins-Regan et al., 1994; Grisham and Arnold, 1995), increased cell number in these regions, larger neurons in HVC and RA (Gurney, 1981), and a more masculine projection from HVC to RA (Simpson and Vicario, 1991). Unlike normal females, adult "E_2 females" will sing if given testosterone in adulthood, and at least one study suggests early E_2 administration alone will allow females to produce male-typical vocalization (Simpson and Vicario, 1991).

Initial studies to determine the cellular mechanisms underlying E_2's ability to masculinize females found ERα expression in the telencephalon but not consistently within any of the song control nuclei (Gahr et al., 1987; Nordeen et al., 1987; Walters et al., 1988; Jacobs et al., 1996). Most of these reports, however, were based on distribution of ERα in adult songbirds, whose song systems are no longer very sensitive to the influence of E_2 (Konishi and Akutagawa, 1988). One study examined ERα mRNA and aromatase mRNA expression in the song control nuclei of animals during early post-hatching development, when the masculinizing effects of E_2 on females are most potent. Cells expressing ERα were found in HVC and RA, and high expression of aromatase mRNA was detected in both the lateral and medial MAN, and along their projections to RA and HVC, respectively (Jacobs et al., 1999).

The importance of trans-synaptic effects of E_2 in the song system has also been documented in two studies- one that showed the attenuation of E_2's masculinizing effects on Area X and RA in females when HVC was lesioned (Herrmann and Arnold, 1991); and another which demonstrated that localized brain implants of estrogen closer to HVC, compared to implants further away, causes morphological masculinization of HVC, RA and LMAN (Grisham et al., 1994). Thus, there is strong support for the idea that the masculinization of the song system is modulated by early post-hatching exposure to E_2, acting either at specific song nuclei, or acting at nearby ones via trans-synaptic signaling.

Androgen

The effects of androgens on development of the song system are less robust than those of estrogen. One study that assessed the masculinizing effects of a variety

of hormones, including E_2, testosterone (T), androstenedione (AE), and progesterone (P) in female hatchlings, found that while T can masculinize some measures of the female song system (volume, soma size), the effects of E_2 are more potent (Grisham and Arnold, 1995). And when dihydrotestosterone (DHT), a non-aromatizable metabolite of T, is administered to hatchling females, it induces only small increases in volume and cell number in RA (Schlinger and Arnold, 1991a), suggesting that most, if not all, masculinizing effects of T treatment in females are attributable to the action of E_2, aromatized from T.

Furthermore, combined treatment of females from hatching to adulthood with E_2 and DHT is no more effective than E_2 treatment alone at masculinizing morphology of the song system (Jacobs et al., 1995). Additionally, treatment of juvenile males with an androgen receptor antagonist, flutamide, starting when androgen receptors are first expressed in the song system, D9, to D60, only slightly de-masculinizes RA cell number, with a trend toward de-masculinizing HVC volume, suggesting that masculine development does not require activation of androgen receptors (Grisham et al., 2007).

Paradoxical Data

The studies above support the hypothesis that the masculine development of the song system occurs in part because it is exposed to E_2 during the early phases of neural development. However, some data are not consistent with this idea. For example, dimorphisms in circulating levels of gonadal steroids have not been reliably detected in developing zebra finches. One study found a higher level of E_2 in males during the first week of hatching when plasma was taken from jugular blood

leaving the brain (Hutchison et al., 1984), but other studies that examined systemic plasma did not replicate this finding (Adkins-Regan et al., 1990; Schlinger and Arnold, 1992). Circulating androgens, which could be converted to E_2 in the brain, either do not differ between males and females during development, or exhibit a female-biased sex difference (Hutchison et al., 1984; Adkins-Regan et al., 1990). Thus, plasma steroid levels do not support the idea that gonadal hormones alone drive sexual differentiation.

Furthermore, if male zebra finches are normally masculinized by their own E_2, then inhibiting estrogen action should prevent masculine development. Several anti-estrogens failed to prevent masculinization in males when administered between D20-D25, a period when E_2 has dramatic masculinizing effects in female zebra finches (Arnold, 1992). Paradoxically, treatment with these anti-estrogens hypermasculinized the neuron soma size in LMAN, RA, and HVC (Mathews and Arnold, 1990). Males castrated in the second week after hatching develop normal song (Arnold, 1975; Adkins-Regan and Ascenzi, 1990). This suggests that masculinization after the first week post-hatching does not require gonadally-derived hormones, an idea supported by the finding that the HVC to RA projection in males grows in vitro in the absence of gonads (Holloway and Clayton, 2001).

Finally, genetic females that are induced to develop functional testicular tissue have fully feminine song control nuclei and do not sing (Wade and Arnold, 1996; Springer and Wade, 1997). These studies demonstrate that functional testicular tissue alone is not sufficient to cause masculine development in genetic females, and suggest that brain sexual differentiation in zebra finches might occur independent of

gonadal secretions. Collectively, these findings suggest that while E_2 is important for masculinizing the song system, its source might be from the brain rather than from the periphery. This idea is, in fact, supported by studies that detect high levels of aromatase activity in the male zebra finch brain, suggesting that in males, the brain is an important site of estrogen synthesis (Schlinger and Arnold, 1991b, 1992).

Role of Sex Chromosome Genes

Sex Chromosomes in Birds

In diploid animals with heterogametic sex chromosomes, males and females have different numbers of copies of sex chromosome genes. In mammals, females have two X chromosomes, whereas males have one X and one Y. Potential differences in the expression of X chromosome genes, both between the sexes and relative to the autosomes, is minimized by dosage compensation (Marin et al., 2000). In birds, females are the heterogametic sex (ZW), and males are homogametic (ZZ), and dosage compensation, if any, is limited, (Itoh et al., 2007).

Thus, in zebra finches, genes on the Z chromosome are likely to be expressed at higher levels in males compared to females. Some of these include genes encoding for secretory carrier membrane protein 1, tyrosine kinase receptor B, and ribosomal proteins L17 and L37, which may be involved in a variety of aspects of song system differentiation (Wade, 2000; Chen et al., 2005; Tang and Wade, 2006; Tang et al., 2007). Thus, in zebra finches, sex differences in the expression of Z genes might underlie sex differences in brain phenotype.

Gynandromorphic Zebra Finch

The study of a rare, naturally-occurring gynandromorphic zebra finch provided evidence for such a role of Z-linked genes in determining sexual phenotype of the song system (Agate et al., 2003). This bird exhibited lateralized genotype and phenotype; it had male plumage and gonads on its right side and female plumage and gonads on its left, sharply divided along the midline. *In situ* hybridization using a W-linked ASW probe, normally expressed only in female tissues, detected expression predominantly on the left half of the brain. The Z-linked gene, PKCIZ, was expressed more on the right side of the brain.

Brain morphology was also lateralized in this bird; song nucleus HVC, in particular, was more masculine on the right side compared to the left. Since both hemispheres were exposed to a common hormonal environment, this difference is attributed to the lateral distribution of sex chromosome genes. Additionally, the genetically female side of this bird's brain was more masculine than that of control females, suggesting that it was masculinized by one or more diffusible factors from the male side of the bird, perhaps E_2. These results, together with paradoxical data described above, provide support for the idea that E_2 alone is not sufficient to masculinize the song system and that sex differences are also mediated by the actions of sex chromosome genes in the brain.

Concluding Remarks

The hypotheses that I set out to test in this dissertation address the contributing roles of E_2 and Z-chromosome genes in mediating sexual differentiation of the neural song circuit. After initial pre-liminary studies on ten Z-genes, originally

detected on a microarray (Tomaszycki et al., 2009), I decided to focus my research questions on one particular Z-gene, tubulin specific chaperone protein A (TBCA).

Summary of Dissertation Experiments

Chapter 1: Investigate the potential contributions of 10 Z-genes that showed male-biased expression in a microarray study. Real-time qPCR was used to first confirm the same pattern of expression, and *in situ* hybridization localized mRNA to song control regions (Qi et al., 2012).

Chapter 2: Time-course study of TBCA transcript and protein expression across development. Double-label immunofluorescence and retrograde tracing were employed to investigate if TBCA+ cells are neurons, and whether they project to RA (Qi and Wade, 2013).

Chapter 3: Evaluate whether E_2 modulates TBCA transcript and protein in LMAN. Determine the functional effects of inhibiting TBCA expression and assess whether TBCA modulates E_2's ability to masculinize the song system (submitted manuscript).

CHAPTER 1

Enhanced Expression of Tubulin Specific Chaperone Protein A, Mitochondrial Ribosomal Protein S27, and the DNA Excision Repair Protein XPACCH in the Song System of Juvenile Male Zebra Finches

Introduction

Sex differences in brain and behavior exist across a wide array of species. Those found in zebra finches are extraordinarily robust. Only adult male birds sing, learning songs from their fathers (Bottjer, 2002), and discrete brain regions that regulate aspects of this behavior are larger in males compared to females. Area X and the lateral magnocellular nucleus of the anterior nidopallium (LMAN) are components of the anterior forebrain pathway; a basal ganglia forebrain circuit largely devoted to song learning and plasticity (Bottjer et al., 1984; Scharff and Nottebohm, 1991; Williams and Mehta, 1999; Brainard and Doupe, 2000). Area X, while large in males, is not detectable in females (Nottebohm and Arnold, 1976). LMAN volume is sexually monomorphic, but nucleoli and neuron soma size are larger in males (Nixdorf-Bergweiler, 2001). HVC (proper name) and the robust nucleus of the acropallium (RA) are in the motor pathway controlling song production (Brenowitz et al., 1997). HVC and RA are about five times larger in volume in male than female zebra finches, and the projection from HVC to RA, which innervates the vocal organ (syrinx), is also substantially more robust in males (Bottjer et al., 1985; Konishi and Akutagawa, 1985; Arnold, 1997; Wade and Arnold, 2004).

The mechanisms regulating sexual differentiation of the zebra finch song circuit are unclear. While early data supported a role for estradiol in masculinizing structure and function (Arnold, 1997), new evidence implicates non-hormonal factors. Some of the most compelling information comes from a rare, naturally-occurring gynandromorphic zebra finch with lateralized male (ZZ) and female (ZW) genotype and phenotype. Data from this bird suggest that direct genetic effects mediate sexual differentiation of the neural song circuit, likely with an influence from diffusible factors such as hormones (Agate et al., 2003). Recently, microarray screens have identified several Z-chromosome genes that exhibit increased expression in particular song control nuclei in juvenile male compared to females zebra finches, including those encoding secretory carrier membrane protein 1, sorting nexin 2, and ribosomal proteins L17 and L37 (Tang and Wade, 2006; Tang et al., 2007; Tomaszycki et al., 2009; Wu et al., 2010).

The present study investigated expression of ten additional genes from our most recent screen (Tomaszycki et al., 2009) in an attempt to supplement our understanding of the genetic mechanisms underlying sexual differentiation of the song circuit. Increased whole telencephalic expression in males originally detected in this cDNA microarray study was first confirmed in a group of new 25-day-old (D25) birds by real-time qPCR. Those genes that consistently showed male-biased expression were then evaluated by in situ hybridization in males and females of the same age for localization to song control nuclei. This developmental stage is when males begin to memorize songs from their fathers, and represents a period of rapid morphological differentiation of the song circuit (Nordeen and Nordeen, 1997;

Doupe et al., 2004; Wade and Arnold, 2004), allowing us to maximize our chances of discovering relevant mechanisms.

Methods

Animals and Tissue Collection

Male and female zebra finches were reared in colony aviaries containing multiple males and females with their offspring. Animals were exposed to a 12:12 light:dark cycle, and provided ad libitum access to drinking water, seed (Kaytee Finch Feed; Chilton WI), gravel and cuttlebone. Their diets were also supplemented weekly with spinach, and egg-bread mixture, and oranges.

For both real-time qPCR and in situ hybridization, birds were rapidly decapitated at D25. For qPCR, whole telencephalons were collected and immediately frozen on dry ice. For in situ hybridization, whole brains were rapidly dissected and frozen in cold methyl-butane. In both cases, samples were stored at -80°C until processing. Gonadal sex of each animal was determined using a dissecting microscope at the time of euthanasia. All procedures were all approved by The Institutional Animal Care and Use Committee (IACUC) of Michigan State University.

Real-time qPCR

RNA from six male and six female telencephalons was extracted with Trizol (Invitrogen, Carlsbad, CA), and DNase treated on RNeasy columns (Qiagen, Valencia, CA) per manufacturer's instructions. RNA was ethanol-precipitated to increase the concentration, which was determined by spectrophotometry. RNA integrity was confirmed on 1% denaturing agarose gels. cDNA was made from the

telencephalic RNA samples using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) per manufacturer instructions. Primers were designed using Primer Express 2.0 (Applied Biosystems, Foster, CA; Table 1), and each pair demonstrated at least 99% efficiency in amplification using a standard curve of multiple known cDNA concentrations.

cDNA for all twelve animals, along with no-template controls, were run in triplicate with each of the ten primer sets. Each qPCR reaction used 100nM of each primer and 25ng of RNA (converted to cDNA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was run in parallel with each reaction and analyzed as a control (Tomaszycki et al., 2009) as its expression is not sexually dimorphic. Power SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA) was used according to manufacturer's instructions. All reactions were run on an ABI Prism PE 7000 (Applied Biosystems).

The triplicate threshold cycles obtained from each animal were averaged, and sex differences were analyzed using one-tailed t-tests with Bonferroni corrections. Fold-differences between the sexes were calculated using $\Delta\Delta$CT (Livak and Schmittgen, 2001), normalized using the GAPDH results.

In Situ Hybridization

Brains were coronally sectioned at 20 μm and thaw-mounted in six series onto SuperFrost Plus slides (Fisher Scientific, Hampton, NH). Tissue was stored at -80°C with dessicant until processing. Each of the ten genes of interest was initially screened on a small number of D25 males and females (n=1-3 per sex). This

GenBank Accession Number for cDNA	Forward	Reverse
CK310968*	**CAAAGTTCAAACCCCAGGACAT**	**CCTTACCCATTAGTTCCCCTTAGC**
CK302334*	**AGGTGTGGCTGAGCTAGGGAA**	**TCCTAGTTCTCTCAGCTCCGCT**
CK304617*	**GCAAGTGATGGAGAATGTGGC**	**TTACAGATTTTGATGTCCCCTGAC**
CK311563	**TGGACAAGTGCATCGGTTCA**	**TCTTTGTCGCTTTTCATCACGA**
CK303438	**CATATCACAGCATCTCTATCAAGCAA**	**ATTTCAGCAACTTATTCAGACCCAT**
CK305515	**GCAGGTGAATAGCATAAGCAAAGA**	**AAGCCATGTGATCATATAGCCAAA**
CK316377	**AAAGCTGGGAAGTGGGAGGT**	**GTTTCTCCTTGTGCACGAACC**
DV945585	**GAAGCCAAGCAGCCCAACT**	**GAGCTGCACTCTGACGCATTT**
DV958715	**TGGATGATGCCCAGGATGTT**	**CGTTGGCATGCACTTCACA**
DV957339	**TTGCCAAAAATAGCACACCCT**	**GCATAGTTCACACATCACTGGGAA**
AF255390 (GAPDH)	**AAACCAGCCAAGTACGATGACAT**	**CCATCAGCAGCAGCCTTCA**

Table 1. Primers Used for qPCR (5' to 3')

* Used for later *In Situ* hybridization studies

procedure resulted in identification of six genes with specific expression in song control nuclei such that the labeled regions stood out from the surrounding tissue (See below). Three of these genes that appeared to exhibit more labeling in males than in females as determined by visual inspection of the slides (Tubulin specific chaperone protein A [TBCA], mitochondrial ribosomal protein S27 [MRPS27], and a DNA excision repair protein [XPACCH]) were selected for more detailed investigation using larger sample sizes. For XPACCH, two adjacent sets of tissue sections (one for antisense and one for sense probes) from six individuals of each sex were used. However, due to tissue damage, one male was omitted for TBCA and one female was eliminated for MRPS27; thus each of these groups had five, rather than six, individuals. Within each gene, all of the tissue was run simultaneously.

Probes for in situ hybridization were produced from glycerol stocks (Replogle et al., 2008) of pBluescript II SK (+), and plasmid DNA was extracted using Qiagen Maxi Prep kit (Valencia, CA), and linearized with XhoI (T3) and NotI (T7). All clones were re-sequenced prior to in situ hybridization from both ends on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Cold transcription reactions confirmed each product's quality and correct size. T3 (anti-sense) and T7 (sense) cRNA probes were generated using the MAXIscript In Vitro Transcription Kit with T3/T7 RNA polymerases (Ambion, Austin, TX), and were labeled with 33P-UTP (Perkin Elmer, Waltham, MA).

The slides were warmed to room temperature for 15 minutes, rinsed in phosphate buffered saline (PBS), fixed in 4% paraformaldehyde for 15 minutes, and rinsed in PBS for 3 minutes. They were then incubated in 0.25% acetic anhydride in

0.1 M triethanolamine for 10 minutes, rinsed in PBS, dehydrated in a series of ethanols, and air-dried. The tissue was pre-hybridized in a solution containing 1X hybridization buffer (2.5M NaCl, 1M Tris, 0.5M EDTA, 1M DTT, 1X Denhardts, 1mg/ml yeast tRNA, and 50% dextran sulfate) and 50% formamide at 55°C for 2 hours. Slides were then hybridized overnight at 55°C with a solution containing 1X hybridization buffer, 10% dextran-sulfate, 50% formamide, and 5 × 106 cpm 33P-UTP-labeled RNA probe (sense or antisense). Hybridization buffer was removed with sequential washes in 4X SSC at room temperature, 2X SSC at 55°C for 10 minutes, and 2X SSC at room temperature for 30 minutes. Slides were incubated in 2X SSC with RNase A (20μg/ml) at 37°C for 30 minutes, then rinsed in 2X SSC at 37°C for 15 minutes and 0.1X SSC at 60°C for 10 seconds. The tissue was dehydrated in ethanol baths of increasing concentration with 0.3M ammonium acetate. Slides were air-dried and three animals per sex from sense and antisense probes were exposed to phosphor-imaging screens (Bio-Rad Kodak #170-7841, Hercules, CA) for 16 hours and scanned with a Molecular Imager FX (Bio-rad Laboratories, Hercules, CA) to confirm the expected signal in song nuclei. All slides were then dipped in NTB emulsion (Eastman Kodak, Rochester, NY) and stored in the dark at 4°C for 4 weeks. They were then developed using Kodak Professional D-19 Developer and Fixer (Eastman Kodak, Rochester, NY), and counter-stained with cresyl violet.

For all three genes, labeling appeared consistent across the two phosphor-imager screens and the emulsion-coated slides. However, for XPACCH, specific labeling was apparent in two regions on the slides that were not obvious on the imaging screens, so it was quantified there as well. As all four primary telencephalic

brain regions (HVC, RA, LMAN and Area X) were analyzed for this gene, an additional region, A, was quantified as a control. As in Tang and Wade (2006), "A" represents a portion of the arcopallium just lateral to RA that is not morphologically distinct or sexually dimorphic.

Each brain region was first located using brightfield microscopy (landmarks identified using the nissl stain), and then captured using Scion Image (NIH Image) in darkfield. The "density slice" function was used to quantify labeling on both sides of the brain in all sections containing each song nucleus of interest. The area covered by silver grains within a 264 μm × 198 μm box (placed in the center of each area) was calculated. For each anti-sense section, an adjacent section exposed to the sense probe was quantified. The sense values were then subtracted from the anti-sense values, and the resulting densities were averaged across sections within individuals.

The effect of sex on the percent area covered by silver grains within each brain region was analyzed separately for each of the genes by two-tailed t-tests. Bonferroni corrections were used to adjust α-levels accordingly. In one case in which variances were not homogenous, due to a bimodal distribution in males, a Mann-Whitney U test was also used (see below).

Results

Real-time qPCR

Whole telencephalic expression was significantly increased in males compared to females for nine of the ten genes (Table 2; α =0.010). GAPDH

GenBank Accession Number	Identification*	Male/Female Ratio using qPCR	qPCR t-value	qPCR p-value
CK310968	Tubulin specific chaperone A	2.16	8.39	<0.001
CK302334	DNA repair protein XPACCH	4.71	3.61	<0.003
CK304617	Mitochondrial ribosomal protein S27	2.54	3.22	<0.005
CK311563	Lsm5 protein	2.34	4.52	<0.001
CK303438	Zinc finger A20 domain	2.24	6.40	<0.001
CK305515	Autophagy protein	2.08	9.37	<0.001
CK316377	Homologue to TGF beta-inducible nuclear protein	1.94	7.02	<0.001
DV945585	Ribosomal protein S23	1.73	4.49	<0.001
DV958715	Thioredoxin	1.73	9.97	<0.001
DV957339	Rasp21 GTPase activating protein	1.1	1.01	0.169

Table 2. Male-Biased Gene Expression in D25 Zebra Finches

*From NCBI blast of nucleotide sequence

expression did not differ between the sexes in any of the ten analyses (all $t < 1.99$, $p > 0.075$).

In Situ Hybridization

For each of the three genes of interest, labeling was generally increased in males compared to females (see phosphor images in figures), consistent with what one might expect for a Z-gene, as dosage compensation in birds is limited. However, labeling within song control nuclei was selective and specific.

TBCA (Genbank accession number CK310968; 99% identity between our 715bp sequence and the zebra finch sequence from NCBI) expression was detected only in LMAN on the phosphor-imaging screen. Qualitative analysis of the slides for all males and females provided the same result, so only this region was quantified. The density of labeling in this area was significantly higher in males than in females ($t_9 = 2.71$, $p = 0.024$; Figure 1.1).

XPACCH (CK302334; 529bp, 100% identity) was detected in Area X and RA on the phosphor-imaging screen (Figure 1.2). Inspection of the tissue sections revealed a similar pattern of specific labeling that defined individual regions with substantially increased expression compared to surrounding tissue in LMAN and HVC of males. Thus, these areas were also analyzed. In each of these song nuclei, labeling was greater in males compared to females (LMAN: $t_{10} = 3.54$, $p = 0.005$; Area X: $t_{10} = 4.20$, $p = 0.002$; HVC: $t_8 = 4.46$, $p = 0.002$; RA: $t_{10} = 3.80$, $p = 0.003$; Figure 1.3). No effect of sex was detected in the control region, A ($t_{10} = 2.15$, $p = 0.057$).

MRPS27 (CK304617; 794bp, 99% identity) was detected in HVC and RA on the phosphor-imaging screen and on the slides. Using parametric analyses, a significant effect of sex was present in HVC ($t_9 = 3.31$, $p = 0.009$; Figure 1.4), but not RA ($t_9 = 1.83$, $p = 0.100$). However, a bimodal distribution existed in the RA of males, such that two birds had silver grains covering over 1% of the analyzed area, while four birds had silver grains covering less than 0.4%. A Mann-Whitney U test detected a trend for an increase in males compared to females ($U= 4.00$, $p= 0.045$).

Discussion

The present study identified selective and specific male-biased expression of three novel genes in the song control system of juvenile zebra finches: TBCA, XPACCH, and MRPS27. These genes map to the Z-chromosome, which is present in two copies in male zebra finches, but only one in females (Ellegren et al., 2007; Itoh et al., 2007). As indicated above, dosage compensation in birds is far more restricted than in mammals, so Z-chromosome genes are likely to exhibit increased expression in males compared to females. However, a variety of other direct or indirect mechanisms might cause increased expression in males compared to females. In fact, the selective nature of this pattern – the fact that sexually dimorphic TBCA, XPACCH and MRPS27 mRNAs were detected in particular song control regions and not throughout the brain – suggests that something in addition to simply their chromosomal location modulates the expression of these genes. It also supports the idea that they could contribute to particular aspects of differentiation of morphology and/or function of the song circuit.

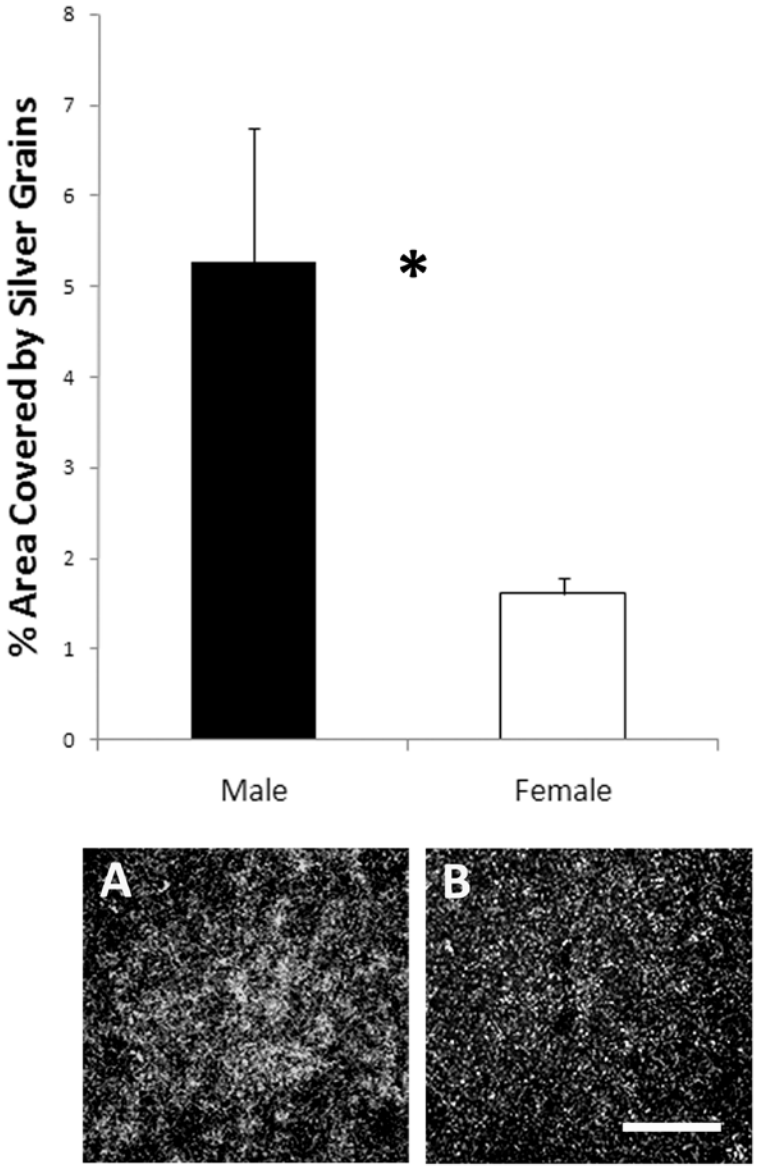

Figure 1.1 Labeling for the gene encoding TBCA was detected only in LMAN and was increased in males compared to females. The top panel represents means ± SEs; *p = 0.024. The photographs are darkfield images from near the center of LMAN in a (A) male and (B) female. Images are approximately twice the size of the area quantified. Scale bar for both darkfield images = 100μm.

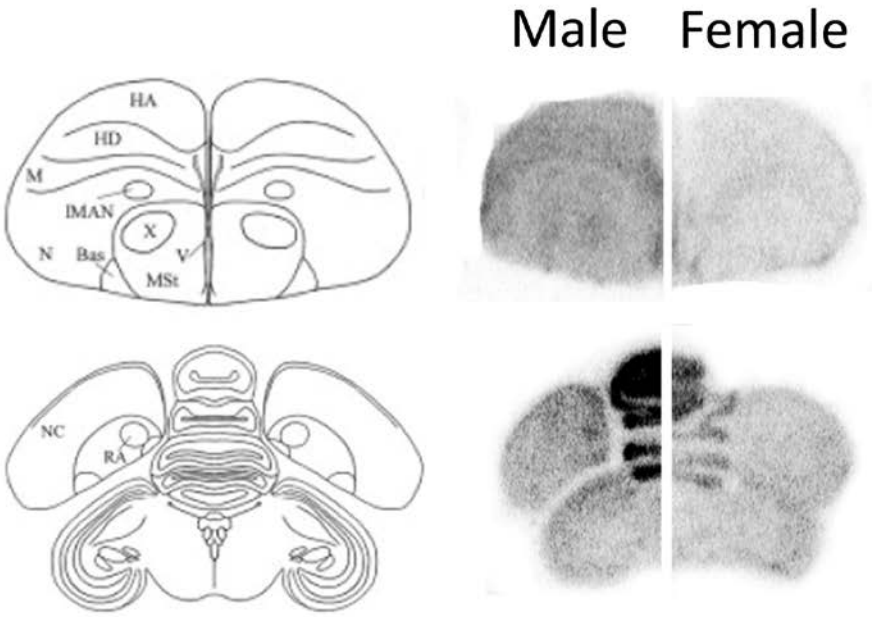

Figure 1.2 The phosphor-imaging screen detected specific, male-enhanced labeling for the gene encoding XPACCH in Area X and RA. The signal was greater overall for male compared to female brains, which can clearly be seen in the large, densely packed Purkinje cells of the cerebellum (midline layered structure in bottom images). However, only in males did it define the two song control regions.

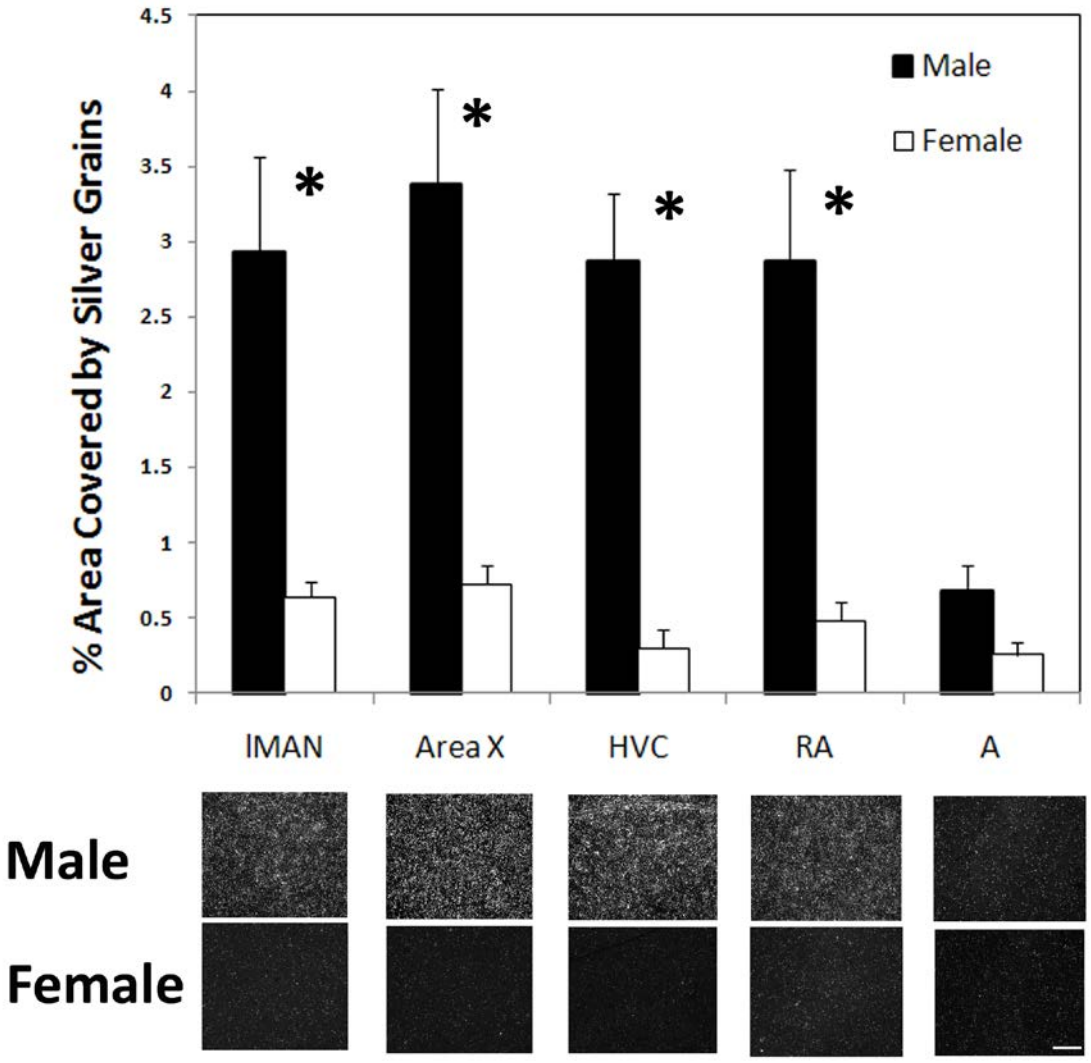

Figure 1.3 Analysis of slides indicated that the expression of the gene encoding XPACCH was specifically detected in four song control nuclei: LMAN, Area X, HVC, and RA. The last region, "A," represents a portion of the arcopallium just lateral to RA. A significant sex difference existed in each of the song control regions, but not in A (graph shows means ± SEs, *p≤ 0.005). Below the graphs are darkfield images depicting the center of each brain region from representative males and females. Scale bar = 100μm for all images.

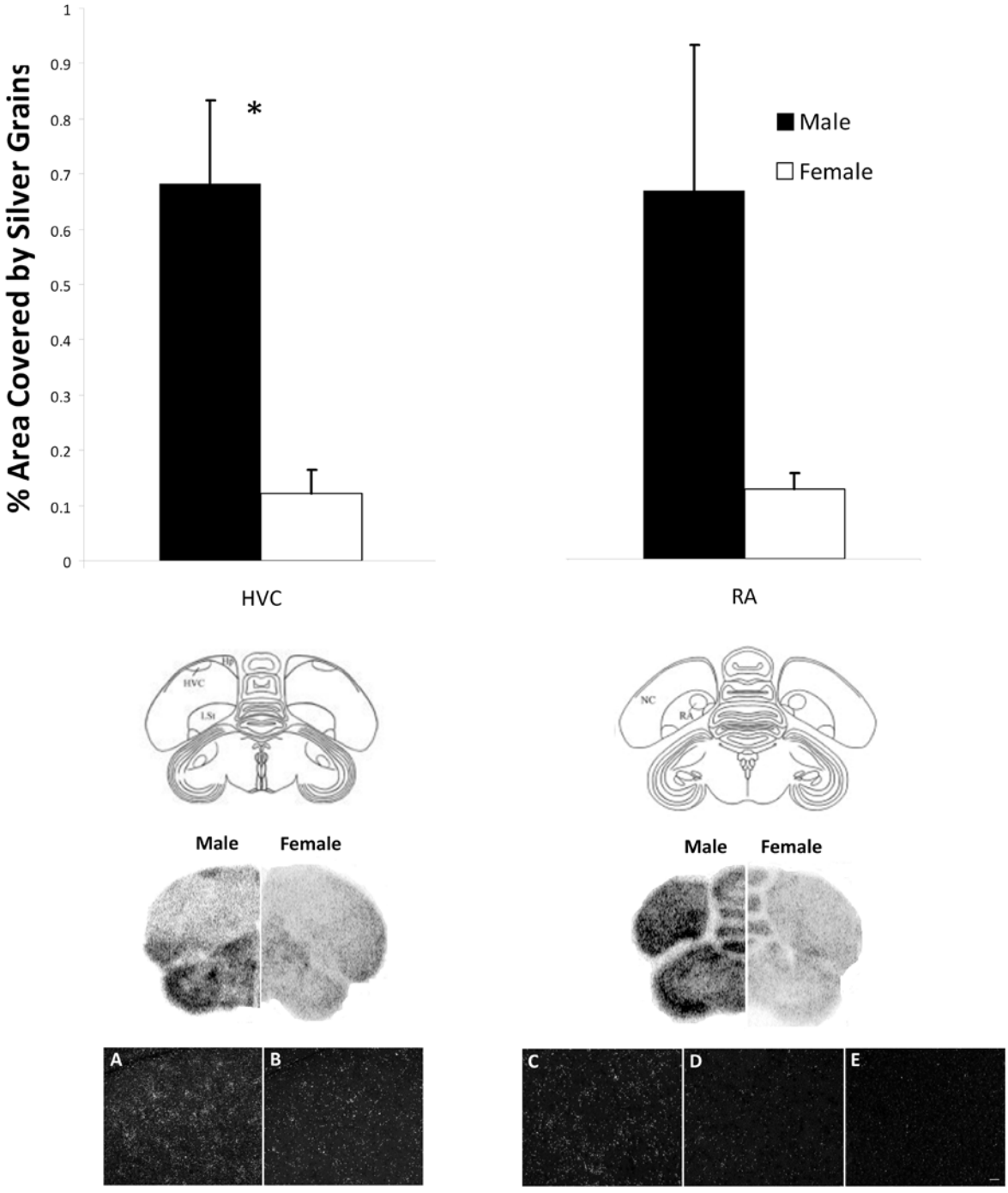

Figure 1.4 The phosphor-imaging screen (middle panels) revealed specific, male-biased labeling of the gene encoding MRPS27 in HVC and RA. Silver grain analysis (t-tests) detected a significant effect of sex only in HVC. However, a bimodal

Figure 1.4 cont'd

distribution existed in the RA of males. Photographs (bottom panels) show silver grain labeling from the center of HVC (left) and RA (right). Values on the graphs (top panel) indicate means ± SEs; *p= 0.009. A and B are darkfield images depicting labeling in a male and a female, respectively. C, D and E represent the bimodal distribution of silver grains in RA of two males (C, D) and a female (E).

TBCA

Enhanced expression of TBCA in D25 males was limited to LMAN. Its morphology, unlike other song nuclei, is not particularly sexually dimorphic (Nixdorf-Bergweiler, 2001). Differences do exist, however, in its projections. Both males and females undergo cell death in this area around 20 days after hatching (Bottjer and Sengelaub, 1989). Despite this loss, the number of RA projecting neurons remains intact in males while a proportion is lost in females (Nordeen et al., 1992). Retention of these projections may support cell survival in RA (Johnson and Bottjer, 1994). TBCA could facilitate maintenance of these projections in males compare to females. The gene is highly conserved among vertebrates and is essential to proper β-tubulin folding (Melki et al., 1996). Together with α-tubulin, β-tubulin forms the basic structural unit for microtubules, which are required for spindle formation during mitosis and meiosis, organelle positioning, and axonal transport (Dutcher, 2001).

Axonal transport of neurotrophins, in particular, has been implicated in the development of the songbird telencephalon (Johnson et al., 1997). Lesions of LMAN remove pre-synaptic input to RA, and cause extensive RA neuron death in juvenile male birds. Infusions of brain-derived neurotrophic factor (BDNF) directly into RA, however, suppress this process in RA. Further, LMAN neurons have the ability to anterogradely transport BDNF into RA, where TrkB (high-affinity receptor for BDNF) is expressed (Johnson et al., 1997). Thus, enhanced TBCA in the LMAN of males could contribute to this sexual differentiation by up-regulating β-tubulin and

microtubule formation to increase axonal transport of neurotrophins into RA from LMAN.

This protein may also influence functional development. While females appear to learn their fathers' songs in a manner similar to males (Miller, 1979), they never produce it. Thus, increased synthesis of TBCA in LMAN might also facilitate the ability of males to learn to produce quality song. The present study documents that this specific, sexually dimorphic mRNA expression exists at D25, when birds are forming templates of their tutors' songs. It will now be important to determine both whether it extends into the later period of sensorimotor integration, as well as whether the protein is also expressed in LMAN's target, RA.

XPACCH

XPACCH was detected in Area X, LMAN, HVC, and RA, and expression was increased in males in each region. While broad, these sex differences in expression are specific, as we did not detect dimorphic labeling in a control portion of the arcopallium, A. XPACCH mediates the assembly of a pre-incision complex during DNA repair, and is the gene compromised in the recessive disorder xeroderma pigmentosum, in which the ability to repair DNA damage by ultraviolet irradiation is inhibited (Cleaver, 1969). DNA repair pathways correct mutagenic and toxic DNA damage (Nyberg et al., 2002), and accumulated DNA damage in the genome may lead to a loss in the fidelity of information transferred from DNA to protein, resulting in transcription of defective proteins that eventually cause cell death (Taddei et al., n.d.; Lu et al., 2004). While specific mechanisms associated with song system differentiation are at this point unclear, it is possible that XPACCH facilitates cell

survival in males during the differentiation process. It is also possible that the increased expression in males is a result of their enhanced structure, and simply reflects an increased need for support of basic cellular processes. Further investigation of the time-course of expression in males and females will be an important step toward elucidating the function of this gene in the song circuit.

MRPS27

MRPS27 was specifically detected in two nuclei of the motor pathway. Expression was clearly enhanced in males in HVC, and a similar trend was detected in RA; it was largely driven by the high expression levels in two males. However, data from only one male was within the range of females. This result and that of the non-parametric Mann-Whitney U test, which analyzes ranks rather than absolute values, suggest that an increase in males might in fact exist. We have no obvious explanation for the variability among the males. They were raised under identical conditions, and their tissues were processed together in the in situ hybridization analysis of this gene. Because alternate sections from the brains of these birds did not exhibit a parallel pattern in analysis of TBCA or XPACCH, a biological rather than technical explanation seems likely. More work will need to be done to determine contributing factors.

MRPS27 is expressed in the mitochondrial ribosome, and thus has potential influences on the translation of proteins in cells of HVC and RA. This idea and subsequent identification of key proteins need to be evaluated. However, it is plausible as three other ribosomal proteins have been associated with song system development. RPL17and RPL37, which like MRPS27 are on the Z chromosome, as

well as RPL7 (Chromosome 2), exhibit specific male-enhanced and selective expression in song nuclei (Tang and Wade, 2006; Duncan et al., 2009; Tang and Wade, 2010). The challenge now is to determine the key proteins that may be regulated by these genes, which can broadly influence translation.

Concluding Ideas

The present study examined sexually dimorphic expression of ten genes that were originally identified from a microarray screen. Three were selected from a pre-liminary in situ hybridization screen for further investigation, based on their location in one or more song nuclei and their apparent sex differences in expression. Each was localized to the song system of D25 zebra finches, and exhibited male-enhanced mRNA expression in one or more song control nuclei. Thus, their protein products may facilitate masculinization. Influences at other critical developmental stages are also possible, and work is currently underway to evaluate the time course of the expression of TBCA, XPACCH, and MRPS27.

CHAPTER 2

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

Introduction

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 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 X 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 X is undetectable in females (reviewed in 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, 2005; Tang and Wade, 2006; Tang et al., 2007; Tomaszycki et al., 2009; Tang and Wade, 2010, 2012; Wu et al., 2010)

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 (reviewed in 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, betweenz 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.

Methods

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.

In Situ Hybridization

Brains (n=4 per age per sex) were coronally sectioned at 20μm 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 33P-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 bright-field microscopy

and then captured in dark-field 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 0.052mm2 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 ($\alpha = 0.0125$).

Western Blot Analysis

Brains (n=6 per age per sex) were coronally sectioned at 300μm, 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 2.1). 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 x 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 Laboratories, 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 (30μg 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 (2μg/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

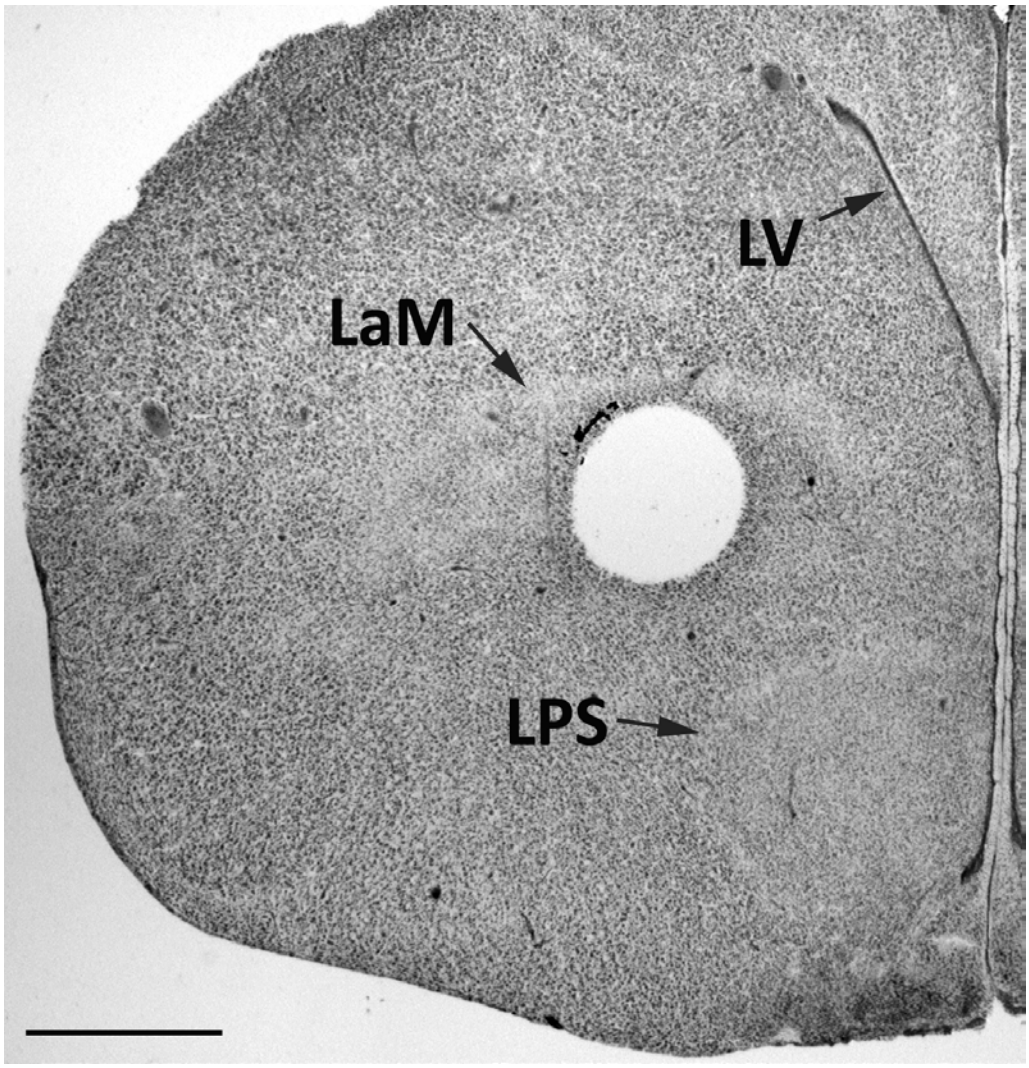

Figure 2.1 Example of a LMAN microdissection taken from a thionin stained coronal hemi-section of an adult male zebra finch brain. LaM = lamina mesopallialis; LPS = lamina pallio-subpallialis; LV = lateral ventricle. Scale bar = 1mm.

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 (1μg/ml, #SC-1615 Santa-Cruz Biotechnology, Santa-Cruz, CA) and a donkey anti-goat HRP-linked secondary (1μg/15ml, #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 2.2).

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

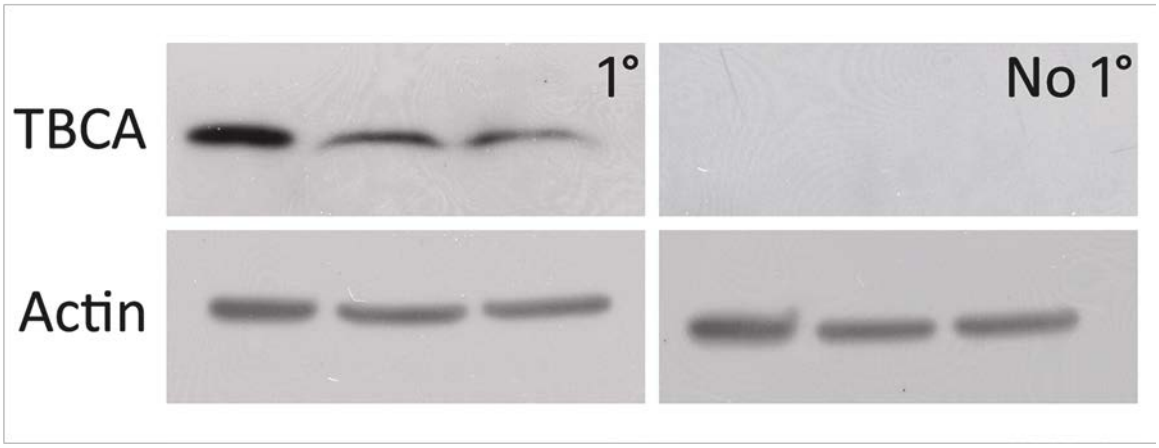

Figure 2.2 Replicate Western blots from the whole telencephalon of an adult female rat (left lane) and LMAN micropunches from two 25 day old male zebra finches (middle and right lanes). The two images on the left show the single 13 kDa band produced by the TBCA primary antibody and the 43 kDa band produced 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.

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 ($\alpha = 0.0125$).

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 (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).

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 (1μg/ml, #DI-1488 Vector Laboratories, Burlingame, CA,) and Rhodamine Red-X-conjugated goat anti-mouse (1μg/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, 1μl of Fluorogold was injected over a five-minute period, at a rate 0.2μl per minute. Birds were allowed a 3-day survival, after which they were rapidly decapitated, and brains were collected. Tissue was

coronally sectioned at 20μm 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 (1μg/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 40X oil objective. A grid of 64 squares (40x40μm 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 60X oil objective, and images were qualitatively examined for co-localization.

Results

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 2.3). 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 2.4). 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 $t_6 \geq 4.31$, $p \leq 0.005$), but not in adulthood ($t_6 = 0.73$, $p = 0.492$).

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 2.5). 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 ($t_{10} = 0.83$, $p = 0.424$).

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 2.6). 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: $F_{1, 20} = 4.82$, $p = 0.040$).

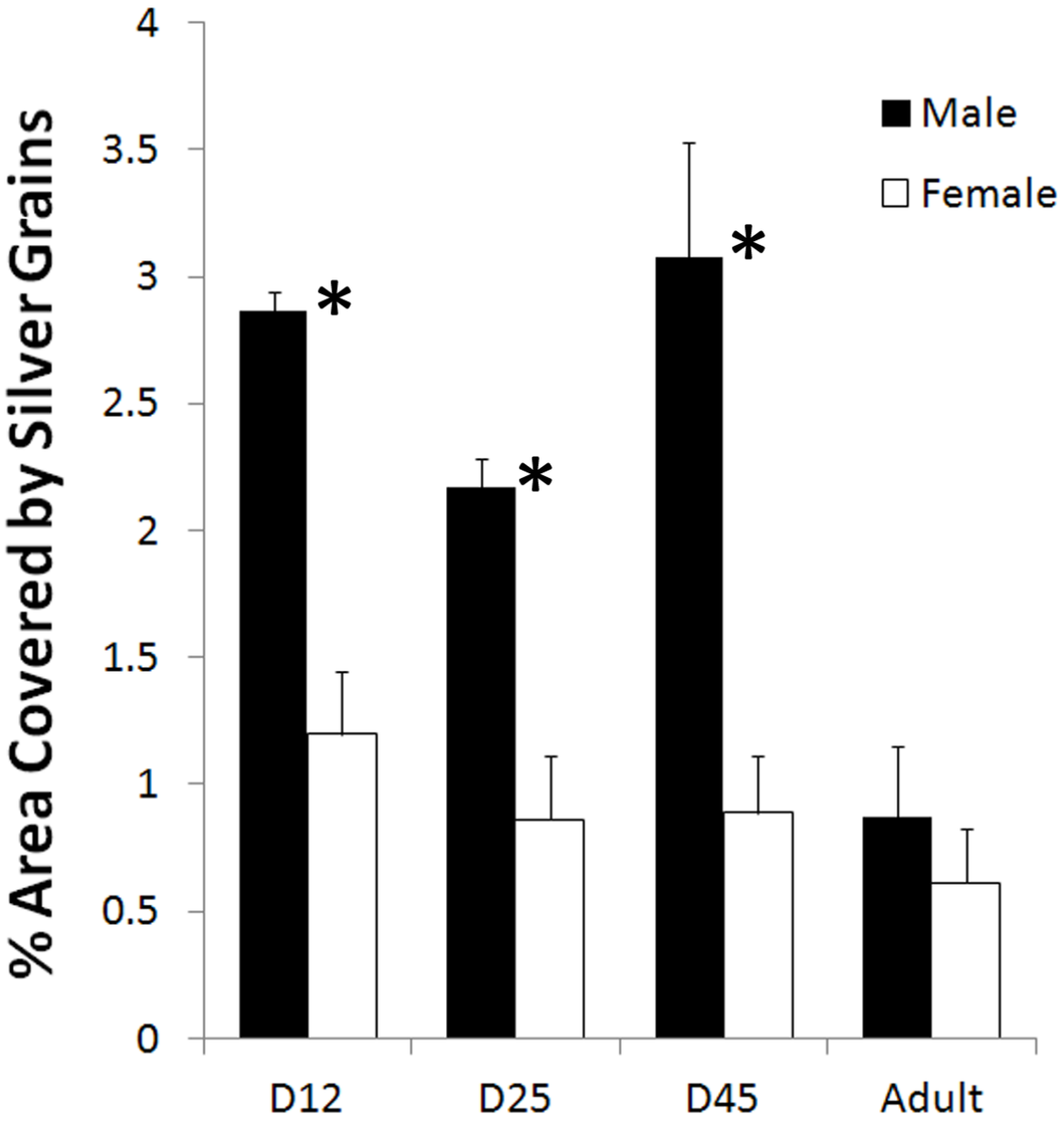

Figure 2.3 TBCA mRNA levels in LMAN from post-hatching day 12 through adulthood. Values represent means + 1 standard error. Main effects of sex and age, as well as an interaction between the two variables, were detected. Asterisks indicate significant effects of sex within age.

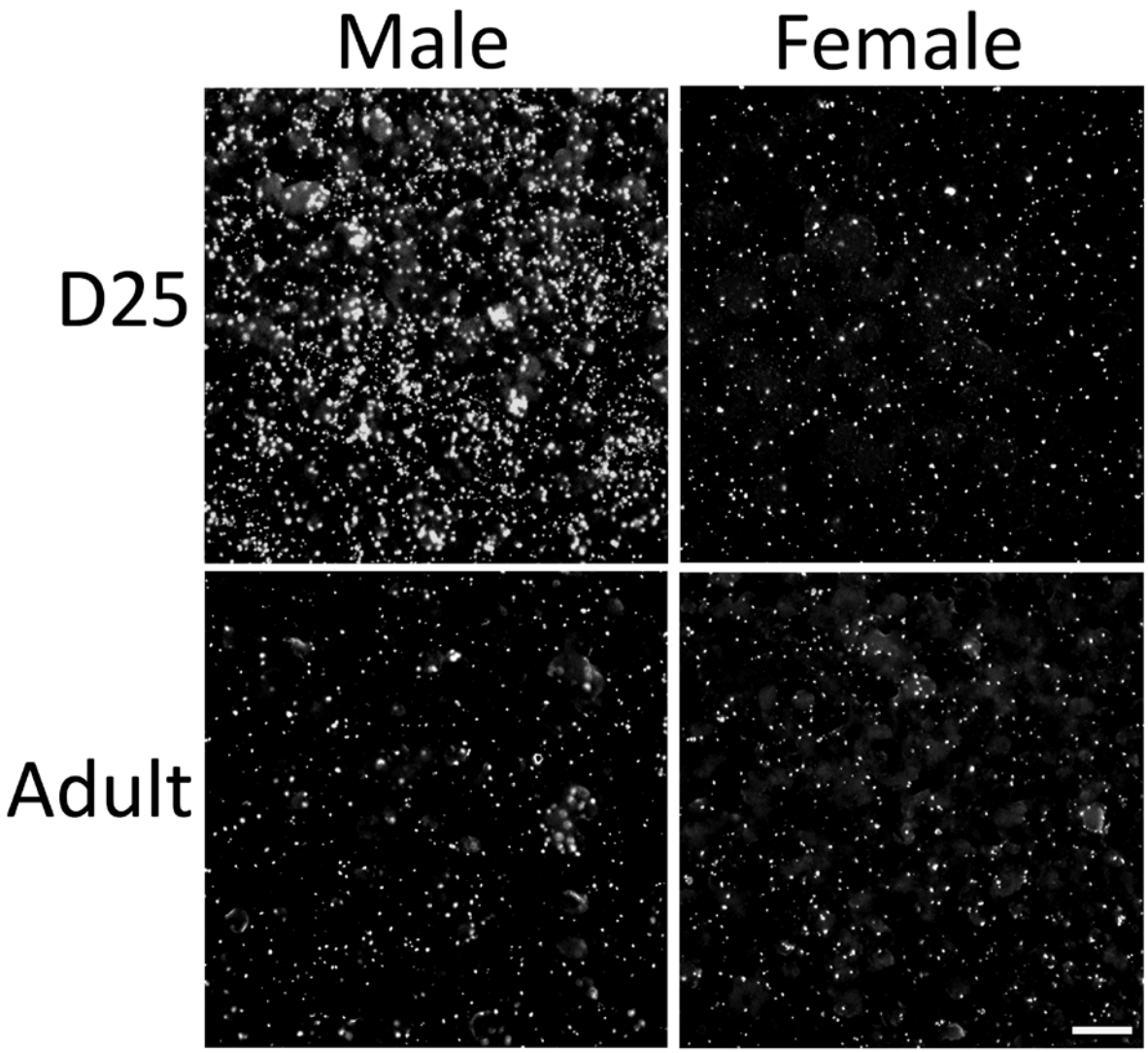

Figure 2.4 Darkfield images depicting representative TBCA mRNA labeling from the center of LMAN. Males exhibited greater expression than females at post-hatching days 12, 25, and 45. The magnitude of the sex difference was similar across these ages, so only post-hatching day 25 is depicted. Scale bar = 20μm for all photographs.

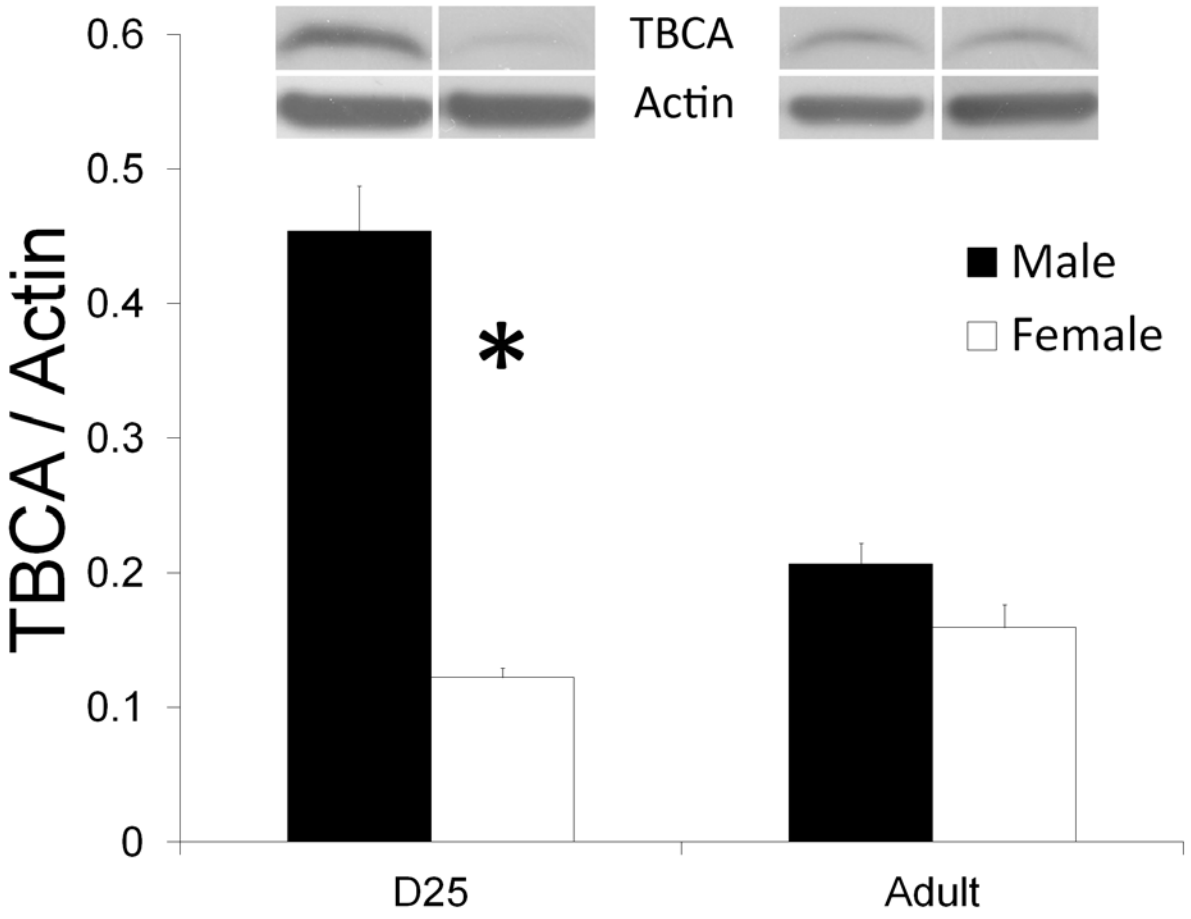

Figure 2.5 Relative TBCA protein levels in LMAN. The histogram indicates mean + 1 standard error for values, corrected for actin. Main effects of sex and age, as well as an interaction between the variables, were detected. The asterisk indicates a significant difference between the sexes at post-hatching 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.

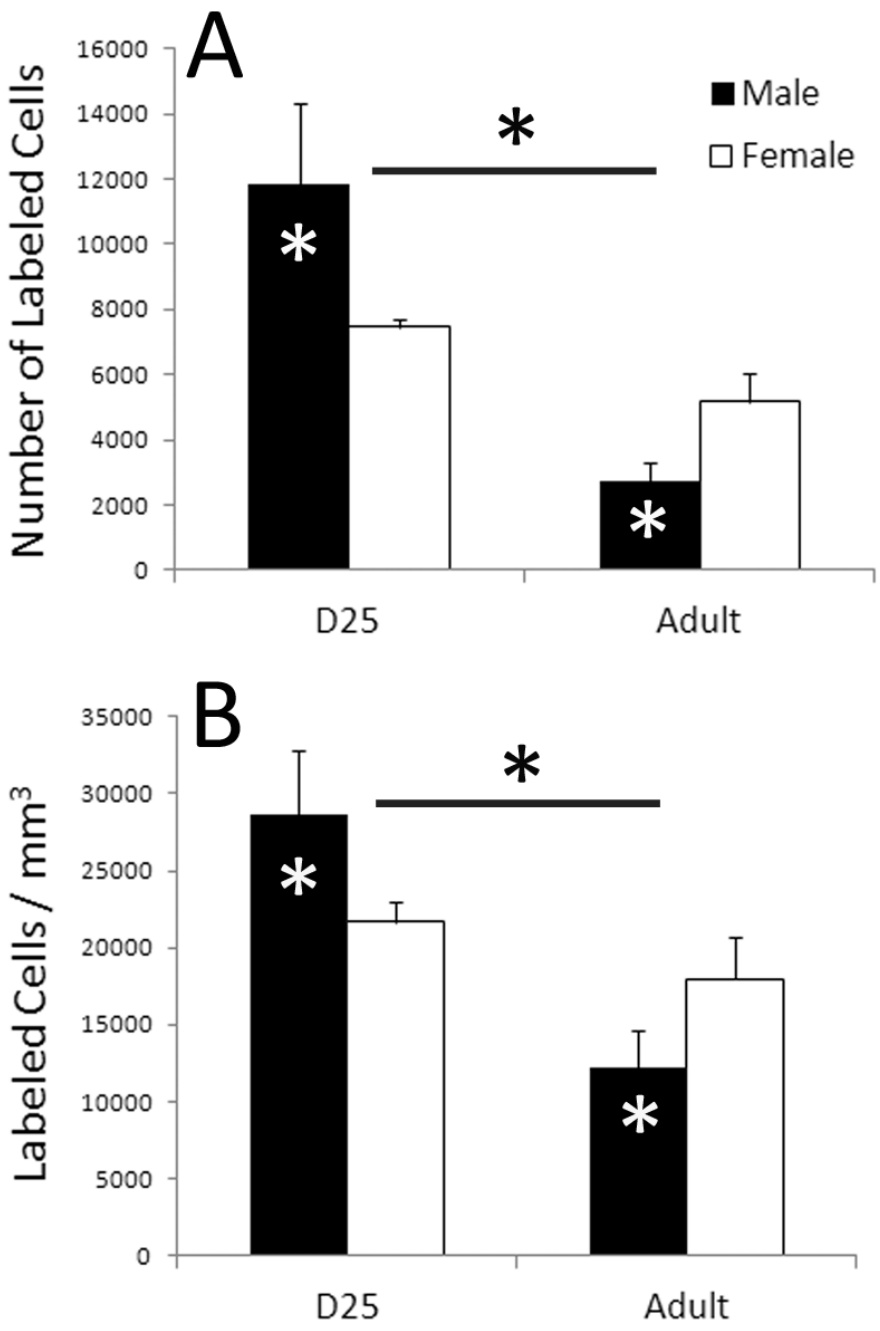

Figure 2.6 Quantification of TBCA immunohistochemical labeling. (A) Estimated total number of TBCA+ cells in LMAN (mean + 1 standard error). (B) Density of TBCA+ cells (mean + 1 standard error). The black asterisks above the bars indicate significant main effects of age. White asterisks indicate significant effects of age within males.

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 2.7). In females, cell number (t10 = 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 immuno-histochemically 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.

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 2.8). 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 2.9).

Discussion

Summary

The present study identified sexually dimorphic and developmentally regulated expression of TBCA in the LMAN of zebra finches. TBCA mRNA was

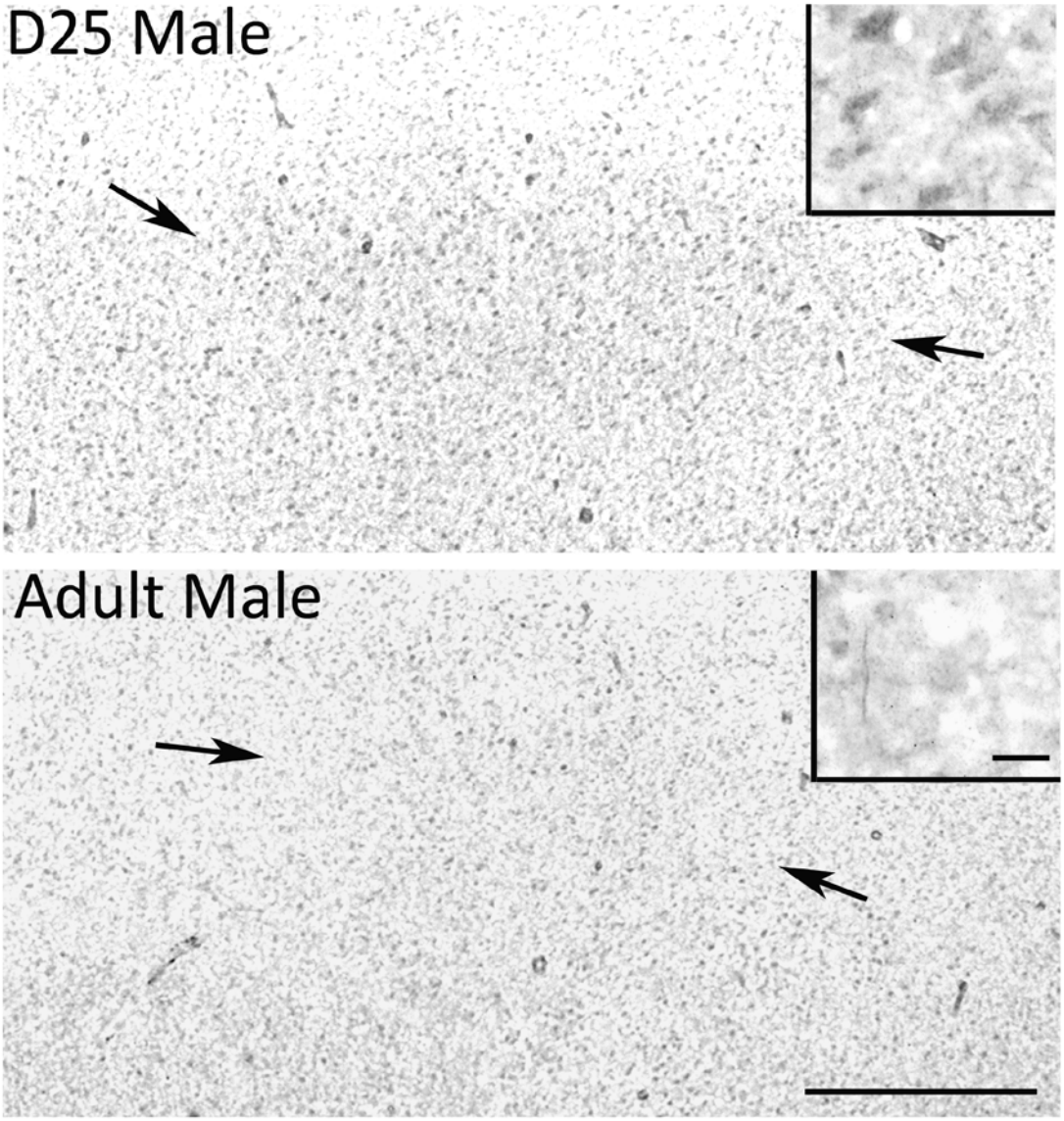

Figure 2.7 TBCA immunohistochemistry in LMAN. Images depict a representative post-hatching day 25 male and an adult male. Arrows indicate the lateral borders of the brain region. Inserts show higher magnification images from the center of LMAN in each photograph. Scale bar for the lower power images = 500μm, higher power = 20μm.

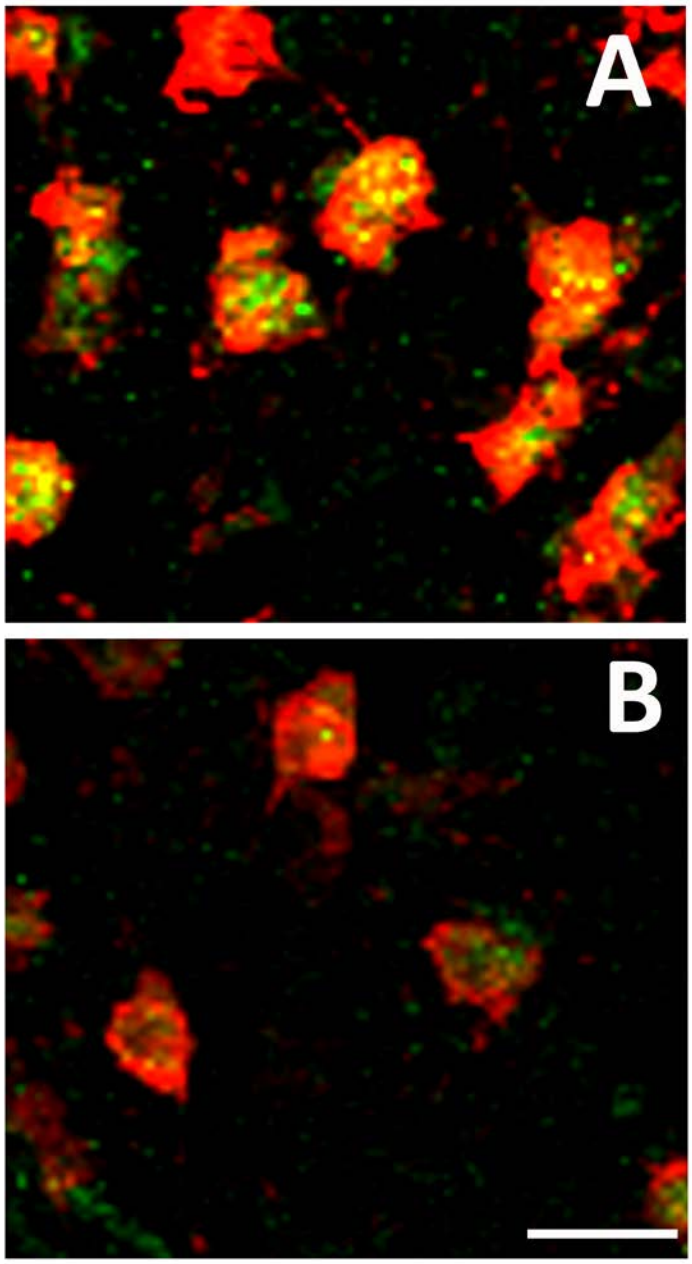

Figure 2.8 TBCA+ and Hu C/D labeling near the center of LMAN in a 45-day-old male (A) and female of the same age (B). These confocal images indicate substantial co-expression, with TBCA in green and Hu C/D in red; more than 80% of the TBCA+ cells are neurons in both sexes. Scale bar = 10μm.

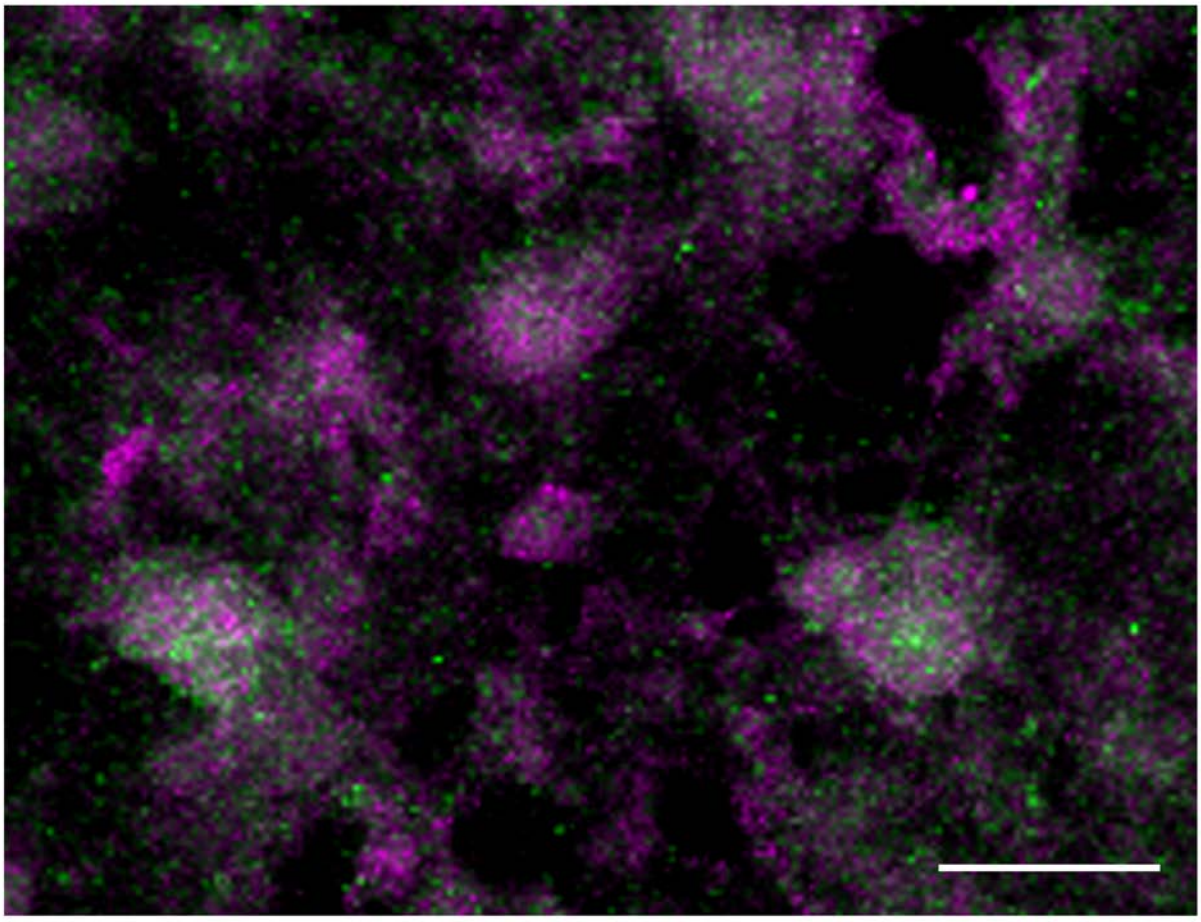

Figure 2.9 Confocal image of LMAN cells expressing TBCA protein (green) and sending projections to RA (magenta; retrograde tracing using Fluorogold). Injections were made into the RA of four 25-day-old males, which were euthanized three days later. The pattern of labeling was very similar across all animals. Scale bar = 10μm.

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 2.8).

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.

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, 1990; Nordeen and Nordeen, 1988; Kirn and DeVoogd, 1989). 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.

Future Directions

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.

CHAPTER 3

Masculinization of the Zebra Finch Song System: Roles of Estradiol and the Z-chromosome Gene Tubulin Specific Chaperone Protein A

Introduction

Sex differences in brain and behavior exist across vertebrates. In many cases, estradiol (E_2) during development masculinizes neural structure and function (reviewed in Wright et al., 2010). However, molecular mechanisms regulating these dimorphisms are largely unknown (reviewed in McCarthy and Arnold, 2011).

Zebra finches represent particularly useful models for investigating these factors. Only males sing, and the brain regions regulating song learning and production are sexually dimorphic. Cortical structures, the HVC (proper name) (Reiner et al., 2004) and robust nucleus of the arcopallium (RA) contain more and larger cells in males (reviewed in Wade and Arnold, 2004). The lateral magnocellular nucleus of the anterior nidopallium (LMAN) is similar in size between the sexes, but the number of LMAN neurons projecting to RA declines in female after post-hatching day 25 (D25) to create a sex difference by adulthood (Nordeen et al., 1992).

As in mammalian systems, E_2 has masculinizing effects when administered to female zebra finches. Treatment of hatchlings partially increases the volume of song nuclei, as well as cell size and number within them (reviewed in Wade and Arnold, 2004). E_2 added to female cultures also facilitates the growth of male-specific HVC projections into RA (Holloway and Clayton, 2001). However, with the exception of this projection, limiting E_2 availability (Wade and Arnold, 1994; Wade et al., 1999) or

action (Mathews et al., 1988; Mathews and Arnold, 1990) in males does not prevent masculine morphological development. Thus, other factors are likely important (Agate et al., 2003).

In zebra finches, males are homogametic (ZZ; females: ZW) and dosage compensation is limited (Itoh et al., 2007), so Z-genes might contribute to masculinization of the brain. Microarray analyses and subsequent studies identified several Z-genes that may be involved (Wade et al., 2004, 2005; Tang and Wade, 2006; Tang et al., 2007; Tomaszycki et al., 2009). One is tubulin specific chaperone A (TBCA), a protein critical for β-tubulin formation (Tian et al., 1996; Conde and Caceres, 2009). In development, but not adulthood, TBCA expression is greater in the LMAN of males compared to females. A majority of the TBCA+ cells are neurons, many of which project to RA (Qi et al., 2012; Qi and Wade, 2013).

The present set of studies represents an initial step in evaluating the idea that both E_2 and Z-gene(s) are required for full masculinization of the song circuit. We tested the possibility that the steroid increases TBCA availability, as well as the idea that TBCA modulate E_2's ability to masculinize. The direct effects of inhibiting TBCA on neural development were also assessed. In addition, because removal of input from LMAN causes RA cell death in juvenile males (Akutagawa and Konishi, 1994; Johnson and Bottjer, 1994), which can be rescued by brain-derived neurotrophic factor (BDNF) (Johnson et al., 1997), we also quantified the effect of inhibiting TBCA in LMAN on BDNF protein in RA.

Methods

Animals and Tissue Collection

Zebra finches were reared in walk-in aviaries, each of which contained 5-7 male and female pairs with their offspring. Nest boxes were checked every day, and new hatchlings were toe-clipped for unique identification. Portions of the removed toes were used to determine the genetic sex of each individual by PCR (Agate et al., 2002). Animals were kept in a 12:12 light: dark cycle, and provided seed (Kaytee Finch Feed; Chilton, WI), water, gravel and cuttlebone *ad libitum*. Each week, their diets were supplemented with spinach, oranges, hard-boiled chicken eggs, and bread.

For all studies, birds were euthanized by rapid decapitation, and their brains were removed and immediately frozen in cold methyl-butane. Tissue was stored at -80°C with desiccant until processing. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Michigan State University.

Experiment 1: Effects of Increasing E_2 on TBCA Expression in LMAN

Hormone Manipulation

On D3, each male and female bird received a subcutaneous implant of either E_2 or a blank control, as in Tang and Wade (2012). Implants were synthesized using a 1:5 uniform mixture of 17β-estradiol (Steraloids, Welton, NH) and silicone sealant (Type A, Dow Corning, Midland, MI), which was extruded through a 3-cc syringe in a straight line onto wax paper and dried overnight. The mixture was cut into 1 mm lengths, which were then further divided to provide 100μg of E_2 per pellet. Blank capsules were produced the same way without the addition of the hormone. Brains

were collected at D25, as noted above, and processed for either immunohistochemistry (IHC) or *in situ* hybridization to detect protein and mRNA, respectively.

IHC

Brains (n = 7 per sex per treatment) were coronally sectioned at 20μm, and thaw-mounted in six series onto SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA). The TBCA primary antibody was validated in Qi and Wade (2013), where the IHC protocol is thoroughly described. Briefly, one series of slides were warmed to room temperature, rinsed in 0.1 M phosphate buffered saline (PBS), and fixed in 4% paraformaldehyde. Tissue was then treated with 0.9% hydrogen peroxide in methanol, incubated in 5% normal goat serum (NGS) in PBS with 0.3% Triton X-100, and finally incubated in TBCA primary antibody (2μg/ml #SAB1100935, Sigma-Aldrich, St. Louis, MO) 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) for two hours at room temperature. The protein was visualized with Elite ABC reagents (Vector Laboratories) per manufacturer's instructions, including diaminobenzidine (Sigma-Aldrich) with 0.0024% hydrogen peroxide. After the color reaction, slides were rinsed in PBS, dehydrated in a series of ethanols and coverslipped with DPX (Sigma-Aldrich).

TBCA protein labeling in LMAN was quantified using Stereo Investigator software (Microbrightfield, Inc., Williston, VT) without knowledge of the animal's sex or treatment. The optical fractionator function was used to estimate the total number

of TBCA positive cells in and the volume of LMAN. Labeled 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 the number of cells within it was 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 two-way ANOVA was used (SPSS 21.0 IBM Armonk, NY) to analyze the effects of sex and treatment on the estimated total cell number, volume defined by TBCA labeling, and density of labeled cells in LMAN.

In Situ Hybridization

Adjacent tissue sections from the same animals used in the IHC study were used for *in situ* hybridization. One E_2 treated female and one control male were not included because those tissues were used for another study (final sample sizes for males: E_2 = 7, blank = 6; females: E_2 = 6, blank = 7). Two adjacent sets of tissue sections from each animal were used - one for anti-sense and one for control, sense probes. The probes and protocol 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 incubated in pre-hybridization buffer for approximately 2 hours before they were exposed to ^{33}P-UTP-labeled RNA probes overnight (20-24 hours). The tissue was washed in saline-sodium citrate (SSC) buffers to remove excess probe and then dehydrated in a

series of ethanols. Representative slides from each group were exposed to a phosphor-imaging screen (Bio-Rad Kodak #170-7841, Hercules, CA) for 16 hours and scanned with a Molecular Imager FX (Bio-Rad Laboratories) to confirm signal in LMAN. Slides were then dipped in NTB emulsion (Eastman Kodak, Rochester, NY) and stored in the dark at 4°C for 5 weeks. They were then developed using Kodak D-19 developer and fixer (Eastman Kodak) and lightly counter-stained with cresyl-violet to facilitate identification of the brain anatomy.

Analysis was also completed in the same manner as in Qi et al. (2012), without knowledge of each animal's sex or treatment group. LMAN was first identified using bright field microscopy, and then captured in dark field using Image J (National Institutes of Health software). The threshold function was used to manually define the amount of silver grains covering a 0.052mm^2 box placed over the center of LMAN. Image J then calculated the area covered by silver grains, and the percentage of TBCA labeling in LMAN was determined. This procedure was used in each section containing LMAN on both the left and right sides of the brain. Background labeling from adjacent sections that were exposed to the sense probe was subtracted from values obtained from anti-sense treated sections. The resulting values were averaged within individuals.

A 2-way ANOVA was used to analyze the effects of sex and treatment on the percent area covered by silver grains in LMAN.

Nissl Staining

A final series of sections (n = 7 per sex per treatment) was stained with thionin to validate that the E_2 treatment had an effect on the brain. Area X is not normally

visible in females, but becomes distinct following post-hatching E_2 treatment (reviewed in Wade, 2001). Females were inspected for the presence or absence of Area X by an observer blind to hormone treatment. In addition, because we are not aware of documentation of effects of early E_2 on juvenile LMAN volume (but see Grisham et al., 2008 which analyzed adult brains), the volume of this region was quantified in all individuals. Cross-sectional areas were determined by tracing the border of LMAN on each side of the brain using Image J in each section that it was visible. Unilateral volumes of the brain region were estimated by multiplying the sum of these areas by the sampling interval (0.12mm). Values for the left and right sides of the brain were averaged. A two-way ANOVA was used to analyze the effects of sex and treatment on LMAN volume.

Experiment 2: Effects of TBCA Inhibition and E_2 Modulation on Song System Morphology

Validation of TBCA siRNA Manipulation

siRNAs were generated by Ambion (Custom Ambion *in vivo* siRNA, Ambion Life Technologies, Carlsbad, CA) based on the TBCA mRNA sequence from GenBank (Accession number DQ214986). Three sequences were designed for maximum efficacy and combined for transfection into a pseudoviral envelope. This hemagglutinating virus of Japan envelope (HVJ-E; GenomeONE-Neo EX HVJ-E, Cosmo Bio Co., LTD, Koto-ku, Tokyo, Japan) is a replication-incompetent vector developed from inactivated Sendai virus (reviewed in Lund et al., 2010). The viral genome is inactive, and only the cell membrane fusion properties are intact, allowing effective delivery of siRNAs into cells without eliciting a cytotoxic response

(reviewed in Kato et al., 2013).

A pilot study (n = 3 males/group) assessed degree of protein knockdown using several siRNA doses (10, 20, 30pM) across survival times (4, 7, 10 days). The three sequences designed by Ambion were first diluted in nuclease free water (Ambion Life Technologies) to achieve the three concentrations, and stored at -20°C until use. Before surgery, each siRNA dose was transfected into a pellet containing the HVJ-E vector, per manufacturer's instructions for treatment of laboratory animals. A control sequence that does not lead to degradation of any known cellular mRNA (Control siRNA-J: sc-44238, Santa Cruz Biotechnology, Santa Cruz, CA) was prepared the same way and transfected at the same time. Based on the results of this study, a more comprehensive analysis was conducted using the 30pM dose and the 10-day survival time that included both males (n = 6) and females (n = 7).

In each case, birds were anaesthetized with isoflurane, and positioned into a stereotaxic instrument (KOPF Instruments, model 900, Tujunga, CA). Injections into LMAN were unilateral, so that one hemisphere received TBCA siRNA while the contralateral side received the control sequence. Injections were 4.8mm anterior to the bifurcation of the midsaggital sinus (lambda), 2.0mm lateral to the midline, and 1.5mm ventral from the surface of the skull. siRNA and the control (1µl each) were infused over 5 minutes, at a rate of 0.2µl per minute. All animals were returned to their home aviaries until euthanasia.

Animals were euthanized by rapid decapitation, and their brains were immediately frozen in cold methyl-butane and stored in -80°C until sectioning. Brains were coronally sectioned at 300µm and thaw-mounted onto SuperFrost Plus

slides. As in Qi and Wade (2013), LMAN was removed using a 17-gauge micropunch (Stoelting, Wood Dale, IL). For each animal, three punches were obtained from the center of LMAN in each hemisphere, and were pooled within animal. Punches from the two sides of the brain were kept separate to distinguish between the siRNA and control treatments. Tissue punches were immediately suspended in cold RIPA lysis buffer (Santa-Cruz Biotechnology) and homogenized using a sonic dismembrator (Fisher Scientific, Pittsburgh, PA). Samples were then centrifuged at 10,000g for 10 minutes at 4°C. The protein supernatants were collected, and a small volume of each sample was used for concentration quantification using the Bio-Rad Protein Assay (Bio-Rad Laboratories). The remainder of each sample was stored at -20°C until Western blot analysis.

Protein (30ug) per sample was loaded into 4-20% mini-protean TGX gels (Bio-Rad Laboratories) along with a Kaleidoscope pre-stained standard (Bio-Rad Laboratories). Samples were divided among seven gels, but the proteins from the two sides of the brain for each individual were always on the same gel. Male and female samples were run on separate gels because of differences in optimal film exposure times. Separated samples were transferred to PVDF membranes (Millipore, Billerica, MA) at 4°C. Membranes were then cut in half so that TBCA (13 kDa) and the loading control, actin (43 kDa), could simultaneously be probed.

Each membrane was incubated in 5% non-fat milk for 1 hour at room temperature to prevent non-specific binding. The TBCA (2μg/ml) and actin primary antibodies (1μg/ml; #SC-1615 Santa-Cruz Biotechnology) were applied to their respective membranes at 4°C overnight. The blots were exposed to HRP-linked

secondary antibodies for TBCA (1:5000; #7074 Cell Signaling Technology Inc., Danvers, MA) and for actin (1μg/15ml; #SC-2020 Santa-Cruz Biotechnology). After washes in PBS, the membranes 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). The film was developed using Kodak Professional D-19 developer and fixer.

A ratio of the optical densities of TBCA and actin, corrected for neighboring background, was calculated for each animal using Image J. Paired t-tests were used to evaluate differences between siRNA and control samples within males and females.

Hormone Manipulations in Experimental Animals

Females were administered either an E_2 or blank implant on D3 as in Experiment 1. Also starting on D3, male birds received daily injections of 20μg of fadrozole hydrochloride (Sigma-Aldrich) in 10μl of 0.75% saline into the breast muscle. This manipulation is highly effective in reducing brain aromatase in developing zebra finches (Wade et al., 1994). Control males received the same volume of 0.75% saline. Each bird then received a manipulation of TBCA in LMAN. Two studies were conducted – one to assess morphology within LMAN and RA, and the other to quantify the projection between these regions.

Morphology of LMAN and RA

Between D15-D17, males and females received stereotaxic injections of TBCA siRNA and control siRNA into LMAN, as described above. One bird was eliminated from analysis due to damaged tissue in LMAN, and two were not used because

injection sites were lateral to LMAN. Final sample sizes are indicated below. Birds were returned to their home aviaries after surgery and euthanized 10 days later. Their brains were removed and immediately frozen in cold methyl-butane and stored in -80°C. They were coronally sectioned at 20μm, and thaw-mounted in six series onto SuperFrost Plus slides.

One series of slides from each animal (final sample sizes for males: fadrozole = 10, saline = 9; females: E_2 = 7, blank = 9) was processed for TBCA immunoreactivity to confirm the efficacy of the siRNA treatment in LMAN. The protocol for TBCA IHC was identical to that used in Experiment 1. Photos of LMAN were captured using Image J and each image was coded so that hormone treatment, side of brain, and sex were blind to the observer. The cross-sectional area of LMAN was determined in each section by tracing its borders with Image J, and the volume based on this TBCA labeling was estimated as above.

To obtain an estimate of relative numbers of TBCA+ cells in LMAN, the cell counter plug-in in Image J was used to mark individual cells within previously traced areas. As in Experiment 1, TBCA+ cells were defined as those with a distinct neuronal morphology exhibiting a brown cytoplasmic reaction product for the DAB. TBCA+ cell number for each side of LMAN was obtained by taking the sum of the cell counts through all LMAN containing sections. The possibility that a cell was counted twice is extremely low, as alternate tissue sections were 0.12mm apart.

A second series of slides from each animal (final sample sizes for males: fadrozole = 11, saline = 9; females: E_2 = 7, blank = 9) was stained with thionin for confirmation of accurate injections into LMAN and for analysis of morphology in both

LMAN and its target, RA. Photographs of each section containing LMAN and RA were captured using Image J, and the files were coded so that hormone treatment, side of brain, and sex were blind to the observer. Cross-sectional areas and volumes of LMAN and RA were determined as in Experiment 1. Cell counts were obtained in the RA of thionin-stained sections in the same manner as TBCA labeled cells were quantified in LMAN.

To obtain an estimate of soma size in RA, the borders of 20 randomly selected cells were traced within each of the three most anterior sections of RA using Image J. Values from the two sides of the brain were kept separate so that comparisons could be made within each animal. Averages were calculated from the 60 cells per hemisphere.

Mixed model ANOVAs (siRNA within animals, hormone manipulation between individuals) were used to analyze each variable: number of TBCA+ cells and the volume defined by them in LMAN, volumes of LMAN and RA based on nissl staining, as well as RA cell size and number. These analyses were conducted separately for males and females as they received different methods of hormone administration (injections versus implants).

LMAN→RA Projection

Between D15-D17, males and females received stereotaxic injections of TBCA siRNA and its control into LMAN, as above. In this set of birds, however, immediately after siRNA injections, cannulae were replaced within their guides and 1μl of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Invitrogen, Carlsbad, CA; 0.1% in dimethylsulfoxide) was infused over 5

minutes at a rate of 0.2μl per minute into both sides of LMAN. Birds were returned to their home aviaries after surgery and euthanized 10 days later. The brains were removed, immediately frozen in cold methyl-butane, and stored in -80°C. They were coronally sectioned at 20μm, and thaw-mounted in six series onto SuperFrost Plus slides.

One series of slides from each animal was stained with thionin to confirm that the injection site was limited to LMAN. Microscopic inspection of the tissue showed that a few animals received DiI ventral to LMAN, and some exhibited unequal amounts of DiI in the two hemispheres. These were not included in the analysis (final sample sizes were males: fadrozole = 7, saline = 6; females E_2 = 11, blank = 9). A second series of slides was processed for quantification of DiI in RA. Slides were warmed to room temperature, rinsed in 0.1M PBS, and fixed in 4% paraformaldehyde. They were then rinsed in PBS, dipped in water, and cover-slipped with ProLong Gold mounting medium (Life Technologies, Carlsbad, CA). Slides cured at room temperature for 48 hours, and were then stored in the dark at 4° until analysis.

Photos of RA were captured using Image J under a TRITC filter, and the images were coded so that treatment and sex were blind to the observer for analysis. Adjacent tissue sections from the nissl-stained slides were used to confirm anatomical resolution of RA. The border was traced in the fluorescent images in every section in which the brain region was visible. The threshold function (default settings) was then used to manually highlight the DiI labeling within each RA trace. Image J calculated the area of each trace and the percent area covered by DiI.

Values within each side of RA in each animal were averaged, and a mixed model ANOVA was used to analyze the effects of siRNA treatment (within animals) and hormone manipulation (between animals). Males and females were also kept separate for these analyses.

Experiment 3: Effects of Inhibiting TBCA in LMAN on BDNF protein in RA

Between D15-D17, eight male birds received stereotaxic injections of TBCA siRNA into LMAN on one side of the brain and control siRNA into the other hemisphere, as in Experiment 2. All birds were returned to home aviaries after surgery and euthanized 10 days later. Their brains were removed and immediately frozen in cold methyl-butane and stored in -80°C until sectioning. Brains were coronally sectioned at 300μm, and thaw-mounted onto SuperFrost Plus slides.

RA was isolated individually from the two sides of the brain (3 sections per side) using a stainless steel micropunch (1.0 mm diameter, Harris Micropunch, Electron Microscope Sciences, Hatfield, PA). Within each side, samples were pooled within individuals, and immediately immersed in RIPA lysis buffer for protein extraction (protocol is the same as in experiment 2). Punches from the two sides of the brain were kept separate to distinguish between the siRNA and control treatments.

The procedures for BDNF Western blot and validation of this antibody in zebra finches are described in Tang and Wade (2012). Briefly, 20μg of total protein was run on pre-cast gels, and separated samples were transferred to PVDF membranes at 4°C. Two gels were used, but samples from the same individual were run together. Membranes were cut just below the 20kDa protein marker (Precision Plus Protein

Standards, Dual Color; Bio-Rad Laboratories) so that the mature form of BDNF (mBDNF; 14 kDa) and the actin loading control (43 kDa) could simultaneously be probed. BDNF is synthesized from precursors, prepro- and then proBDNF, which is cleaved and released into the mature form. The pro and mature forms of BDNF may exert opposing effects in the brain, with mBDNF facilitating cell proliferation and survival, synaptogenesis, and long-term potentiation, and proBDNF inhibiting these events (reviewed in Lu et al., 2005). Since we were particularly interested in effects potentially related to cell survival, we probed exclusively for mBDNF.

All membranes were incubated in 5% non-fat milk for 60 minutes at room temperature to block non-specific binding. The BDNF primary antibody (1μg/ml; sc-546; Santa Cruz Biotechnology) was diluted in the milk blocking solution and applied to the BDNF membranes overnight at 4°C. The same actin primary antibody (0.5 μg/ml) from experiment 2 was used and applied the same way. For BDNF blots, a goat anti-rabbit-HRP secondary antibody (1:5000; #7074 Cell Signaling Technology Inc.) was next applied to the membranes for one hour at room temperature. For actin blots, the same donkey anti-goat-HRP secondary antibody (1 μg/30 ml) from experiment 2 was used. Immunoreactivity was detected by chemiluminescence (Clarity Western ECL Substrate, Bio-Rad Laboratories), followed by exposure to HyBlot CL autoradiography film. The film was developed using Kodak Professional D-19 developer and fixer.

A ratio of the optical densities of the bands labeling mBDNF and actin, corrected for background, was calculated for each animal using Image J. Paired t-tests were used to evaluate differences between TBCA siRNA and control samples

across the two sides of the brain using SPSS.

Results

Experiment 1: E_2 Does Not Increase TBCA mRNA or Protein

A main effect of sex existed in silver grain density, indicating an increase in TBCA mRNA in the LMAN of males compared to females ($F_{1, 22}$ = 21.92, P < 0.001; Figure 3.1A, B). However, no effect of treatment was detected ($F_{1, 22}$ = 0.470, P = 0.500), and no interaction was observed between sex and treatment ($F_{1, 22}$ = 0.832, P = 0.372).

Main effects of sex ($F_{1, 24}$ = 2.455, p = 0.130) and treatment ($F_{1, 24}$ = 0.135, p = 0.717) were not detected on the estimated number of cells expressing TBCA protein, and the variables did not interact ($F_{1, 24}$ = 2.908, p = 0.101; Figure 3.1C). However, the volume defined by this labeling was greater in males than in females ($F_{1, 24}$ = 5.844, p = 0.024; Figure 3.1D). Neither an effect of E_2 ($F_{1, 24}$ = 1.141, p = 0.296) or an interaction between E_2 and sex was detected on this measure ($F_{1, 24}$ = 2.253, p = 0.146). There were no main effects of sex ($F_{1, 24}$ = 0.641, p = 0.431) or hormone treatment ($F_{1, 24}$ = 0.000, p = 0.986) on the density of labeled cells, and the variables did not interact on this measure ($F_{1, 24}$ = 1.655, p = 0.210; not shown).

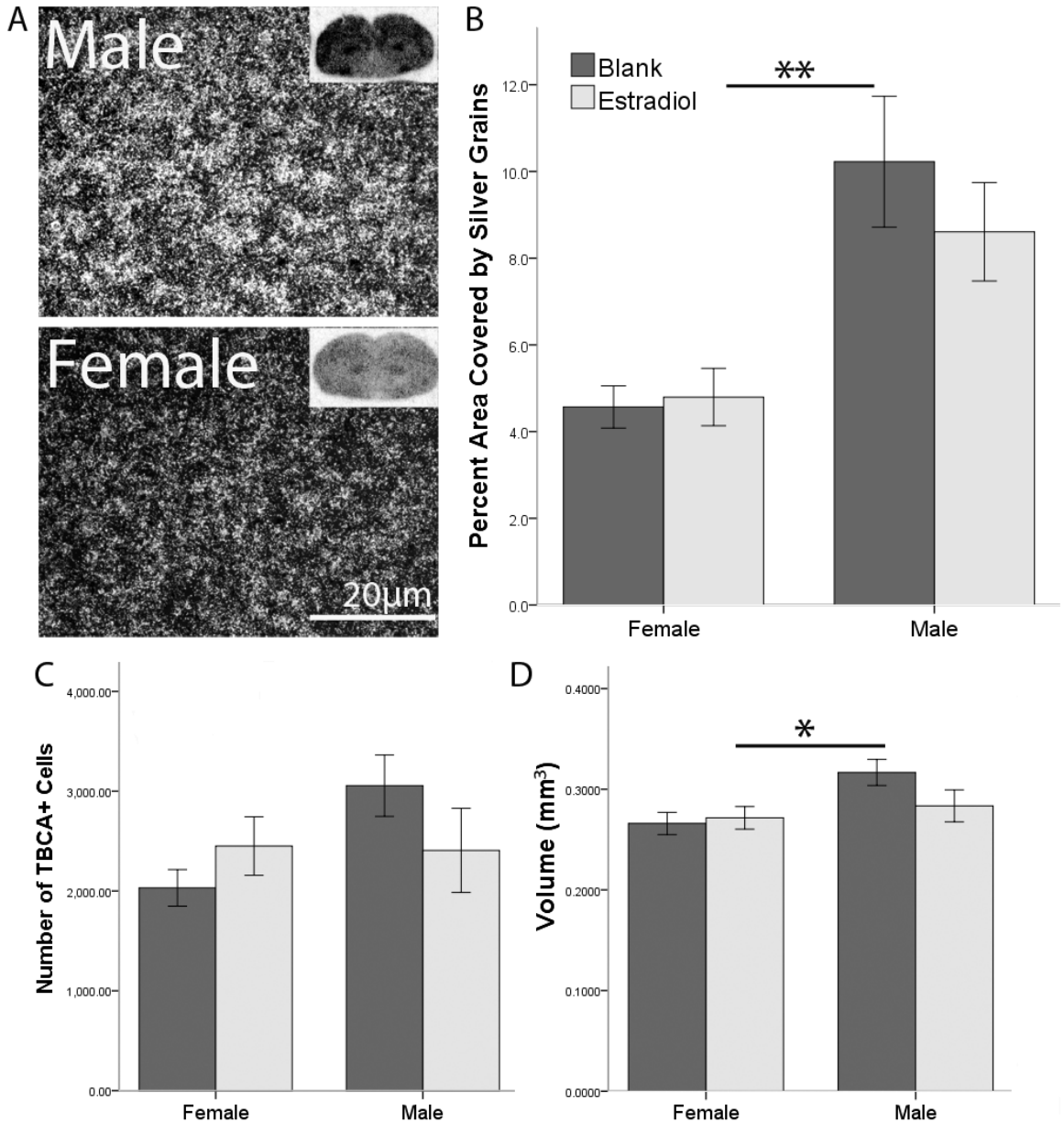

Figure 3.1 *In situ* hybridization and immunohistochemistry for TBCA in LMAN after E_2 manipulation. (A) Darkfield images of silver grains from the center of LMAN with inserts from a phosphor-imager screen of full coronal sections. (B) Relative densities of silver grain labeling. (C) Stereologically estimated number of TBCA+ cells. (D) Volume of LMAN defined by TBCA immunoreactivity. All values represent means + 1 standard error; main effects of sex: *p = 0.024, ** p < 0.001

The volume of LMAN identified by nissl staining was not affected by sex ($F_{1, 24}$ = 0.113, p = 0.740) or E_2 treatment ($F_{1, 24}$ < 0.001, p = 0.997), and no interaction between the variables existed ($F_{1, 24}$ = 2.466, p = 0.129; not shown).

Experiment 2: TBCA siRNA in LMAN effectively inhibits local protein expression, and demasculinizes LMAN and RA, as well as the projection between them

Western blot analyses used to validate the treatment paradigm indicated that the siRNA significantly decreased relative TBCA protein levels, compared to the control treatment, in both females (t_6 = 4.144, p = 0.006) and males (t_5 = 2.631, p = 0.046; Figure 3.2). In females, protein was decreased by 59% on average, and in males it was decreased by 71%. Analysis of the control, actin, alone indicated no effect of the siRNA manipulation in either females (t_6 = 0.493, p =0.640) or males (t_5 = 1.256, p = 0.265), suggesting specificity of the manipulation.

The siRNA treatment also affected immunohistochemical labeling of TBCA in experimental animals. It significantly reduced the number of TBCA+ cells in LMAN in both females ($F_{1, 14}$ = 8.234, p = 0.012; Figure 3.3A) and males ($F_{1, 17}$ = 15.81, p = 0.001; Figure 3.3B). In females, no main effect of hormone ($F_{1, 14}$ = 1.969, p = 0.182) or interaction between hormone and siRNA existed ($F_{1, 14}$ = 0.175, p = 0.682). In males, fadrozole did not affect TBCA+ cell number ($F_{1, 17}$ = 0.878, p = 0.362), but an interaction was detected between siRNA and hormone manipulation ($F_{1, 17}$ = 6.527, p = 0.021). In saline-treated birds, TBCA+ cell number was significantly reduced due to siRNA treatment (t_8 = 4.477, p = 0.002). However, this effect did not exist within fadrozole-treated males (t_9 = 1.038, p = 0.326). Effects of the siRNA (t_{17} = 0.902, p =

0.379) and its control (t_{17} = 1.934, p = 0.070) were not significantly different across the two hormone manipulations.

The volume of LMAN defined by TBCA labeling was reduced in both females ($F_{1, 14}$ = 8.128, p = 0.013; Figure 3.3C) and males ($F_{1, 17}$ = 19.99, P < 0.001; Figure 3.3D). In females, no main effect of hormone ($F_{1, 14}$ = 0.335, p = 0.572) or interaction between hormone and siRNA existed ($F_{1, 14}$ = 0.024, p = 0.880). In males, there was no effect of fadrozole treatment ($F_{1, 17}$ = 2.028, p = 0.173). However, an interaction between siRNA and hormone was detected ($F_{1, 17}$ = 10.07, p = 0.006). Within saline-treated birds, the effect of the siRNA was statistically significant (t_8 = 5.553, p = 0.001), whereas it was not within fadrozole-treated males (t_9 = 0.904, p = 0.389). Also parallel to the results on TBCA+ cell number, effects of the siRNA (t_{17} = 0.138, p = 0.892) and its control (t_{17} = 2.358, p = 0.031; Bonferroni α = 0.0125) were not significantly different across the two hormone manipulations.

In females, TBCA siRNA significantly decreased LMAN volume in thionin-stained tissue ($F_{1, 14}$ = 5.325, p = 0.037), but there was no main effect of hormone treatment ($F_{1, 14}$ = 0.377, p = 0.549) or interaction between the variables ($F_{1, 14}$ = 0.142, p = 0.712; Figure 3.4A). In males, the TBCA siRNA manipulation did not reduce LMAN volume ($F_{1, 18}$ = 2.261, p = 0.150; Figure 3.4B), and no interaction between siRNA and hormone treatments was detected ($F_{1, 18}$ = 2.375, P = 0.141). There was, however, a main effect of hormone treatment such that the volume of LMAN was greater in males treated with fadrozole than saline ($F_{1, 18}$ = 8.672, p = 0.009).

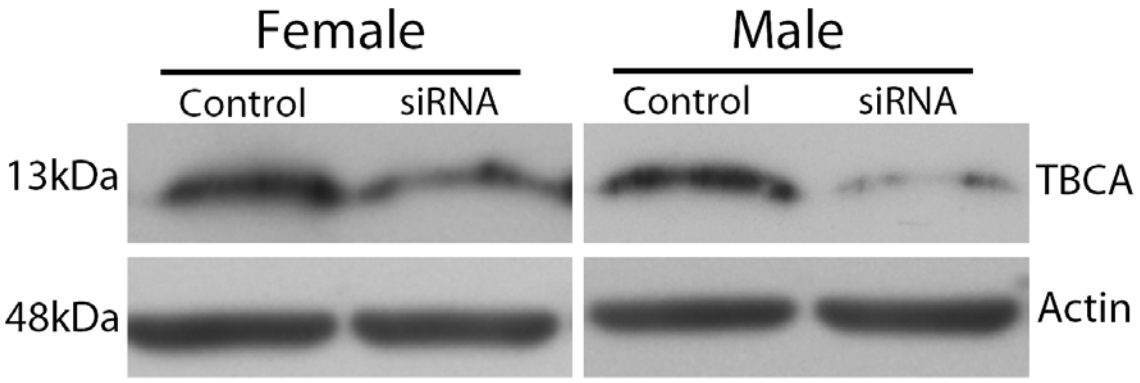

Figure 3.2 Western blots using protein extracted from micropunches of LMAN from two zebra finches. In each bird, LMAN on one side of the brain received a control sequence and the other received the TBCA siRNA.

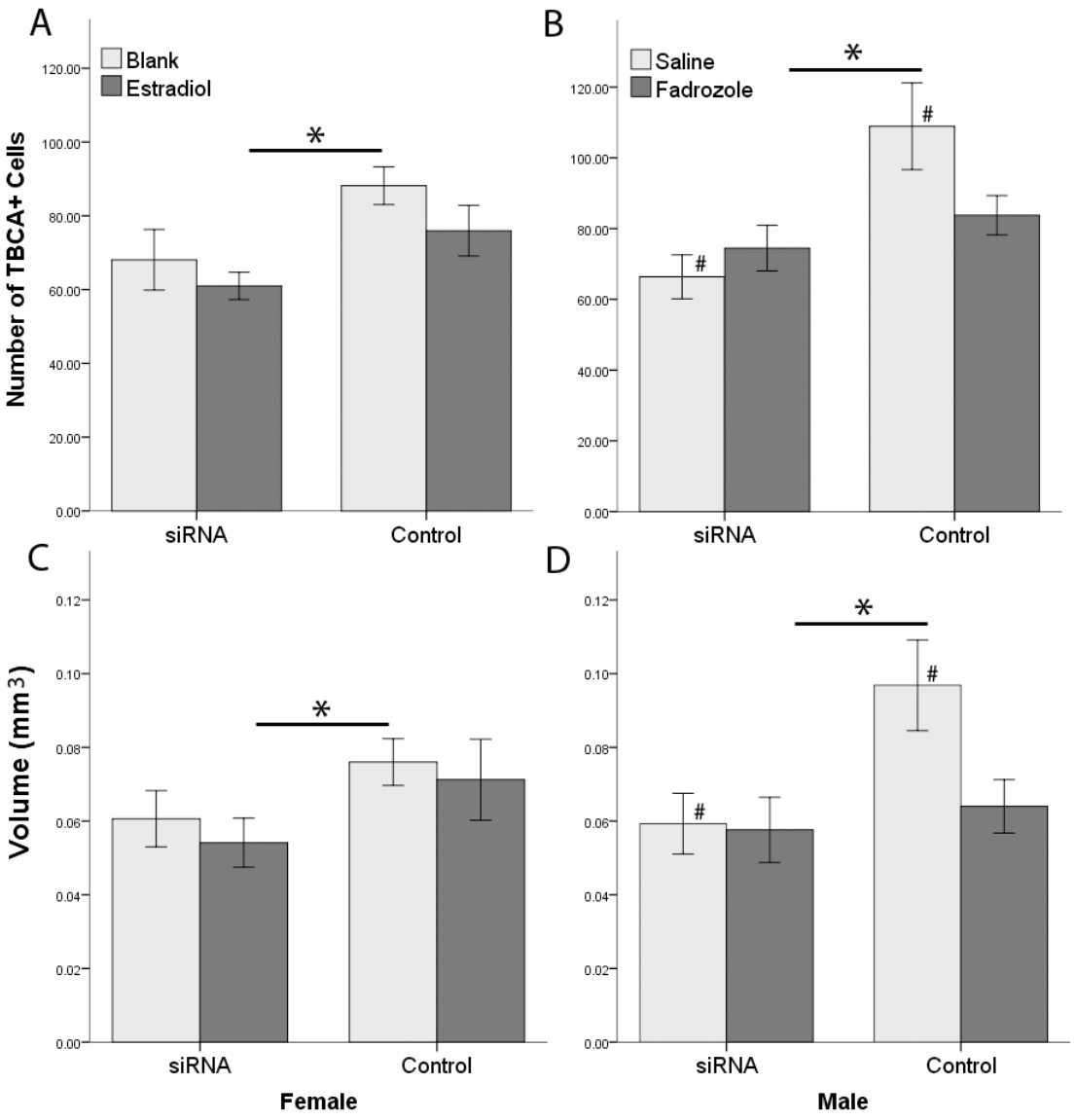

Figure 3.3 Estimated relative numbers of TBCA+ cells in LMAN are shown for (A) females and (B) males. The volume defined by this labeling is depicted in (C) females and (D) males. All values represent means + 1 standard error; *indicates a significant main effect of the siRNA manipulation in all analyses, and # represents a decrease due to the siRNA within saline-treated males.

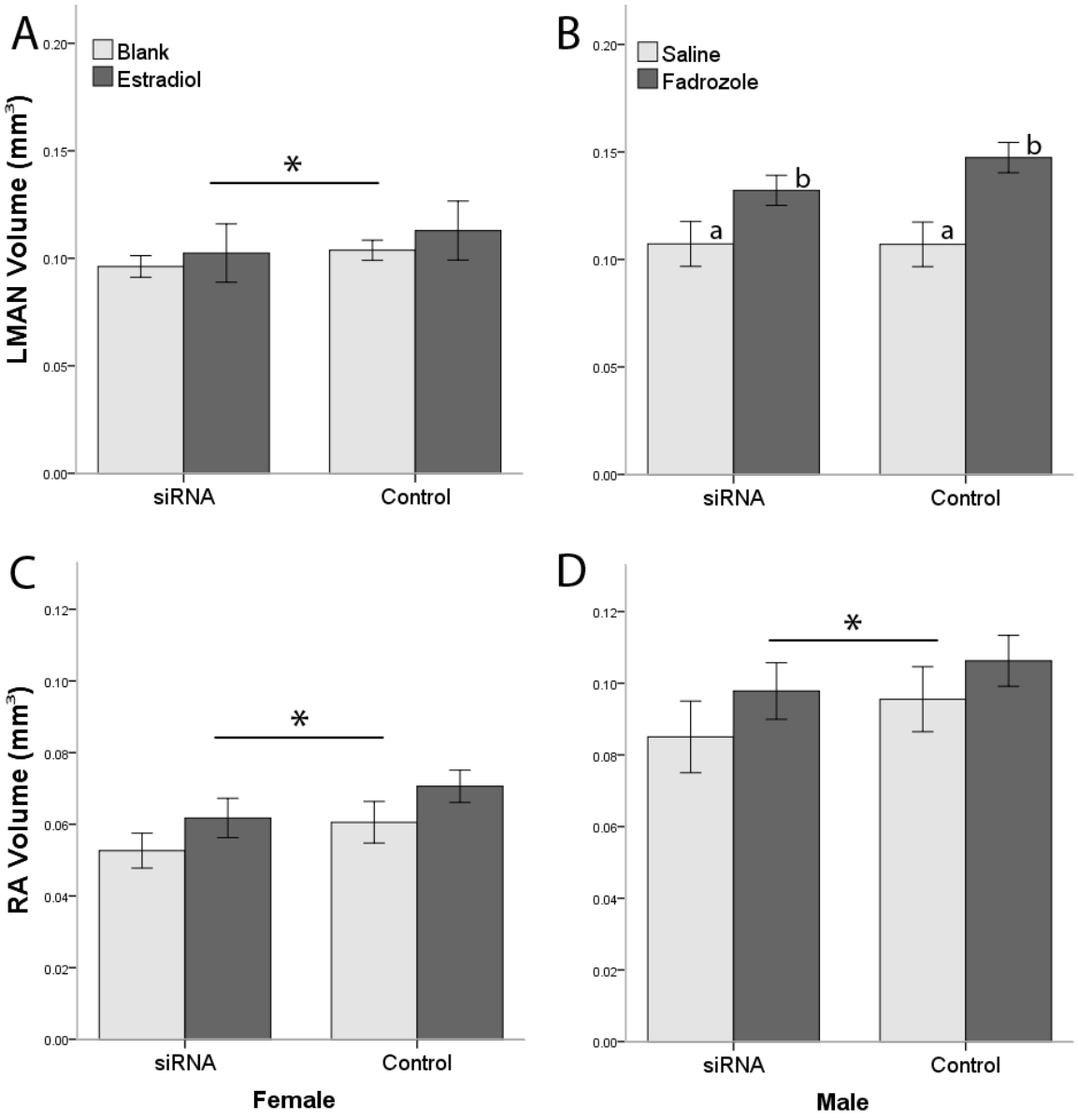

Figure 3.4 Brain region volumes after TBCA siRNA and hormone manipulations. (A) Represents LMAN values in females, and (B) shows data from this area in males. RA volumes are also depicted for (C) females and (D) males. All values represent means + 1 standard error; *indicates significant main effect of siRNA, and the different letters in (B) represent a main effect of fadrozole.

TBCA siRNA also reduced RA volume in females as quantified in nissl-stained tissue ($F_{1, 14}$ = 23.14, $p < 0.001$) without a main effect of hormone treatment ($F_{1, 14}$ = 1.710, p = 0.212) or an interaction between the variables ($F_{1, 14}$ = 0.078, p = 0.784; Figure 3.4C). The effect was the same in males, with TBCA siRNA reducing RA volume ($F_{1, 18}$ = 13.01, p = 0.002). No main effect of fadrozole ($F_{1, 18}$ = 1.016, p = 0.327) or interaction between the siRNA and fadrozole manipulations existed ($F_{1, 18}$ = 0.163, p = 0.691; Figure 3.4D).

Parallel to the effects on volume, the estimate of relative cell number in RA was decreased in females by the TBCA siRNA manipulation ($F_{1, 14}$ = 10.78, p = 0.005), without a main effect of hormone treatment ($F_{1, 14}$ = 0.258, p = 0.620) or an interaction ($F_{1, 14}$ = 0.124, p = 0.730; Figure 3.5A). The pattern was the same in males, with a main effect of siRNA treatment reducing RA cell number ($F_{1, 18}$ = 21.22, $p < 0.001$; Figure 3.5B) and no effect of hormone treatment ($F_{1, 18}$ = 0.669, p = 0.424) or interaction between the two variables ($F_{1, 18}$ = 1.223, p = 0.283).

RA soma size was also reduced in females due to the siRNA compared to control treatment ($F_{1, 14}$ = 16.97, p = 0.001), with no effect of hormone treatment ($F_{1, 14}$ = 1.324, p = 0.269) or interaction between the variables ($F_{1, 14}$ = 1.664, p = 0.218; Figure 3.5C). A parallel main effect of the siRNA existed in males ($F_{1, 18}$ = 47.33, $p < 0.001$; Figure 3.5D, E). Fadrozole also increased this measure ($F_{1, 18}$ = 5.290, p = 0.034), but no interaction between siRNA and hormone treatment was detected ($F_{1, 18}$ = 1.366, p = 0.258).

The projection from LMAN to RA, as measured by the percentage of area covered by DiI, was diminished by TBCA siRNA treatment in both females ($F_{1, 18}$ =

5.378, p = 0.032; Figure 3.6A, C-E) and males ($F_{1, 11}$ = 7.105, p = 0.022; Figure 3.6B). In females, there was no main effect of E_2 treatment ($F_{1, 18}$ = 0.102, p = 0.753) or interaction between siRNA and hormone ($F_{1, 18}$ = 3.953, p = 0.062). However, in males, fadrozole significantly increased the percent area of RA covered by DiI ($F_{1, 11}$ = 10.26, p = 0.008). No interaction existed between siRNA and fadrozole treatment ($F_{1, 11}$ = 1.760, p = 0.211).

Experiment 3: Inhibition of TBCA in LMAN does not affect mBDNF protein in RA

The TBCA siRNA manipulation did not significantly alter mBDNF levels in RA (t_7 = 2.237, p = 0.060). A substantial amount of variability existed, particularly with the control manipulation; data from one sample is largely responsible for the apparent trend of a reduction (Figure 3.7).

Discussion

To our knowledge, this is the first set of studies to document specific effects of a sex chromosome gene on avian brain development. Inhibition of TBCA in LMAN demasculinized soma size and cell number in RA, as well as RA volume. It also diminished the projection from LMAN to RA. Parallel effects occurred in females, with an additional decrease in LMAN volume. The role of E_2 remains unclear, however. While we had considered additive or interactive effects of this hormone and TBCA on masculinization, the results suggest that neither occurs.

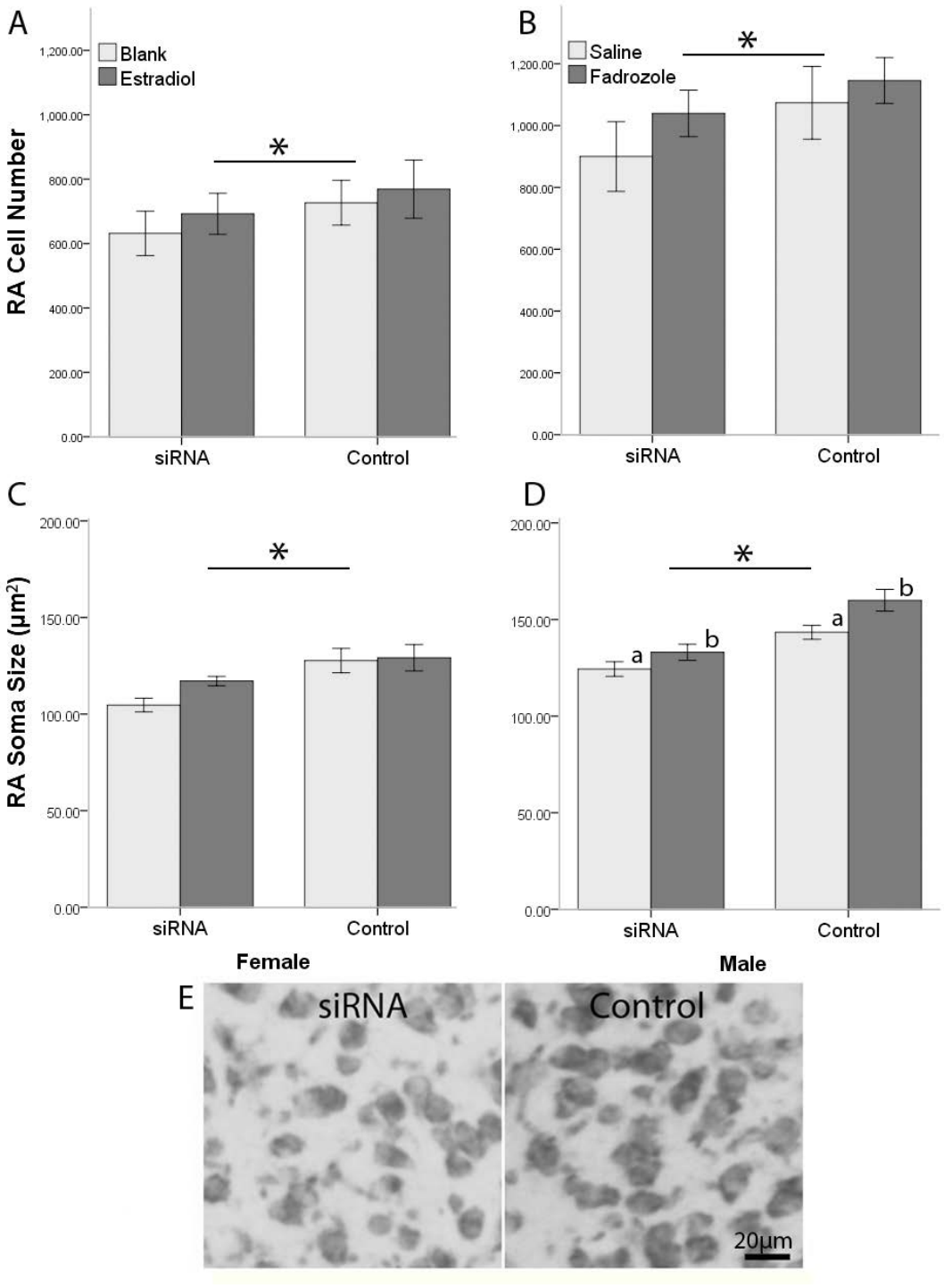

Figure 3.5 Estimated relative numbers of cells in RA are depicted in (A) females and (B) males. Soma size in this region is indicated for (C) females and (D) males. (E) Shows representative images of nissl-stained sections

Figure 3.5 cont'd

through the center of RA in a saline-treated male that received TBCA siRNA (left) and its control on the opposite side of the brain (right). All values represent means + 1 standard error; *indicates significant main effect of siRNA, and the different letters in (D) represent a main effect of fadrozole.

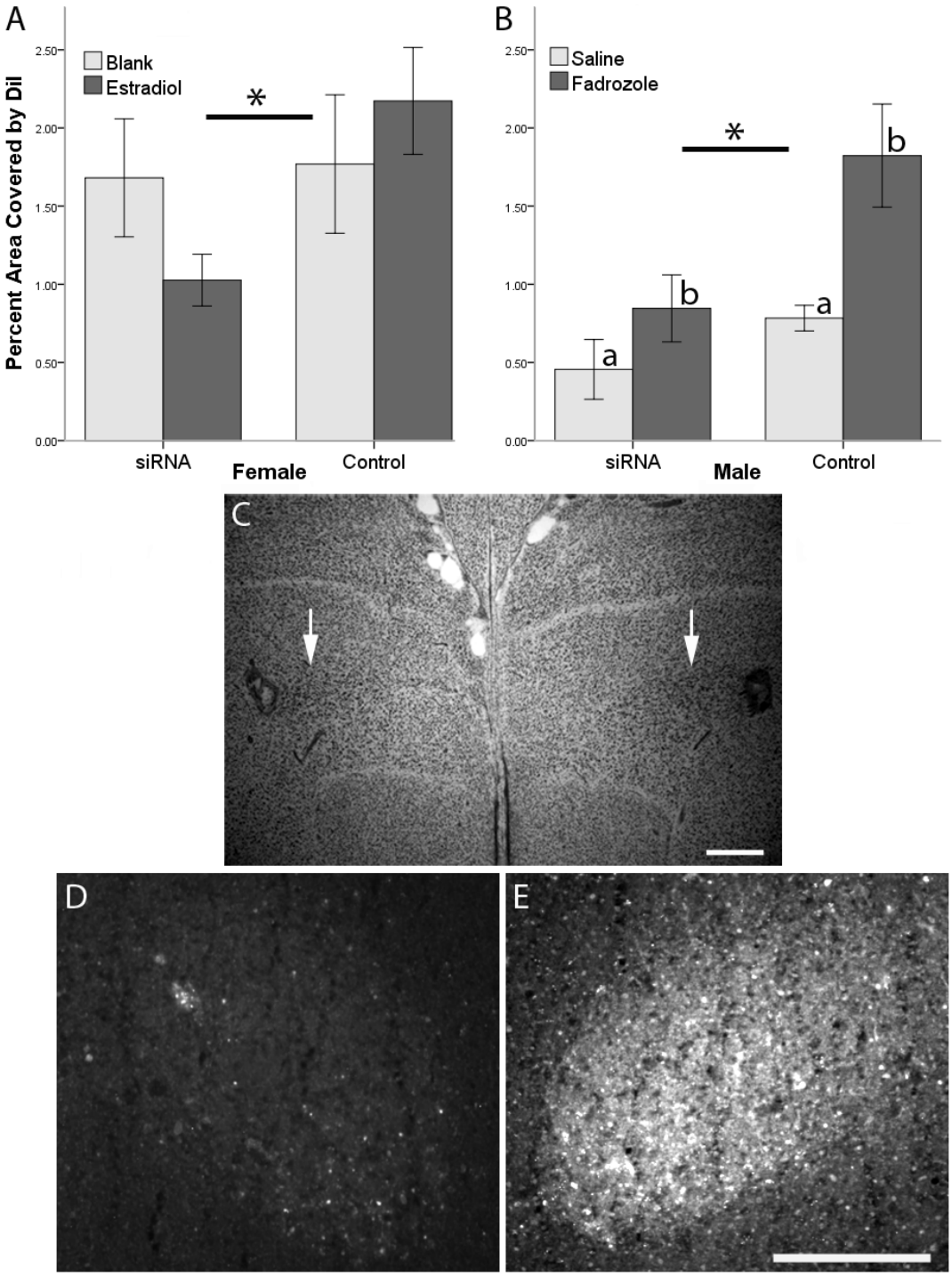

Figure 3.6 Percent of RA's area filled by DiI following injection in LMAN in (A) females and in (B) males. All values represent means + 1 standard error;

Figure 3.6 cont'd

*indicates a main effect of siRNA treatment in both sexes, and different letters in (B) represent an effect of fadrozole in males. (C) Is a low magnification photo of a nissl-stained coronal section in an E_2-treated female, where the white arrows point to dorsal borders of LMAN; injection sites are visible in each hemisphere of LMAN near the edges of the image. Fluorescent images of RA from the same animal are depicted for the (D) siRNA treated side of the brain and (E) control. The scale bar in (C) = 500 μm, and the one in (E) represents 200 μm for both (D) and (E).

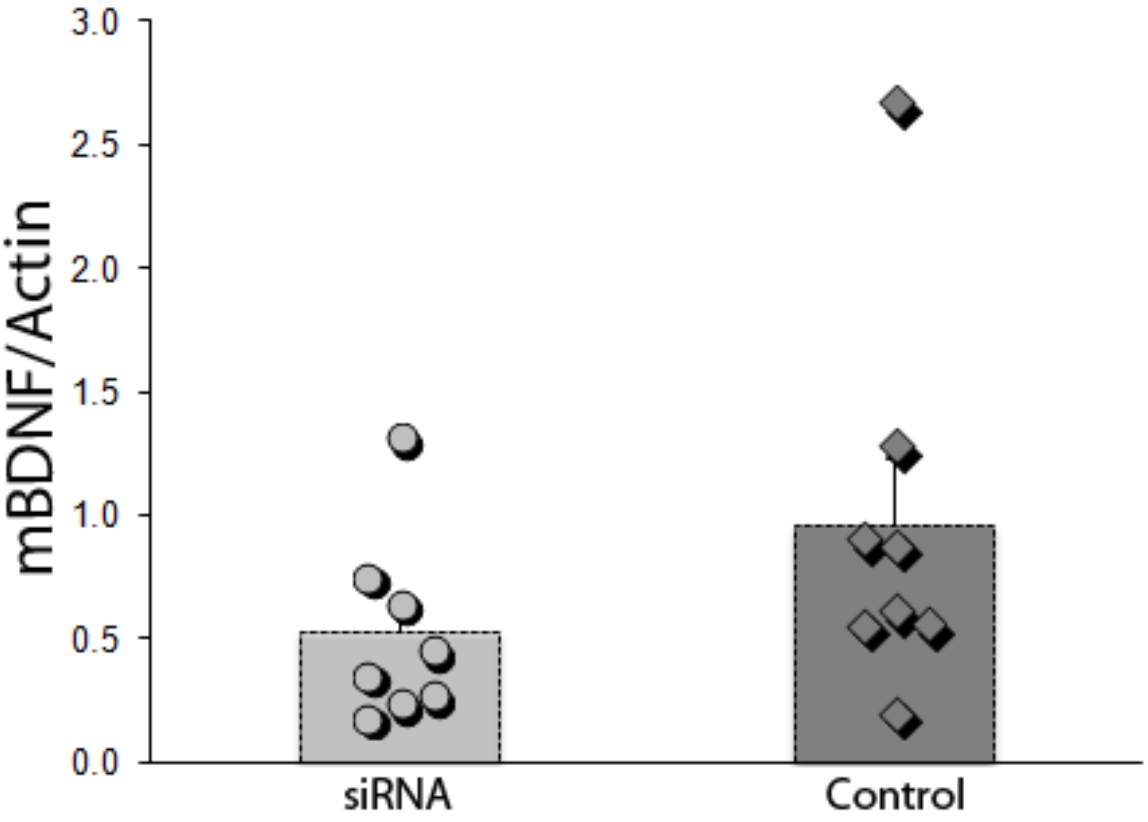

Figure 3.7 Relative levels of mBDNF in RA following siRNA or control manipulation in LMAN. Symbols indicate values for individual samples, and the histogram shows means + 1 standard error.

TBCA Is Involved In Song System Masculinization

TBCA is conserved across vertebrates and one of several chaperones required for the generation of α/β tubulin (reviewed in Tian and Cowan, 2013). Human mutations in a β-tubulin gene, *TUBB2B*, are associated with defective TBCA and result in reduced tubulin heterodimers and abnormal brain development (Jaglin et al., 2009). TBCA silencing in mammalian cell lines causes a reduction in soluble tubulin and eventually death of these cells (Nolasco et al., 2005). Thus, TBCA in mammalian systems is important for cell survival and normal brain maturation.

The present data on TBCA inhibition are consistent with similar roles in zebra finches. Effects were detected in both sexes despite lower endogenous TBCA levels in females (Qi et al., 2012; Qi and Wade, 2013), suggesting that this Z-gene exerts similar effects regardless of the genetic sex of the brain. The results parallel those from electrolytic lesions of LMAN in juvenile males, which reduce the volume of and cell number in RA, likely due to apoptosis (Johnson and Bottjer, 1994). LMAN cells can transport neurotrophins, including BDNF, to RA, and infusion of these proteins into RA rescues cell death (Johnson et al., 1997). Thus, the projection from LMAN to RA likely facilitates RA cell survival by providing trophic support.

As an initial test that TBCA knockdown in LMAN interfered with neurotrophin transport, we evaluated relative BDNF content in RA following TBCA siRNA infusion into LMAN. The data are not consistent with this hypothesis, but other techniques are required to fully test the idea. The mBDNF in RA detected here and in earlier studies (Tang and Wade, 2012, 2013) likely derives from both local synthesis and transport from LMAN. LMAN projections reach the RA of both males and females by D15

(Mooney and Rao, 1994) and are not sexually dimorphic until after D25 (Nordeen et al., 1992). Thus, decreasing TBCA in LMAN probably facilitated regression of this pathway in contrast to inhibiting growth. It is possible that the loss of the projections from LMAN to RA resulted in diminished availability of neurotrophins other than BDNF. NT-3 and NT-4/5 can also inhibit apoptosis in RA following lesions of LMAN (Johnson et al., 1997).

TBCA Expression in LMAN is Sexually Dimorphic, but E_2 Does Not Affect its Expression

Consistent with previous results from juvenile zebra finches (Qi and Wade, 2013), TBCA mRNA was increased in the LMAN of males, but the number of TBCA immunoreactive cells in this brain region did not differ between the sexes. In Experiment 1, the volume defined by this labeling, but not nissl staining, was increased in males. Similarly, previous assessment of changes in the volume of LMAN in nissl-stained tissue produced no main effect of sex and parallel developmental trajectories in males and females (Nixdorf-Bergweiler, 1996). In our earlier study (Qi and Wade, 2013), Western blot analysis showed a greater concentration of protein in LMAN of males compared to females at D25. Collectively, these results suggest that the quantity of TBCA per cell may be greater in males. Such a result might make individual cells appear larger in immunostained tissue, which could affect the detection of the borders of LMAN.

E_2 treatment did not modulate TBCA mRNA or protein in the LMAN of juvenile birds of either sex. We had hypothesized that one way E_2 might facilitate masculinization, at least when administered to females, is via up-regulation of this

protein important for neural development. However, the data from Experiment 1 indicate that increasing circulating levels of this steroid has no effect on TBCA expression in the LMAN of either sex. Consistent results were detected in Experiment 2. E_2 in females had no effect on TBCA+ cell number or the volume defined by this marker. Similarly, main effects of the estrogen synthesis inhibitor fadrozole were not detected in males on these measures.

Manipulation of E_2 Availability Produced Some Novel Results

Many studies have documented potent effects of post-hatching E_2 in females on masculinizing RA morphology (reviewed in Wade and Arnold, 2004) that were not seen in Experiment 2. All females treated with E_2 in the present experiment had an Area X, which is normally not visible without early exposure to E_2, whereas none of those receiving blank capsules did. Thus, the steroid functioned in the brains of these birds. One possibility for the lack of effects in RA is that mechanical injury caused by inserting cannulae into LMAN up-regulated aromatase (Peterson et al., 2001). Recent studies in mice suggest that such an increase in estrogen synthesis can even propagate to beyond the site of injury (Saldanha et al., unpublished observations). Thus, it is possible that E_2 levels were increased in both LMAN and RA in all of the subjects, which minimized any effects that might have been due to exogenous hormone manipulation.

In males, aromatase inhibition increased RA cell size and LMAN volume, as well as the transport of DiI from LMAN into RA. LMAN volume and the projection to RA had not been evaluated following post-hatching fadrozole treatment. However, previous reports of RA morphology following fadrozole administration are partially

inconsistent with the current data (Wade and Arnold, 1994). That is, no effect of aromatase inhibition on RA volume was detected in either study, but unlike the present work, the earlier experiment also found no effect on RA cell size. The reason for the difference across the studies is not obvious. The doses were identical across the two studies, but the earlier one treated males from D1 to D30, whereas the present birds were injected from D3 to D25-27. The ages of injected birds are different, although it seems unlikely that a shorter duration would have a more potent outcome.

Hypermasculinizing effects following manipulation of estrogenic systems have been detected in zebra finches. Administering the estrogen receptor blockers tamoxifen, LY117018, or CI628 to males increases a variety of aspects of song system morphology (Mathews et al., 1988; Mathews and Arnold, 1990). While the mechanisms are unclear, it appears that increased estrogen availability or action has some opposing effects in males and females. These warrant further investigation.

Fadrozole might also affect brain morphology by increasing androgen. Inhibiting aromatase activity increases available testosterone (T) because less of this substrate is metabolized (Schlinger et al., 1999; Soma et al., 2000). Like the data from studies on E_2, the results from manipulations of androgen or its ability to act are somewhat paradoxical. Treatment of juvenile female zebra finches with 5α-dihydrotestosterone (DHT), a non-aromatizable metabolite of T, can increase RA soma size and cell number (Gurney, 1981; Schlinger and Arnold, 1991a). However, data following administration of the anti-androgen flutamide are inconsistent. In one study, treatment of developing males with this drug had no effect on numerous

measures of song system morphology, including RA soma size (Grisham et al., 2007). In another it hypermasculinized volume and neuron number in RA (Schlinger and Arnold, 1991a). In a third, castration of juvenile males combined with treatment with the anti-androgen, flutamide, decreased LMAN volume (Bottjer and Hewer, 1992). Some, but not all of these data, are consistent with the hypermasculinizing effects we detected in LMAN and RA following fadrozole treatment; more work is needed to isolate specific mechanisms. Details regarding the increase in the projection from LMAN to RA due to fadrozole treatment need to be further evaluated. However, we can conclude that the mechanisms are different than those involved in masculinizing the projection from HVC to RA. This male-specific innervation (Konishi and Akutagawa, 1985; Mooney and Rao, 1994) requires E_2 produced in the brain (Holloway and Clayton, 2001).

Future Directions

The history of research into the mechanisms underlying sexual differentiation of the zebra finch song system suggest that both Z-genes and E_2 may be important for masculinization. The current studies document that a specific Z-gene, TBCA, influences song system development. While TBCA does not appear to modulate E_2's effects in the brain, other Z-genes might. One candidate is tyrosine kinase receptor B (TrkB), the high affinity receptor for BDNF. Expression of both the ligand and receptor in the song system are sexually dimorphic and modulated by E_2 (Chen et al., 2005; Tang and Wade, 2012). Not only is it possible that multiple Z-genes influence masculinization, but W-genes, which are specific to females, may also facilitate feminization. The present work represents an initial series of steps in

determining the role of sex chromosome genes in the development of brain and behavior.

CONCLUSION

Sex differences in human physiology have now forced biological sex into the spotlight as a main predictor of susceptibility to disease and disease progression. To this end, the Institute of Medicine published a report, "Exploring the biological contributions to human health: Does sex matter?" to facilitate development of sex-specific approaches to medicine. Before this occurs, critical gaps in our knowledge need to be further addressed with use of strategic animal models, such as the Australian zebra finch. The sequencing of the genome has allowed us to identify potential candidate Z-genes that might influence sex-specific development of neural structures by mediating events such as neuronal survival and maintenance of axonal projections.

The experiments in this dissertation set out to understand the role of one Z-gene, TBCA, and its interactions with E_2, in facilitating sexually dimorphic development of the zebra finch song system. TBCA was initially detected on a microarray screen that found enhanced expression of its sequence in the forebrain of juvenile males. In the studies that followed, I localized this male-biased expression to a specific region in the song system, LMAN, where I found its expression to be regulated by both genetic sex and age of the animal. The discrete nature of this expression and TBCA's conserved role in microtubule biosynthesis and integrity allowed me to formulate specific hypotheses regarding its role, perhaps in concert with E_2, in neural development.

The data suggest that TBCA is implicated in mechanisms underlying the control of brain region volume, cell number and size, and the development and/or

maintenance of neural projections. While the scope of this dissertation cannot address the downstream mechanisms that mediate these morphological effects, we now have at our disposal a variety of avenues for future investigation.

For instance, how does increased TBCA in the LMAN of males facilitate the maintenance of their projections to RA? What implications might this have on the development of sex differences within RA? For instance, are neurotrophins or other molecules transported to RA of males to mediate cell survival, and does natural cell death in females reflect a paucity of these same factors? By further examining these questions, we can begin to uncover what elements seemingly protect one sex while leaving the other vulnerable.

In terms of potential interactions of TBCA with E_2, I found that E_2 does not modulate TBCA transcript or protein levels, nor does TBCA influence effects of E_2 in the song system. First, this suggests that the increased levels of TBCA in the male LMAN reflect inherent differences in the sexes, due to either lack of dosage compensation or another factor independent of E_2. Second, it informs us that male brains are not more sensitive to E_2 merely because they express higher levels of TBCA. Finally, I did not see evidence for additive effects of both TBCA and E_2 in masculinization of neural structures.

The role of E_2 in masculinization of the song system remains unclear. Treatment of male hatchlings with the aromatase inhibitor, fadrozole, hyper-masculinized several morphological parameters. Regardless of the mechanism by which this occurred, we can conclude that limiting estrogen synthesis in males might

not mimic increasing estrogen provisioning in females. Future work will need address potential reasons for this paradox.

The implications of this body of work are broad, and expand beyond the study of sex differences in a songbird species. By exploiting sex differences in the expression of conserved proteins in the song system, we can advance our understanding of their functions in other vertebrates, including humans. Even though TBCA is not on a sex chromosome in humans (chromosome 5), elucidating its role in nervous system development, especially in conjunction with other factors such as steroid hormones, is extraordinarily valuable.

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