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Tracheosyringeal Nerve Transection in Juvenile Male Zebra Finches Decreases BDNF in HVC and RA and the Projection Between Them

Yu Ping Tang^{1,3,*} and Juli Wade^{1,2,3}

¹Michigan State University, Department of Psychology, East Lansing, MI 48824

²Michigan State University, Department of Zoology, East Lansing, MI 48824

³Michigan State University, Department of Neuroscience Program, East Lansing, MI 48824

Abstract

This study investigated relationships among disruption of normal vocal learning, brain derived neurotrophic factor (BDNF), and the morphology of song nuclei in juvenile male zebra finches. The tracheosyringeal nerves were bilaterally transected at post-hatching day 20–25, so that the animals could not properly develop species-typical vocalizations. BDNF protein and the projection from HVC to the robust nucleus of the arcopallium (RA) were quantified during the sensorimotor integration phase of song development. The manipulation decreased the number of BDNF cells in HVC and RA, the volume of these areas defined by BDNF labeling, and the projection from HVC to RA. BDNF was not affected in Area X or the lateral magnocellular nucleus of the anterior nidopallium (LMAN). Thus, inhibition of a bird's ability to practice and/or to hear its own typically developing song during development specifically diminishes BDNF expression in cortical motor regions required for song production.

Keywords

Song System; Sexual Differentiation; Neurotrophin; Motor System

1. Introduction

Songbirds provide an excellent model to study the relationship between the development of brain morphology and function. Song learning in juveniles and its production in adults are controlled by a well-defined set of neural structures [43]. These regions include the HVC (proper name) and robust nucleus of the arcopallium (RA), cortical components in the motor pathway for song production. HVC projects to RA, which in turn innervates a portion of the

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*Corresponding Author: Yu Ping Tang, Michigan State University, East Lansing, MI 48824-1101, Phone: 517-432-5113, tangyupi@msu.edu.

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hypoglossal nucleus, which controls the syrinx muscles via the tracheosyringeal nerve. Area X and the lateral magnocellular nucleus of the anterior nidopallium (LMAN) are more important for song learning and plasticity than motor control of the vocalizations [3, 26, 42].

A variety of parallels exists between the morphology of these brain regions and aspects of the behavior. For example, in many species males sing more frequently or more complex songs than females and the song nuclei are larger in males [43]. In duetting species, these brain regions tend not to be sexually dimorphic ([6], but see [13]). In seasonally breeding songbirds, volumes of song nuclei, as well as the medial preoptic nucleus, are often larger during the breeding season, when males sing a more stereotyped song with greater frequency [7, 15, 18, 30, 32, 35].

Juvenile zebra finches learn songs from adult tutors [5, 27]. In males, the process begins about three weeks after hatching. From approximately day 20–60, they form a template of tutor song and practice during the sensorimotor integration period (days 30 to 90), and eventually match their songs to the stored templates [31, 33, 39, 45]. Songs become crystallized by 90 days after hatching [27]. The song nuclei in zebra finches are first visible around 9–11 days of age [12, 17], and they mature through about day 50. During that period, cells are added to the male HVC from about days 20 to 50 [19], and the projection from HVC enters RA in males at about 30 days of age [21, 28].

The song nuclei not only regulate vocal learning and production, but they are also responsive to the behavior. For example, auditory exposure to bird's own song stimulates HVC activity more effectively than its tutor's song, especially in juvenile males [24, 29, 40], consistent with the idea that the neural activity associated with the practice and/or hearing one's own song facilitates the learning process [4].

Several pieces of evidence suggest that brain derived neurotrophic factor (BDNF) may facilitate song learning and/or that this protein could be increased by males producing and/or hearing their own song. Functions of BDNF include facilitating cell proliferation and survival, axon guidance, as well as learning and memory formation [9, 25]. Transient expression of the protein has been found in the song nuclei of male zebra finches during sensorimotor integration [2]. Dittrich, et al. [10] also reported BDNF mRNA expression in the HVC of juvenile male zebra finches, in which BDNF facilitates cell survival [16]. The high affinity receptors for BDNF are also present during this developmental period in the forebrain song control nuclei [10, 37, 41]. We reported a male-specific developmental increase of BDNF protein expression in the HVC and RA of juvenile zebra finches during the period that males learn song [38].

As an initial means of addressing the idea that the behavioral and perceptual functions that occur during sensorimotor integration modulate BDNF, here we investigated whether disruption of the normal process of vocal development affects BDNF protein expression. The tracheosyringeal nerves were transected prior to the time when males begin to produce song. In adulthood, this manipulation dramatically affects the overall structure of song and the characteristics of individual syllables [34, 46]. In juveniles, this surgery results in the production of extremely abnormal songs which contain simple syllables consisting of

harmonically related notes; this developing song is far less complex than the plastic song of unmanipulated birds and very different from the tutor's [36]. BDNF expression and morphology of the song circuit (size of brain regions and the extent of the projection from HVC to RA) were quantified following the nerve cut.

2. Materials and Methods

2.1 Animals

Zebra finches were raised in aviaries containing about seven adults of each sex and their offspring. A 12:12 light:dark cycle was maintained, and seed and water were available *ad libitum*. Once a week, the diet was supplemented with bread mixed with hard-boiled chicken eggs, as well as spinach and oranges. Nests in each aviary were checked daily; the day a hatchling was found was considered day 1 (d1). DNA extractions from toe clips used as unique identifiers on the day of hatching were processed using PCR to identify each bird's genetic sex [1]. Only males were used in the present study. All procedures were conducted in accordance with NIH guidelines and approved by the Michigan State University IACUC.

2.2 Treatment and Tissue Collection

Tracheosyringeal nerve transections (TSNX) were performed on male zebra finches (n=8) between post-hatching d20–25. Birds were anesthetized under isoflurane (Abbot Laboratories, Abbot Park, IL), and an incision was made in the skin of the neck to expose the trachea. The nerves run along its sides, and 3–5 mm was removed bilaterally. Control birds (SHAM; n=8) received the surgical manipulations without cutting the nerves. After recovery, birds were returned to their home aviaries. Between 30–34 days following the nerve manipulation, 1 µl of 0.1 % 1,1'-Diocetadecyl-3,3',3'-Tetramethylindocarbocyanine Perchlorate (DiI, D-282, Molecular Probes, Inc., Eugene, OR) diluted in DMSO was injected into the HVC on the right side of each bird's brain to assess the projection from HVC to RA. The left side remained unmanipulated so that morphology and BDNF expression could be evaluated in intact sections of HVC. During this procedure, animals were anesthetized with isoflurane and placed in a stereotaxic apparatus. An incision was made in the skin over the skull. The bifurcation of the sinus at the point where the telencephalic hemispheres meet the cerebellum was used as the origin, and injection coordinates were: 0.7 mm posterior, 2.5 mm lateral, and 0.7 mm ventral from the top of the brain.

Brains and tracheas were collected 7 days after the DiI injection following rapid decapitation; all birds were 60–65 days old. The left and right hemispheres were separated, frozen in ice cold 2-methylbutane and stored at –80 °C. Sagittal sections (20 µm) were cut through the entire brain on a cryostat in 6 series and stored at –80 °C with desiccant. One series from the left side of the brain was used for BDNF immunohistochemistry; a second series was stained with cresyl violet. Two series from the right side were used – one for analysis of DiI in RA and the other to confirm the location of the HVC injection in nissl-stained sections. Tracheas were also examined under a dissecting microscope to confirm that the nerve cuts were maintained.

2.3 BDNF Immunohistochemistry

Procedures were described and the primary antibody was validated in Tang and Wade [37]. Briefly, tissue from the left side of the brain was rinsed in 0.1 M phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde, and washed again in PBS. Brain sections were treated with 0.9% H₂O₂/methanol followed by 3% normal goat serum in PBS with 0.3% Triton X-100, and incubated overnight at 4°C with the BDNF antibody (0.5 µg/ml; sc-546; Santa Cruz Biotechnology, Santa Cruz, CA) in 0.1 M PBS containing 0.3% Triton X-100 and 3% normal goat serum. A biotin-conjugated goat anti-rabbit secondary antibody (1 µg/ml; Vector Labs, Burlingame, CA) and *Elite* ABC reagents (Vector Labs, Burlingame, CA) were used, followed by diaminobenzidine containing 0.0024% hydrogen peroxide to produce a brown reaction product.

2.4 Analysis of cell number and brain region volume

HVC, RA, LMAN, and Area X were analyzed. For LMAN and Area X, tissue from all animals was quantified. In a few cases, tissue could not be quantified due to damage; final sample sizes for HVC were 8 TSNX and 6 SHAM animals, and 7 TSNX and 8 SHAM birds for RA. The optical fractionator function in StereoInvestigator (Microbrightfield Inc., Williston, VT) was used by an individual blind to the manipulation of each bird to estimate the total number of BDNF labeled cells. As in Tang and Wade [37], the border of each region defined by BDNF labeling was traced in all sections in which they were present. All cells exhibiting neuronal morphology and a clear reaction product were manually tallied in the 30µm × 30µm counting frame, which provided both an estimated total number of cells within each brain region and the overall volume of each song nucleus evaluated (Gundersen Coefficient 0.1).

Based on the results (see below), the same procedure was used in adjacent nissl-stained sections to quantify the volumes of HVC and RA. Although individual cells were not counted, the outline of each song nucleus was traced in each section in which it was visible to produce a volume estimated by the software based on these cross sectional areas and the distance between them.

The estimated total number of BDNF positive (BDNF+) cells and the volume defined by this labeling were each compared between the SHAM and TSNX groups using t-tests separately for the four song nuclei (SPSS 20, IBM, Armonk, NY). T-tests were also used to compare the effects of the manipulation on the nissl-defined volumes of each of HVC and RA. Because analyses of the volumes of HVC and RA were calculated using two different markers, α was set at $0.05/2 = 0.025$ (Bonferroni correction).

2.5 DiI Tracing

The RA from each section in one series from the right side of the brain ($n = 7$ per group) was captured using Image J (National Institutes of Health) under a TRITC filter. The border of RA was traced to obtain its cross-sectional area. The 'threshold' function was used to mark the DiI labeling and the area covered by it within RA was also generated by the software. The percentage of RA covered by DiI was then calculated, and a mean determined for each bird. These values were compared between the TSNX and SHAM groups by t-test.

An adjacent series of sections from the right side of each brain was stained with cresyl violet and viewed under brightfield illumination to confirm that the injections were all appropriately located in, and limited to, HVC. One bird receiving the nerve transection and three sham controls were eliminated from the analysis because the DiI injections either did not fill the majority of HVC or were not located completely within the brain region. Final sample sizes were 6 TSNX and 4 SHAM.

3. Results

In HVC, the TSNX manipulation decreased the estimated total number of BDNF+ cells compared to the SHAM procedure ($t_{1,12}=5.66$, $p<0.001$; Fig. 1). A parallel significant effect of treatment was detected on the volume of this brain region defined by BDNF labeling ($t_{1,12}=3.00$, $p=0.011$; $\alpha=0.025$). The number of BDNF+ cells ($t_{1,13}=4.41$, $p=0.001$) and the volume defined by them ($t_{1,13}=3.73$, $p=0.003$; $\alpha=0.025$) were also significantly decreased in RA of TSNX-treated birds (Fig. 1). No effects of the TSNX manipulation were detected on BDNF labeling in either LMAN (cell number: $t_{1,14}=1.85$, $p=0.085$; volume: $t_{1,14}=1.03$, $p=0.317$) or Area X (cell number: $t_{1,14}=0.60$, $p=0.561$; volume: $t_{1,14}=0.51$, $p=0.615$; not shown).

To get a sense of whether the effects in HVC and RA were specific to cells expressing BDNF, the volumes of these brain regions were compared across treatments in nissl-stained tissue. This measure did not differ significantly in either region (HVC: $t_{1,12}=0.39$, $p=0.704$; RA: $t_{1,13}=2.16$, $p=0.050$; $\alpha=0.025$; not shown).

TSNX surgery significantly decreased the percentage of the area covered by DiI in RA following injection into HVC ($t_{1,8}=4.541$, $p=0.002$; Fig. 2).

4. Discussion

Bilateral transection of the tracheosyringeal nerves significantly decreased the number of BDNF+ cells in both HVC and RA. These data are consistent with our hypothesis, that practicing and/or hearing one's own typically developing song increases BDNF in juvenile male zebra finches. Specific localization is suggested, as this effect was not detected in LMAN or Area X. This pattern of results indicates that expression of this protein is more directly affected in the motor pathway than in the anterior forebrain pathway, which is interesting in light of data suggesting that HVC is not required for 'subsinging' at 33–44 days of age although is important for normal production of plastic (post-hatching days 45–73) and mature song [3]. The present data suggest that neurochemical changes relating to song may be induced in this premotor region prior to the age at which it is necessary for vocal production. This idea warrants further investigation.

The change in BDNF expression could be either to reduced song production or auditory feedback, or both may be critical. As the TSNX birds could hear typical song of other conspecifics, including their tutors, the effects are not due to an absence of hearing song altogether. Data from birds that were deafened as juveniles suggest that vocal production may be more important than the auditory experience for structural change in the song system

[8]. Future studies should assess the quantity and quality of vocalizations produced by birds with TSNX manipulations in the context of BDNF expression in their song systems.

It will also be important to evaluate potential relationships to cell survival. Some effects on the recruitment of new neurons into HVC can be detected in birds that received unilateral tracheosyringeal nerve transection as juveniles [44]. However, the effects are transient and are not observed on the side of the cut nerve or in birds that were deafened or received bilateral axotomies. These data are consistent with those on the volume of song nuclei in the present study. That is, while the volumes of HVC and RA defined by BDNF labeling were decreased by the nerve cuts, the effect was not detected in nissl-stained tissue. They also parallel data of Solis and Doupe [36], who showed that the volume of nissl-stained RA is not affected in 60 day old males that received the nerve transection at days 26–33 post-hatching. Collectively, the data suggest a change in expression of the BDNF protein specifically due to nerve transection, rather than as a result of a change in the structure of these brain regions. Thus, while additional work is needed to draw a firm conclusion, the present study is consistent with the idea that BDNF expression was decreased in existing cells, and the manipulation may not have affected the recruitment or survival of cells more generally.

TSNX treatment also significantly diminished the projection from HVC into RA, as indicated by the percentage of area in RA covered by DiI. It is presently unclear whether BDNF expression in either HVC or RA has a causal relationship to the growth or maintenance of this projection. In juvenile of zebra finches [16] and mammalian species [9, 22], BDNF is important for cell survival and axon growth. Experiments are currently underway to determine whether BDNF supports the structure of this pathway in development.

In adult male canaries, singing facilitates survival of new HVC neurons. This behavior also increases BDNF expression in RA-projecting HVC neurons, and BDNF expression in HVC correlates positively with song rate [23]. In adult white crowned sparrows, infusion of BDNF into RA increases soma size and decreases the density of neurons [47]. Thus, relationships exist in adult birds among song production, BDNF expression and the morphology of the vocal motor pathway. These relationships now must be examined more fully during development. In addition, experimental up-regulation of BDNF in HVC during sensorimotor integration facilitates song learning [11]. The present and our previous [38] results suggest that BDNF protein expression in HVC and RA may play a role in song learning during sensorimotor integration, and perhaps in the maintenance of song and song system structure in adulthood.

One question that remains regarding the current work is whether the decreases in BDNF expression detected in HVC and RA were due to damage of the tracheosyringeal nerve in a manner that was independent of the ability of the bird to produce normal song. We are not aware of studies documenting changes in the expression of BDNF or other neurotrophic factors in the forebrain following peripheral nerve injury, although transient increases in motoneurons following axotomy are common in other species (for example, see [14, 20]).

In sum, the present data provide novel indications of potential relationships among song development, BDNF expression and the maturation of the neural circuit regulating production of these courtship vocalizations. Earlier research has shown that this protein can be transported from LMAN to RA during development, and that it can rescue cells in this circuit from apoptosis following deafferentation [16]. We now must determine the specific role(s) that BDNF may play in development of structure and function of the motor pathway including HVC and RA.

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Highlights

- Brain derived neurotrophic factor (BDNF) is expressed in the developing song system.
- Tracheosyringeal nerve transection in juvenile males decreases BDNF in HVC and RA.
- The manipulation does not affect BDNF in LMAN or Area X.

Figure 1.

BDNF labeling in HVC and RA. The photographs provide representative examples of immunohistochemistry; arrows point to the ventral border of HVC and outside edges of RA. Scale bar = 50 μ m for all images. The graphs indicate the estimated total number of BDNF+ cells within each brain region (top) and volume defined by this labeling (bottom; all mean + SEM). Asterisks indicate significant effects of treatment: *** $p < 0.001$, ** $p=0.003$, * $p=0.011$

Figure 2.

Tract tracing. (A) representative example of the DiI injection site in HVC; (B) adjacent nissl-stained section. The arrow shows the location of the manipulation, and arrowheads indicate the ventral border of HVC. Scale bar = 200 μm for these two images. DiI labeling in RA and adjacent nissl-stained sections are depicted in representative SHAM (C,D) and TSNX (E,F) animals. Arrowheads in these photographs point to the borders of RA; scale bar = 100 μm for all four images. The histogram depicts relative levels of DiI in RA for the two treatment groups (mean + SEM), ** p=0.002.