Published in final edited form as:

Neuroscience. 2013 October 10; 250: 578–587. doi:10.1016/j.neuroscience.2013.07.062.

Developmental Changes in BDNF Protein in the Song Control Nuclei of Zebra Finches

Yu Ping Tang^{*,a,c} and Juli Wade^{a,b,c}

^aMichigan State University, Department of Psychology, East Lansing, MI 48824, USA

^bMichigan State University, Department of Zoology, East Lansing, MI 48824, USA

^cMichigan State University, Department of Neuroscience, Program East Lansing, MI 48824, USA

Abstract

The zebra finch song system provides an excellent model to study the mechanisms underlying the development of sex difference in brain structure and function. Only male zebra finches sing and the brain nuclei controlling song learning and production are considerably larger than in females. Sexual differentiation may in part be regulated by estrogen, but other molecules including neurotrophic factors likely also affect masculinization. Brain derived neurotrophic factor (BDNF) plays crucial roles in numerous aspects of vertebrate brain development and function, including neurogenesis, cell survival, growth of axonal projections, synaptogenesis and processes linked to learning and memory. The current study investigated expression of BDNF protein in juvenile males and females at four ages, as well as in adults, to begin to evaluate the potential roles of endogenous BDNF in particular stages of structural and functional development of the song system. In both HVC and the robust nucleus of the arcopallium (RA), males had more BDNF+ cells than females. The number of immunopositive cells increased in males and decreased in females as they matured, in a pattern generally consistent with a role for BDNF in sensorimotor integration of song learning. In addition, in HVC (but not RA) the ratio of mature BDNF (mBDNF) compared to its precursor proBDNF was greater in adult males than those at post-hatching day 25, indicating a region-specific shift in the relative availability of the two forms. Collectively, the data suggest that changes in BDNF protein expression across development may be associated with song system maturation, particularly during the sensorimotor integration of masculine vocalizations.

Keywords

neurotrophic factor; songbird; sexual differentiation

Introduction

Sex differences in brain structure and function exist across vertebrate groups, particularly characteristics associated with reproductive behaviors such as courtship and copulation (Cooke et al., 1998, De Vries et al., 2002, Simerly, 2002, De Vries, 2004). Among

© 2013 IBRO. Published by Elsevier Ltd. All rights reserved.

*Corresponding Author: Yu Ping Tang, Michigan State University, Neuroscience Program, 108 Giltner Hall, 293 Farm Lane, East Lansing, MI 48824-1101, USA, Tel: + 1-517-432-5113, Fax: + 1-517-432-2744, tangyupi@msu.edu (Y.P. Tang).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

songbirds, the degree of sexual dimorphism in neural structure grossly parallels the differences detected in singing behavior between males and females (Brenowitz et al., 1985). For example, zebra finches exhibit among the largest male-biased sex differences in neural structure and under normal conditions only males sing.

The motor pathway controlling singing behavior includes the HVC (proper name, Reiner et al., 2004) and robust nucleus of the arcopallium (RA), which projects to a portion of the hypoglossal nucleus that controls the muscles of the vocal organ (syrinx). The lateral magnocellular nucleus of the anterior nidopallium (LMAN) and Area X, in the striatum, are associated with the process through which song is learned from tutors (reviewed in Wade and Arnold, 2004). The anatomy of each song nucleus is sexually dimorphic. HVC and RA contain more and larger cells, and the projection between the regions is more robust in males compared to females. Area X is not detectable in females with a variety of anatomical and biochemical markers (Wade and Arnold, 2004). LMAN is similar in overall size between the sexes, but some of the projections to RA are lost in females (Nordeen et al., 1992, Mooney and Rao, 1994, Johnson et al., 1997), and cells in this area also become larger in males during development (Nixdorf-Bergweiler, 1998).

The mechanisms regulating sexual differentiation of the zebra finch song system are not fully understood. In rodents, early exposure to estradiol (E2) can permanently masculinize both the size of forebrain structures and the display of reproductive behaviors (Sakuma, 2009). Similarly, E2 administered to hatchling female zebra finches can increase the size of song nuclei and permits individuals to sing in adulthood (Adkins-Regan and Ascenzi, 1990, Simpson and Vicario, 1991, Adkins-Regan et al., 1994, Adkins-Regan, 1999, Wade, 2001, Sakuma, 2009). However, unlike rodents, exogenous E2 alone only induces partial masculinization in female zebra finches, suggesting that other factors may affect the process as well (Wade and Arnold, 2004). One potential candidate is brain-derived neurotrophic factor (BDNF). BDNF is a member of nerve growth factors family which play crucial roles in development and maintenance of the central nervous system (McAllister et al., 1999, Cohen-Cory et al., 2010). In both mammalian and zebra finch systems, expression of this protein can be modulated by E2 (Miranda et al., 1993, Dittrich et al., 1999, Gibbs, 1999, Solum and Handa, 2002, Sohrabji and Lewis, 2006, Aguirre and Baudry, 2009, Tang and Wade, 2012).

BDNF is synthesized via precursors, first prepro- and then proBDNF, which is cleaved and secreted in a mature form (mBDNF; Lessmann and Brigadski, 2009, Cohen-Cory et al., 2010, Cunha et al., 2010). This secretion can occur in a regulated, activity dependent manner, or via more passive, constitutive mechanisms (Nagappan et al., 2009, Je et al., 2012). Functions of mBDNF in the brain include facilitating cell proliferation and survival, axon guidance, synaptogenesis, dendritic branching, neuronal activity, long-term potentiation, and memory formation. These functions appear to occur via action at tyrosine kinase receptors (TrkB; Abraham and Williams, 2008, Lu et al., 2008). However, proBDNF can also be released in the brain, and appears to induce effects opposite to mBDNF, via the p75 pan-neurotrophin receptor (p75NTR). These effects include facilitating cell death and inhibiting neuronal migration (Lu et al., 2005, Yang et al., 2009, Je et al., 2012).

We previously reported a sex difference in the number of cells expressing BDNF protein in HVC at post-hatching day 25 (d25; Tang and Wade, 2012). This pattern is consistent with the expression of BDNF mRNA in this brain region, which develops in males between d20 and d35 (Dittrich et al., 1999), and suggests a potential role in masculinization. We also detected greater expression of BDNF protein in the RA of males compared to females at d25 (Tang and Wade, 2012), despite the fact that the mRNA was below the limit of detectability in both sexes at ages ranging from post-hatching day 20 to 35 (Dittrich et al., 1999).

Akutagawa and Konishi (1998) described transient expression of BDNF protein across the song nuclei of male zebra finches during vocal development, particularly at d45 and d65, also indicating BDNF might be involved in the process of maturation of these brain regions.

The goal of the present work was to create a more complete picture of BDNF protein expression in both males and females at key stages of song system development. We chose four time points: d25, d35, d45 and d65. At d25 song templates are memorized by males and likely females (reviewed in Konishi, 2010) and morphology of the song control nuclei is rapidly differentiating (Nordeen and Nordeen, 1997, Doupe et al., 2004, Wade and Arnold, 2004). At d35, the projection from HVC has entered RA in males but not females (Konishi and Akutagawa, 1985). This age, along with d45 and d65, spans the period of sensorimotor integration in males, during which song production is initiated and modified based on comparison to the stored template (reviewed in Brainard and Doupe, 2000). Adults were also investigated in the present study, as the song of males has crystallized and morphology of the song system has also become stable (Wade and Arnold, 2004). An antibody that recognizes both the mature and pro forms of BDNF was used for immunohistochemistry to quantify the number of cells within song control nuclei. Based on these data, reports of a developmental increase in the ratio of mBDNF to proBDNF in the mouse brain (Yang et al., 2009), and the idea that neuronal activity may increase cleavage of proBDNF to mBDNF (Je et al., 2012), we also used Western blot analyses with the same antibody to compare the ratio of mBDNF:proBDNF in HVC and RA between d25 and adult male zebra finches.

Experimental Procedures

Animals

Male and female zebra finches were raised in mixed sex group aviaries from our colony at Michigan State University. A 12:12 light:dark cycle was maintained, and seed and water were available *ad libitum*. Once a week, the birds were also fed bread mixed with hard-boiled chicken eggs, spinach and orange. Nests in each aviary were checked daily, and the day a hatchling was found was considered d1. At that time, the toe(s) of birds within each nest were clipped in a unique pattern to provide individual identification. They were given unique leg bands once they were large enough, prior to fledging. All procedures were conducted in accordance with NIH guidelines and approved by the Michigan State University IACUC.

BDNF Immunohistochemistry

Brains of both sexes were collected on post-hatching days 25, 35, 45, 65 and in adulthood (greater than 100 days of age; n=6/sex/age). They were removed following rapid decapitation, frozen in ice cold 2-methyl-butane, and stored at -80 °C. Coronal sections (20 µm) of the entire telencephalon were cut on a cryostat, thaw-mounted onto six alternate sets (Super-Frost Plus slides; Fisher Scientific, Hampton, NH, USA) and stored at -80 °C with desiccant.

Procedures paralleled those in Tang and Wade (2012). Briefly, one set of slides from each animal was rinsed in 0.1 M phosphate-buffered saline (PBS) after being allowed to reach room temperature, fixed in 4% paraformaldehyde, and washed again in PBS. Brain sections were treated with 0.9% H₂O₂/methanol followed by 3% normal goat serum (NGS) in PBS with 0.3% Triton X-100, and then incubated with the BDNF (N-20) primary antibody (0.5 µg/ml; sc-546; Santa Cruz Biotechnology, Santa Cruz, CA) in 0.1 M PBS containing 0.3% Triton X-100, 3% NGS overnight at 4°C. A biotin-conjugated goat anti-rabbit secondary antibody (1 µg/ml; Vector Labs, Burlingame, CA) and *Elite* ABC reagents were used, followed by diaminobenzidine containing 0.0024% hydrogen peroxide to produce a brown

reaction product. The tissue sections were rinsed in PBS, dehydrated in a series of ethanols, and coverslipped with DPX (Fluka, St. Louis, MO). We verified the specificity of BDNF antibody in zebra finch brain in a previous study. Preadsorption of the antibody with the immunizing peptide eliminated labeling in both Western blots and immunohistochemistry (see figure 2 in Tang and Wade, 2012).

HVC, RA, LMAN and Area X were analyzed in all animals in which BDNF labeling defined border could be distinguished from surrounding tissue. Stereo Investigator software (Microbrightfield Inc., Williston, VT) was used to estimate the total number of cells labeled with the BDNF antibody by an individual blind to sex and age of the animals. As in Tang and Wade (2012), the border of each region defined by BDNF labeling was traced throughout its rostrocaudal extent. All cells exhibiting neuronal morphology and a clear reaction product were manually counted in sections using the Optical Fractionator function.

While BDNF+ cells were present in the song control nuclei of females, at some ages they were quite limited and, importantly, the borders of these brain regions could not be defined by BDNF labeling (see below). This characteristic suggested that any effects of BDNF would not be specific to the region(s) of interest at particular ages in females. It also precluded stereological quantification in tissue sections from these females at some developmental stages. Thus, statistical analyses included effects of sex only when an accurate estimate of BDNF+ cell number could be obtained from both males and females. A two-way ANOVA (sex $\times$ age), one-way ANOVA (age within sex), or t-test (sex within each age) was applied to analyze the estimated total BDNF+ cells in each song nucleus, as appropriate. Pairwise comparisons were also conducted when significant sex $\times$ age interactions existed, using Bonferroni corrections.

BDNF Western Blot Analyses

Additional d25 and adult male zebra finches (n=8 per age) brains were collected as above. Frozen coronal sections (300 μ m) were thaw-mounted onto a slide, and stored at -80 °C. HVC and RA were individually punched out with a stainless steel cannula (0.5 mm diameter; Stoelting C., Wood Dale, IL) from both sides of the brain in each section in which they were visible (4-5 sections for HVC, 2-3 for RA). Punches were pooled within individual and brain region in RIPA lysis buffer (sc-24948; Santa Cruz Biotechnology, Santa Cruz, CA). Protein was extracted per manufacturer's instructions. Concentrations were quantified with the Bradford assay method (Bio-Rad; Hercules, CA). Total protein from each sample of HVC and RA (10 and 5 μ g, respectively) was run on precast gels (4-20% Mini-PROTEAN TGX; Bio-Rad; Hercules, CA) and transferred to polyvinylidene difluoride (PVDF) membranes at 4°C. PVDF membranes were treated with the SuperSignal Western Blot Enhancer kit (Part # 46641, Thermo Scientific; Rockford, IL) according to manufacturer's instructions, and blocked with SuperBlock Blocking Buffer (Product # 37516, Thermo Scientific; Rockford, IL) for 60 minutes at room temperature to eliminate non-specific binding. The same BDNF (N-20) primary antibody (sc-546; Santa Cruz Biotechnology, Santa Cruz, CA) used for immunohistochemistry (validated in Tang and Wade, 2012; see above) was prepared in primary antibody diluent (1 μ g/ml) from SuperSignal Western Blot Enhancer kit and then applied to the membranes for overnight incubation at 4°C. A goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (1:5,000; Cell Signaling, Danvers, MA) was applied to the membranes at room temperature for 1 hour. Immunoreactivity was detected by chemiluminescence (SuperSignal West Pico, Thermo Scientific; Rockford, IL) followed by exposure to HyBlot CL autoradiography film (Denville Scientific Inc.; Metuchen, NJ).

In a previous experiment, we did not detect mBDNF in 8 μ g of protein from HVC and RA (Tang and Wade, 2012). However, the technique was optimized for the current study by

cutting the PVDF membrane just under 25 kDa protein marker (Precision Plus Protein Standards, Dual Color; Cat# 161-0374; Bio-Rad; Hercules, CA), which allowed the mature (14 kDa) and pro (38 kDa) forms of BDNF to be processed separately. Under these conditions, we could provide different exposure times and then could detect both mBDNF and proBDNF consistently in 5 µg of total protein. Membranes were stripped and reprobed for actin (as in Tang and Wade, 2012) to ensure consistency of loading (see Discussion).

The mean optical density for each band of interest was quantified using Image J (National Institutes of Health). A value was also obtained for an immediately adjacent region of the same size, which was subtracted to control for background (as in Tang and Wade, 2012). The ratio of mBDNF:proBDNF was calculated and analyzed by Bonferroni corrected t-tests in each brain region. All statistics for the present works were computed using SPSS version 19 (IBM; Armonk, NY).

Results

BDNF Immunohistochemistry

HVC—While BDNF labeling defined the HVC in all males, only at d25 did females exhibit a clear HVC based on this characteristic (Figure 1, top). A one-way ANOVA within males revealed a significant main effect of age on the estimated total number of BDNF+ cells ($F_{4,21}=5.746$, $p=0.003$; Figure 1, bottom). *Post hoc* comparisons indicated that adult males had more BDNF+ cells in HVC than d25, 35, and 45 (Tukey HSD; $p=0.014$, 0.008 , and 0.010 , respectively). At d25, more BDNF+ cells were detected in the HVC of males than females ($t_7=5.411$, $p=0.001$).

RA—Adult female zebra finches did not have RAs definable by BDNF labeling, although distinct borders could be detected with this marker at all juvenile stages (Figure 2, top). A two-way ANOVA in juveniles (sex $\times$ age, d25 through d65) showed a significant main effect of sex ($F_{1,34}=179.766$, $p<0.001$) and an interaction between age and sex ($F_{3,34}=7.539$, $p=0.001$; Figure 2, bottom). A main effect of age was not detected ($F_{3,34}=0.661$, $p=0.582$). Pairwise comparisons between males and females within each age showed that males had significantly more BDNF+ cells than females in RA at d35, 45, and 65 (d35, $t_7=9.535$, $p<0.001$; d45, $t_{11}=8.208$, $p<0.001$; d65, $t_9=9.882$, $p<0.001$; Bonferroni correction = 0.0125). At d25, males displayed a trend for more BDNF+ cells than females ($t_7=2.574$, $p=0.037$; Bonferroni correction = 0.0125). One-way ANOVAs across juvenile animals within each sex revealed main effects of age on the estimated total number of BDNF+ cells (males: $F_{3,17}=4.918$, $p=0.012$; females: $F_{3,17}=3.890$, $p=0.028$). *Post hoc* comparisons (Tukey HSD) showed that d65 males had more BDNF+ cells than d25 males ($p=0.015$) and d65 females had fewer BDNF+ cells than d25 females ($p=0.021$).

LMAN—BDNF labeling outlined a distinguishable LMAN in all males at all ages, whereas in females LMAN was only identifiable with this marker at d25 (Figure 3, top). At this age, the number of BDNF+ cells was equivalent between the sexes ($t_9=0.049$, $p=0.962$). A one-way ANOVA on data from males revealed no effect of age on the estimated total number of BDNF+ cells ($F_{4,25}=1.314$, $p=0.292$; Figure 3, bottom).

Area X—As in our previous study (Tang and Wade, 2012), Area X was not visible in unmanipulated females. The estimated total numbers of BDNF+ cells in the Area X was not different among males across ages (one-way ANOVA, $F_{4,24}=0.134$, $p=0.968$; data not shown).

Western Blot Analyses

Both mBDNF (14 kDa) and proBDNF (38 kDa) were detected in each sample from the HVC and RA of d25 and adult male zebra finches. In HVC, adult males exhibited a significantly higher ratio of mBDNF:proBDNF than d25 males ($t_{14}=3.679$, $p=0.002$; Figure 4, left). Unlike HVC, the ratio of mBDNF:proBDNF was not different between these two ages in RA ($t_{14}=1.078$, $p=0.299$; Figure 4, right).

Discussion

Developmental changes in the expression of BDNF in HVC and RA are consistent with the idea that this protein could play a role in masculinization of song production, as the increase of BDNF+ cells in the HVC and RA of males parallels the period of sensorimotor integration and production of mature song. In contrast, stability of relative BDNF levels from d25 through adulthood in LMAN and Area X suggests that a regulated function of BDNF in these regions specific to this developmental stage is unlikely. The difference in patterns of BDNF+ cells in males and females also suggests a sexual dimorphism in its contribution to development across the song circuit. In addition to qualitatively fewer labeled cells, this difference is characterized by lack of distinguishable song nuclei based on immunohistochemical detection of BDNF protein in females in many cases. This characteristic limits the ability to determine an accurate estimate of total BDNF+ cells number by stereology in females at particular ages, which requires a traceable border of individual brain regions. While an option would be to perform this procedure in Nissl-stained alternate sections, the estimate would likely be less accurate and obtaining it would not add to the ability to interpret the patterns we detected. That is, the fact that labeling in these females was not distinct from the surrounding tissue suggests that any influence that the limited BDNF expression might have within a song control nucleus is not specific to that region. Our interpretations are discussed below, separately for the motor and anterior forebrain pathways within the song circuit.

HVC and RA

In HVC, BDNF+ cells increased in males between d45 and adulthood, a period associated with the later stages of sensorimotor integration, which leads to the production of mature song. These results are consistent with a role for BDNF in the development and maintenance of crystallized vocalizations. The present results differ from those of Akutagawa and Konishi (1998), who documented a peak in the optical density of BDNF immunolabeling in HVC at d45 in males and disappearance of the labeling by adulthood. It is difficult to reconcile the findings of the two studies. One possibility relates to the quantification technique. A decrease in optical density across a brain region does not necessarily mean that fewer cells are labeled. It reflects the amount of protein per cell, as well as what is present in fibers projecting into the region. In fact, Akutagawa and Konishi (1998) describe “many” labeled cells at day 20, and a transition at day 65 to primarily labeling fibers. Both that study and the present are consistent with BDNF playing a role in sensorimotor integration of song. However, our work also indicates a function in maintenance of adult song, whereas data obtained by Akutagawa and Konishi (1998) suggest that BDNF in HVC may no longer be critical.

A variety of studies provides support for an adult function of BDNF in HVC, and suggests that it is integrally related to song production. In fact, this protein may both influence and be influenced by the behavior. For example, testosterone-induced song by female canaries requires BDNF signaling in HVC (Hartog et al., 2009). Studies examining the expression of immediate early genes (Kimpö and Doupe, 1997, Velho et al., 2005) and using electrophysiological recording (Crandall et al., 2007, Day et al., 2009) document increases

in HVC neuronal activity in juvenile and adult male zebra finches with singing. In adult canary, song production can also increase BDNF expression and cell survival in HVC (Li et al., 2000). Thus, the enhanced BDNF expression we detected in males during the period of sensorimotor integration and into adulthood could result from this behavior. Similarly, the lack of distinct expression in the HVC of female zebra finches may be due to the absence of singing behavior. The increased expression in males may also be the result of individuals hearing their own song. A bird's own song is more effective than their tutor's song in stimulating HVC neural activity, particularly in young birds (Margoliash, 1983, Volman, 1993, Reiner et al., 2004, Nick and Konishi, 2005), suggesting that when juvenile males practice singing and hear their own song, it may increase neuronal activity and BDNF syntheses in HVC. This activity may be associated with the strength of song learning (Hartog et al., 2009, Bolhuis et al., 2012).

The pattern of BDNF labeling across the two sexes suggests that BDNF may not play a primary role in sexual differentiation of HVC morphology. Immunohistochemical labeling of BDNF defines HVC at d25, but not in older females, despite the fact that the brain region is clearly identifiable with a Nissl stain throughout juvenile development and adulthood. Even at this early age, males on average have more than twice the number of BDNF+ cells compared to females. These data are consistent with our earlier work (Tang and Wade, 2012) and suggest that this effect could be a result, rather than a cause, of the substantial sex difference in total cell number that exists by this age (Kirn and DeVoogd, 1989). The rate of overall cell loss in the female HVC is particularly high from d20 to d30, peaking around d25 (Kirn and DeVoogd, 1989). This developmental pattern supports the idea that the decline in BDNF labeling in females is due to the decrease in cells in females during this period. Previous research on BDNF mRNA in the juvenile HVC is generally consistent with the present data. Little, if any, expression was detected in the HVC of d20 and d35 female zebra finches, while expression was clear in d35 males (Dittrich et al., 1999). Collectively, the data imply that BDNF expression becomes prominent in the HVC of males between d20 and d25, and the disappearance of a BDNF-defined HVC in females older than d25 results from either apoptosis of BDNF expressing cells or decrease of BDNF synthesis within remaining cells which become undetectable in current study. Further investigations are needed to investigate those possibilities.

The pattern in RA is somewhat different than in HVC, with the total number of BDNF+ cells in the male RA increasing steadily across development and peaking at day 65 rather than in adulthood. In females, the quantity of these cells decreased gradually, and the labeling eventually became indistinguishable from the surrounding tissue as they reached maturity. Our previous work documented a sex difference in RA at d25, and the current study showed the same pattern with the average total number of BDNF+ cells in males almost 2-fold higher than females. While this effect did not reach statistical significance in the present study with a Bonferroni correction, the results support the idea of more BDNF+ cells in the RA of males compared to females as early as d25 and certainly at d35. The peak of BDNF labeling at day 65 agrees with RA data from Akutagawa and Konishi (1998). However, as in HVC, the labeling described in their paper declined to baseline in adulthood. The difference in adulthood between the two studies could reflect the idea that we assessed only cell bodies and their study measured relative optical density across the brain region. However, our Western blot data from males are also inconsistent with a decrease in adults. The punches from which we extracted protein included both fibers projecting into regions and the cell bodies originating there. Neither the pro nor mature form of BDNF decreased in adulthood compared to post-hatching day 25. In RA, relative levels of both forms, corrected for actin, were equivalent in the juveniles and adults (both $t_{14} < 1.06$, $p > 0.308$). In HVC, proBDNF was very similar at the two ages ($t_{14}=0.344$, $p=0.736$), and mBDNF exhibited a trend for an *increase* in adults (on average 2.1-fold; $t_{14}=1.949$, $p=0.072$).

Our results on protein also differ from quantification of BDNF mRNA (Dittrich et al., 1999), which was not detected in the RA of either sex at day 20 or d35. Results from Akutagawa and Konishi (1998) are consistent with the mRNA data; overall optical density in RA was lower than surrounding tissue at day 20, but substantial at day 45. The trajectory of the change between these two points is unknown. It is not clear how to bring together these results, although the discrepancies for mRNA vs. protein could exist for either technical (assay sensitivity) or biological (*e.g.*, rate of translation or turnover) reasons.

Our results on BDNF protein expression in RA parallel studies showing some cell addition in the male and primarily loss in the female RA as animals mature, especially at 30 days after hatching (Konishi and Akutagawa, 1985, Kirn and DeVoogd, 1989). In males only, HVC fibers enter RA around d30 (Konishi and Akutagawa, 1985). At this age, a substantial number of RA-projecting neurons in the male HVC express BDNF mRNA (Dittrich et al., 1999), suggesting that transport of BDNF from HVC to RA might be important for cell survival in RA. In parallel, atrophy of female RA might be due to a lack of BDNF release from the terminals of HVC projections that do not enter RA. Manipulations of local levels of BDNF should be done to test these hypotheses.

The developmental changes in immunohistochemical detection of BDNF in HVC and RA led us to question whether they reflected the pro or mature form of the peptide. The proBDNF and mBDNF peptides appear to facilitate opposite biological functions through different receptor systems (p75NTR and trkB, respectively; Lu et al., 2005). Neural activity can also modulate relative expression of pro vs. mBDNF. For example, in the rodent hippocampus, high frequency stimulation increases extracellular mBDNF whereas low frequency stimulation increases extracellular proBDNF but not mBDNF (Nagappan et al., 2009). Similarly, activity dependent conversion of pro to mBDNF in *Xenopus* motoneurons appears to regulate the process of synapse elimination, with proBDNF mediating retraction of relatively inactive synapses and mBDNF supporting stabilization of active ones (Je et al., 2012).

In the present study, adult males exhibited a significant increase in the ratio of mBDNF to proBDNF compared to d25 males in HVC but not RA. The result suggests a regionally specific shift as these animals mature, which may be important for song crystallization or maintenance of HVC structure or both. This increase in the mBDNF:proBDNF ratio could result from HVC neuronal activity induced by singing, an idea that warrants investigation. As proBDNF can be cleaved both intracellularly (Seidah et al., 1996, Mowla et al., 2001) and extracellularly (Lee et al., 2001) into mBDNF, more detailed studies with form-specific antibodies are also needed to evaluate more detailed avenues regarding mechanism. Regardless, our results for HVC are similar to a transition detected in the mouse brain, in which the highest expression of proBDNF and the p75NTR occur in perinatal animals and then decline in adulthood, whereas mBDNF becomes the major isoform in the adult (Yang et al., 2009).

LMAN and Area X

Similar to HVC, BDNF labeling defined LMAN in males at all ages and in females only at d25. However, effects of age within males and of sex at d25 on the total number of BDNF+ cells were not detected in this brain region. The similarity of early BDNF expression in males and females parallels the fact that, unlike other song nuclei, LMAN does not exhibit major sex differences in overall morphology (Bottjer et al., 1985, Bottjer and Sengelaub, 1989, Johnson and Bottjer, 1992, Nixdorf-Bergweiler, 1996). Early in juvenile development, both male and female zebra finches appear to form a memory of their tutor's song (Miller, 1979, Nixdorf-Bergweiler, 2001). It is not clear whether LMAN is involved in this process, but if so, the equivalent BDNF expression we detected at d25 in the two sexes might support

or result from it. In parallel, the qualitative change in expression resulting in lack of a definable border of LMAN based on immunohistochemical BDNF labeling in older females might due to their lack of song development. These results suggest that if BDNF plays a functional role in the LMAN of females it is more likely to be involved in the formation of song memories (which begins around d20) rather than singing behavior (which occurs later). In contrast, the consistent number of BDNF+ cells we detected in the male LMAN across ages suggests an ongoing role or set of roles. This idea is consistent with data indicating that this brain region plays a role in plasticity during both song development and in adulthood (Johnson and Bottjer, 1992, McDonald and Kirn, 2012 and references therein).

Like LMAN, Area X plays a crucial role in song learning during early development (Konishi, 1994, Margoliash, 1997), so potential functions of BDNF in this process are parallel. Overall, the number of cells in Area X increases from post-hatch day 10 to day 40 in male zebra finches (Nordeen and Nordeen, 1988a, Burek et al., 1991). The lack of an effect of age in the present study suggests that these newly added cells may not express BDNF and that any role this peptide might have in morphological development likely occurs earlier than our first point of analysis at day 25. One possibility relates to the innervation of this area from HVC, which occurs from d20 to d25 (Nordeen and Nordeen, 1997). While a considerable number (16%) of HVC neurons project to Area X, only about 6% of those neurons are born after d20 (Nordeen and Nordeen, 1988b).

Similar to HVC and RA, Akutagawa and Konishi (1998) observed developmental changes of BDNF labeling in LMAN and Area X, including decreases in adulthood to levels present in surrounding tissue. Here too, our results differ. The collection of disparate results raises the question of the specificity of antibody used by Akutagawa and Konishi (1998). Limited information is available on this reagent, and its validation involved only omitting the primary or replacing it with rabbit IgG. Johnson et al. (2000) suggest that at least some of the labeling detected by Akutagawa and Konishi (1998) is endogenous biotin, rather than BDNF. The antibody used in the present study was validated by Western blot analysis, and included a preadsorption control for both that and the immunohistochemistry (Tang and Wade, 2012), which provide more confidence that the labeling is specific to the peptide of interest.

Conclusions

The present study identified sex differences in BDNF protein expression across HVC, RA, LMAN and Area X, and developmental changes in HVC and RA. Collectively the data suggest that this peptide is of potential importance in the functional maturation of the motor pathway involved in song production. Particularly in HVC, our results support the idea of relationships of BDNF to changes in neuronal activity and behavior, which should be investigated in future studies.

Acknowledgments

This work was supported by a grant from the National Institutes of Health R01-MH096705 to J.W.

References

- Abraham WC, Williams JM. LTP maintenance and its protein synthesis-dependence. *Neurobiol Learn Mem.* 2008; 89:260–268. [PubMed: 17997332]
- Adkins-Regan E. Testosterone increases singing and aggression but not male-typical sexual partner preference in early estrogen treated female zebra finches. *Horm Behav.* 1999; 35:63–70. [PubMed: 10049604]

- Adkins-Regan E, Ascenzi M. Sexual differentiation of behavior in the zebra finch: effect of early gonadectomy or androgen treatment. *Horm Behav.* 1990; 24:114–127. [PubMed: 2328966]
- Adkins-Regan E, Mansukhani V, Seiwert C, Thompson R. Sexual differentiation of brain and behavior in the zebra finch: critical periods for effects of early estrogen treatment. *J Neurobiol.* 1994; 25:865–877. [PubMed: 8089662]
- Aguiarre CC, Baudry M. Progesterone reverses 17 β -estradiol-mediated neuroprotection and BDNF induction in cultured hippocampal slices. *Eur J Neurosci.* 2009; 29:447–454. [PubMed: 19175406]
- Akutagawa E, Konishi M. Transient expression and transport of brain-derived neurotrophic factor in the male zebra finch's song system during vocal development. *Proc Natl Acad Sci U S A.* 1998; 95:11429–11434. [PubMed: 9736753]
- Bolhuis JJ, Gobes SM, Terpstra NJ, den Boer-Visser AM, Zandbergen MA. Learning-related neuronal activation in the zebra finch song system nucleus HVC in response to the bird's own song. *PLoS One.* 2012; 7:e41556. [PubMed: 22848527]
- Botter SW, Glaessner SL, Arnold AP. Ontogeny of brain nuclei controlling song learning and behavior in zebra finches. *J Neurosci.* 1985; 5:1556–1562. [PubMed: 4009245]
- Botter SW, Sengelaub DR. Cell death during development of a forebrain nucleus involved with vocal learning in zebra finches. *J Neurobiol.* 1989; 20:609–618. [PubMed: 2794995]
- Brainard MS, Doupe AJ. Auditory feedback in learning and maintenance of vocal behaviour. *Nat Rev Neurosci.* 2000; 1:31–40. [PubMed: 11252766]
- Brenowitz EA, Arnold AP, Levin RN. Neural correlates of female song in tropical duetting birds. *Brain Res.* 1985; 343:104–112. [PubMed: 4041847]
- Burek MJ, Nordeen KW, Nordeen EJ. Neuron loss and addition in developing zebra finch song nuclei are independent of auditory experience during song learning. *J Neurobiol.* 1991; 22:215–223. [PubMed: 1890414]
- Cohen-Cory S, Kidane AH, Shirkey NJ, Marshak S. Brain-derived neurotrophic factor and the development of structural neuronal connectivity. *Dev Neurobiol.* 2010; 70:271–288. [PubMed: 20186709]
- Cooke B, Hegstrom CD, Villeneuve LS, Breedlove SM. Sexual differentiation of the vertebrate brain: principles and mechanisms. *Front Neuroendocrinol.* 1998; 19:323–362. [PubMed: 9799588]
- Crandall SR, Aoki N, Nick TA. Developmental modulation of the temporal relationship between brain and behavior. *J Neurophysiol.* 2007; 97:806–816. [PubMed: 17079340]
- Cunha C, Brambilla R, Thomas KL. A simple role for BDNF in learning and memory? *Front Mol Neurosci.* 2010; 3:1. [PubMed: 20162032]
- Day NF, Kinnischtzke AK, Adam M, Nick TA. Daily and developmental modulation of “premotor” activity in the birdsong system. *Dev Neurobiol.* 2009; 69:796–810. [PubMed: 19650042]
- De Vries GJ. Minireview: Sex differences in adult and developing brains: compensation, compensation, compensation. *Endocrinology.* 2004; 145:1063–1068. [PubMed: 14670982]
- De Vries GJ, Rissman EF, Simerly RB, Yang LY, Scordalakes EM, Auger CJ, Swain A, Lovell-Badge R, Burgoyne PS, Arnold AP. A model system for study of sex chromosome effects on sexually dimorphic neural and behavioral traits. *J Neurosci.* 2002; 22:9005–9014. [PubMed: 12388607]
- Dittrich F, Feng Y, Metzdorf R, Gahr M. Estrogen-inducible, sex-specific expression of brain-derived neurotrophic factor mRNA in a forebrain song control nucleus of the juvenile zebra finch. *Proc Natl Acad Sci U S A.* 1999; 96:8241–8246. [PubMed: 10393979]
- Doupe AJ, Solis MM, Kimpo R, Boettiger CA. Cellular, circuit, and synaptic mechanisms in song learning. *Ann N Y Acad Sci.* 2004; 1016:495–523. [PubMed: 15313792]
- Gibbs RB. Treatment with estrogen and progesterone affects relative levels of brain-derived neurotrophic factor mRNA and protein in different regions of the adult rat brain. *Brain Res.* 1999; 844:20–27. [PubMed: 10536257]
- Hartog TE, Dittrich F, Pieneman AW, Jansen RF, Frankl-Vilches C, Lessmann V, Lilliehook C, Goldman SA, Gahr M. Brain-derived neurotrophic factor signaling in the HVC is required for testosterone-induced song of female canaries. *J Neurosci.* 2009; 29:15511–15519. [PubMed: 20007475]
- Je HS, Yang F, Ji Y, Nagappan G, Hempstead BL, Lu B. Role of pro-brain-derived neurotrophic factor (proBDNF) to mature BDNF conversion in activity-dependent competition at developing

- neuromuscular synapses. *Proc Natl Acad Sci U S A*. 2012; 109:15924–15929. [PubMed: 23019376]
- Johnson F, Bottjer SW. Growth and regression of thalamic efferents in the song-control system of male zebra finches. *J Comp Neurol*. 1992; 326:442–450. [PubMed: 1469121]
- Johnson F, Hohmann SE, DiStefano PS, Bottjer SW. Neurotrophins suppress apoptosis induced by deafferentation of an avian motor-cortical region. *J Neurosci*. 1997; 17:2101–2111. [PubMed: 9045737]
- Johnson F, Norstrom E, Soderstrom K. Increased expression of endogenous biotin, but not BDNF, in telencephalic song regions during zebra finch vocal learning. *Dev Brain Res*. 2000; 120:113–123. [PubMed: 10727739]
- Kimpo RR, Doupe AJ. FOS is induced by singing in distinct neuronal populations in a motor network. *Neuron*. 1997; 18:315–325. [PubMed: 9052801]
- Kim JR, DeVoogd TJ. Genesis and death of vocal control neurons during sexual differentiation in the zebra finch. *J Neurosci*. 1989; 9:3176–3187. [PubMed: 2795159]
- Konishi M. An outline of recent advances in birdsong neurobiology. *Brain Behav Evol*. 1994; 44:279–285. [PubMed: 7842286]
- Konishi M. From central pattern generator to sensory template in the evolution of birdsong. *Brain Lang*. 2010; 115:18–20. [PubMed: 20955898]
- Konishi M, Akutagawa E. Neuronal growth, atrophy and death in a sexually dimorphic song nucleus in the zebra finch brain. *Nature*. 1985; 315:145–147. [PubMed: 3990816]
- Lee R, Kermani P, Teng KK, Hempstead BL. Regulation of cell survival by secreted proneurotrophins. *Science*. 2001; 294:1945–1948. [PubMed: 11729324]
- Lessmann V, Brigadski T. Mechanisms, locations, and kinetics of synaptic BDNF secretion: an update. *Neurosci Res*. 2009; 65:11–22. [PubMed: 19523993]
- Li XC, Jarvis ED, Alvarez-Borda B, Lim DA, Nottebohm F. A relationship between behavior, neurotrophin expression, and new neuron survival. *Proc Natl Acad Sci U S A*. 2000; 97:8584–8589. [PubMed: 10890902]
- Lu B, Pang PT, Woo NH. The yin and yang of neurotrophin action. *Nat Rev Neurosci*. 2005; 6:603–614. [PubMed: 16062169]
- Lu Y, Christian K, Lu B. BDNF: a key regulator for protein synthesis-dependent LTP and long-term memory? *Neurobiol Learn Mem*. 2008; 89:312–323. [PubMed: 17942328]
- Margoliash D. Acoustic parameters underlying the responses of song-specific neurons in the white-crowned sparrow. *J Neurosci*. 1983; 3:1039–1057. [PubMed: 6842281]
- Margoliash D. Functional organization of forebrain pathways for song production and perception. *J Neurobiol*. 1997; 33:671–693. [PubMed: 9369466]
- McAllister AK, Katz LC, Lo DC. Neurotrophins and synaptic plasticity. *Annu Rev Neurosci*. 1999; 22:295–318. [PubMed: 10202541]
- McDonald KS, Kirn JR. Anatomical plasticity in the adult zebra finch song system. *J Comp Neurol*. 2012; 520:3673–3686. [PubMed: 22473463]
- Miller DB. Long-term recognition of father's song by female zebra finches. *Nature*. 1979; 280:3.
- Miranda RC, Sohrabji F, Toran-Allerand CD. Presumptive estrogen target neurons express mRNAs for both the neurotrophins and neurotrophin receptors: A basis for potential developmental interactions of estrogen with the neurotrophins. *Mol Cell Neurosci*. 1993; 4:510–525. [PubMed: 19912958]
- Mooney R, Rao M. Waiting periods versus early innervation: the development of axonal connections in the zebra finch song system. *J Neurosci*. 1994; 14:6532–6543. [PubMed: 7965057]
- Mowla SJ, Farhadi HF, Pareek S, Atwal JK, Morris SJ, Seidah NG, Murphy RA. Biosynthesis and post-translational processing of the precursor to brain-derived neurotrophic factor. *J Biol Chem*. 2001; 276:12660–12666. [PubMed: 11152678]
- Nagappan G, Zaitsev E, Senatorov VV Jr, Yang J, Hempstead BL, Lu B. Control of extracellular cleavage of ProBDNF by high frequency neuronal activity. *Proc Natl Acad Sci U S A*. 2009; 106:1267–1272. [PubMed: 19147841]

- Nick TA, Konishi M. Neural song preference during vocal learning in the zebra finch depends on age and state. *J Neurobiol.* 2005; 62:231–242. [PubMed: 15459895]
- Nixdorf-Bergweiler BE. Divergent and parallel development in volume sizes of telencephalic song nuclei in male and female zebra finches. *J Comp Neurol.* 1996; 375:445–456. [PubMed: 8915841]
- Nixdorf-Bergweiler BE. Enlargement of neuronal somata in the LMAN coincides with the onset of sensorimotor learning for song. *Neurobiol Learn Mem.* 1998; 69:258–273. [PubMed: 9707489]
- Nixdorf-Bergweiler BE. Lateral magnocellular nucleus of the anterior neostriatum (LMAN) in the zebra finch: neuronal connectivity and the emergence of sex differences in cell morphology. *Microsc Res Tech.* 2001; 54:335–353. [PubMed: 11668647]
- Nordeen EJ, Grace A, Burek MJ, Nordeen KW. Sex-dependent loss of projection neurons involved in avian song learning. *J Neurobiol.* 1992; 23:671–679. [PubMed: 1279116]
- Nordeen EJ, Nordeen KW. Sex and regional differences in the incorporation of neurons born during song learning in zebra finches. *J Neurosci.* 1988a; 8:2869–2874. [PubMed: 3411358]
- Nordeen KW, Nordeen EJ. Projection neurons within a vocal motor pathway are born during song learning in zebra finches. *Nature.* 1988b; 334:149–151. [PubMed: 3386754]
- Nordeen KW, Nordeen EJ. Anatomical and synaptic substrates for avian song learning. *J Neurobiol.* 1997; 33:532–548. [PubMed: 9369458]
- Reiner A, Perkel DJ, Bruce LL, Butler AB, Csillag A, Kuenzel W, Medina L, Paxinos G, Shimizu T, Striedter G, Wild M, Ball GF, Durand S, Gunturkun O, Lee DW, Mello CV, Powers A, White SA, Hough G, Kubikova L, Smulders TV, Wada K, Dugas-Ford J, Husband S, Yamamoto K, Yu J, Siang C, Jarvis ED. Revised nomenclature for avian telencephalon and some related brainstem nuclei. *J Comp Neurol.* 2004; 473:377–414. [PubMed: 15116397]
- Sakuma Y. Gonadal steroid action and brain sex differentiation in the rat. *J Neuroendocrinol.* 2009; 21:410–414. [PubMed: 19226349]
- Seidah NG, Benjannet S, Pareek S, Chretien M, Murphy RA. Cellular processing of the neurotrophin precursors of NT3 and BDNF by the mammalian proprotein convertases. *FEBS Lett.* 1996; 379:247–250. [PubMed: 8603699]
- Simerly RB. Wired for reproduction: organization and development of sexually dimorphic circuits in the mammalian forebrain. *Ann Rev Neurosci.* 2002; 25:507–536. [PubMed: 12052919]
- Simpson HB, Vicario DS. Early estrogen treatment of female zebra finches masculinizes the brain pathway for learned vocalizations. *J Neurobiol.* 1991; 22:777–793. [PubMed: 1765783]
- Sohrabji F, Lewis DK. Estrogen-BDNF interactions: implications for neurodegenerative diseases. *Front Neuroendocrinol.* 2006; 27:404–414. [PubMed: 17069877]
- Solum DT, Handa RJ. Estrogen regulates the development of brain-derived neurotrophic factor mRNA and protein in the rat hippocampus. *J Neurosci.* 2002; 22:2650–2659. [PubMed: 11923430]
- Tang YP, Wade J. 17 β -Estradiol regulates the sexually dimorphic expression of BDNF and TrkB proteins in the song system of juvenile zebra finches. *PLoS One.* 2012; 7:e43687. [PubMed: 22952738]
- Velho TA, Pinaud R, Rodrigues PV, Mello CV. Co-induction of activity-dependent genes in songbirds. *Eur J Neurosci.* 2005; 22:1667–1678. [PubMed: 16197507]
- Volman SF. Development of neural selectivity for birdsong during vocal learning. *J Neurosci.* 1993; 13:4737–4747. [PubMed: 8229196]
- Wade J. Zebra finch sexual differentiation: the aromatization hypothesis revisited. *Microsc Res Tech.* 2001; 54:354–363. [PubMed: 11668648]
- Wade J, Arnold AP. Sexual differentiation of the zebra finch song system. *Ann N Y Acad Sci.* 2004; 1016:540–559. [PubMed: 15313794]
- Yang J, Siao CJ, Nagappan G, Marinic T, Jing D, McGrath K, Chen ZY, Mark W, Tessarollo L, Lee FS, Lu B, Hempstead BL. Neuronal release of proBDNF. *Nat Neurosci.* 2009; 12:113–115. [PubMed: 19136973]

Highlights

- BDNF protein expression is sexually dimorphic in the developing zebra finch brain.
- In HVC and RA, BDNF increases in males and decreases in females as they mature.
- The ratio of mBDNF/proBDNF in HVC increases in males as they mature.

Figure 1.

Photographs from representative animals showing immunohistochemical labeling of BDNF in HVC and adjacent sections with Nissl stain (top two rows). Arrows point to the ventral border of HVC, where it is visible. Insets show higher magnification images from the center of each HVC, with arrowheads pointing to the labeled cells that were counted (D25 male and female, D35 male). The inset in the D35 female also shows two BDNF+ cells, however they were not quantified because labeling in this brain region was not distinct from the surrounding tissue in females at this age. Scale bar = 200 μ m for each of the large photos; 20 μ m for all insets. The estimated total number of BDNF+ cells (mean + SEM) in HVC is depicted in the histogram (bottom). A one-way ANOVA was used to analyze the effect of age within males; a t-test evaluated the effect of sex at d25. Lowercase letters indicate significant differences between ages within each sex. * represents a significant effect of sex at post-hatching day 25. NVB: no visible border in females at 35 days of age through adulthood.

Figure 2.

Photographs from representative animals showing immunohistochemical labeling in RA and adjacent sections with Nissl stain (top two rows). Arrows point to the border of RA where it is visible. Scale bar = 200 μ m for all images. The estimated total number of BDNF+cells (mean + SEM) in RA is depicted in the histogram (bottom). A two-way ANOVA (sex $\times$ age) was used to analyze labeling in juveniles (post-hatching days 25 to 65). Lowercase letters indicate significant differences between ages within each sex. * represents a significant sex differences within an age. NVB: no visible border in adult females.

Figure 3.

Photographs from representative animals showing immunohistochemical labeling of BDNF in LMAN and adjacent sections with Nissl stain (top two rows). Arrows point to the border of LMAN where it is visible. Scale bar = 200 μ m for all images. The estimated total number of BDNF+ cells (mean + SEM) in LMAN is depicted in the histogram (bottom). NVB: no visible border in females at d35 through adulthood.

Figure 4. Western blot analyses of mature (mBDNF) and proBDNF of 25-day-old and adult male zebra finches. Data (mean + SEM) and representative images from each group are from the punches of HVC (left) and RA (right). * indicates a significant difference in the ratio of mBDNF:proBDNF between day 25 and adult males in HVC.