TrkB-like immunoreactivity in the song system of developing zebra finches

Juli Wade *

Departments of Psychology and Zoology, 235 Psychology Research Building, Michigan State University, East Lansing, MI 48824-1117, USA

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Abstract

The neural song system in zebra finches develops for approximately the first 2 months after hatching. During that time, male-biased sexual dimorphisms emerge in the volume of song control nuclei as well as in the number and size of neurons within them. Brain derived neurotrophic factor (BDNF) has been documented in song control nuclei at various stages of development. Its high affinity receptor (tyrosine kinase B; trkB) is also in the song system, at least at around 1 month of age. The present study was designed to more completely describe the timing and potential location of BDNF action by investigating trkB expression during sexual differentiation of the song control nuclei. The pattern of immunoreactivity to a trkB antibody was examined in male and female zebra finches at post-hatching days 3–60. Labeling in somata and neuropil appeared to define the telencephalic components of the motor pathway (high vocal center and robust nucleus of the archistriatum) for song production in males from days 30 to 60, and in females on days 45 and 60 (high vocal center). These results are consistent with the hypothesis that the receptor, and its ligand BDNF, play a role in processes related to song learning in both sexes, including perhaps the motor component exhibited by developing males. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Morphology of the neural song system of zebra finches develops from roughly post-hatching days 10–60 (Nixdorf-Bergweiler, 1996), although stabilization of singing behavior continues for another 20–30 days (Bottjer, 1997; Bottjer and Arnold, 1997). During the first 2 months after hatching, telencephalic regions including the high vocal center (HVC) and robust nucleus of the archistriatum (RA) form in both males and females and then sexually differentiate. The brain regions become substantially larger in volume, and eventually contain more neurons that are larger and have more extensive dendritic arborization in males than in females (Arnold, 1992, 1997). These morphological differences parallel a behavioral dimorphism; males sing and females do not (Arnold et al., 1996). A number of studies have shown that pharmacological treatment of females with estradiol in the first few weeks after hatching can increase the volume of song control regions, as well as the number and size of neurons within the brain areas (Arnold, 1992). This treatment also organizes the capacity for song production in adulthood (Gurney, 1982; Simpson and Vicario, 1991). However, the mechanisms involved in the normal development of both males and females have remained elusive. Gonadal hormones are unlikely to be directly responsible for the process of sexual differentiation (Wade and Arnold, 1996; Wade et al., 1996, 1999), but critical, non-steroidal factors have yet to be identified.

One approach to this problem is to investigate factors known to be estrogen-sensitive; the hypothesis being that estrogen treatment of females artificially upregulates proteins important for neural song system development that are normally high in males. Some neurotrophins, including nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF), and their high affinity receptors, tyrosine kinase A and B (trkA and trkB), respectively, interact with estradiol in mammalian systems (Gibbs and Pfaff, 1992; Gibbs et
ences and the developmental time course of $^{125}$I-NGF in telencephalic homogenates (Contreras and Wade, 1999) and BDNF mRNA expression in HVC specifically (Dittrich et al., 1999) have recently been shown to increase in response to estradiol treatment and decrease in response to treatment with the estrogen synthesis inhibitor Fadrozole. Thus, these neurotrophins and their receptors seem good candidates for factors involved in maturation of the neural song system. Unfortunately, a lack of substantial sex differences and the developmental time course of $^{125}$I-NGF binding suggest that trkA receptors are probably not critical to the sexual differentiation process (Contreras and Wade, 1999).

However, BDNF immunoreactivity and mRNA are present in the song control nuclei of males during development, and the neurotrophin can rescue neurons in RA from cell death induced by removing input from the lateral magnocellular nucleus of the anterior neostriatum (IMAN) (Johnson et al., 1997; Akutagawa and Konishi, 1998; Dittrich et al., 1999).

In canaries, BDNF infusion also increases the recruitment or survival of new neurons in adulthood (Rasika et al., 1999). TrkB mRNA has been detected in HVC, RA and IMAN of both male and female zebra finches between post-hatching days 20 and 35 (Dittrich et al., 1999).

The present study was designed to investigate in more detail potential sites of BDNF action in the development of the song system by mapping the distribution of immunoreactivity to an antibody raised against the trkB receptor in both males and females from 3 to 60 days post-hatching.

2. Materials and methods

2.1. Animal rearing and tissue collection

Zebra finches were housed in communal aviaries with approximately five breeding pairs and their young. Nest boxes were checked daily, and new hatches in each clutch received a unique toe-clip for identification. Before they fledged, each bird was banded so that it could be tracked during development. The day an individual hatched was considered day 1. Males and females were deeply anesthetized with Equithesin and perfused with 0.75% saline containing 0.1% heparin, followed by a solution of 4% paraformaldehyde and 0.4% glutaraldehyde at the following ages: day 3, 15, 30, 45, 60 ($n = 2–3$ of each sex at each age). The gender of birds can be readily identified by plumage coloration at the latter two ages, but the gonad(s) of each individual were checked under a dissecting microscope at the time of perfusion to confirm sex.

Brains were postfixed overnight in 4% paraformaldehyde and then embedded in sucrose/gelatin. The block containing the tissue was stored overnight in 4% paraformaldehyde with 20% sucrose. Tissue was sectioned coronally at 20 or 30 μm and preserved in cryoprotectant at $-20^\circ$C until further use.

2.2. Immunohistochemistry

Every third brain section that contained tissue from the rostral through the caudal poles of the telencephalon, was processed as follows. Tissue was rinsed $6 \times 10$ min in phosphate buffered saline (PBS), and then incubated in 0.5% H$_2$O$_2$ for 15 min. Following three PBS rinses, the sections were then blocked in 5% normal donkey serum and 0.3% Triton-X100 for 1 h.

The tissue was rinsed again, and incubated at 4°C overnight in primary trkB antibody (Santa Cruz Biotechnology sc-12) at a final concentration of 0.2 μg/ml with 2% normal donkey serum and 0.3% Triton-X100. This antiserum was raised in rabbit against amino acids 794–808 of the mouse trkB receptor. It also reacts with rat and human trkB, and has been successfully used in songbirds (Rasika et al., 1999) at the same (1:500) dilution employed in the present study. The primary antibody was rinsed off using PBS (3 × 5 min), and the tissue incubated overnight at 4°C in secondary antibody (donkey anti-rabbit, Jackson Labs) that was diluted 1:500 and contained 0.2% Triton-X100. The tissue was rinsed again in PBS, and reaction products were visualized using an ABC kit (Elite, Vector) followed by rinses in PBS, Tris buffered saline (TBS), and incubation with diaminobenzidine (0.5 mg/ml TBS) plus 0.0075% H$_2$O$_2$. Sections were rinsed in PBS, mounted on gelatin-coated slides, rinsed in dH$_2$O, dehydrated, cleared in xylene and coverslipped with Permount.

Specificity of trkB labeling was confirmed in two ways. First, primary antibody was omitted in alternate sections from some individuals, in some cases being replaced with control rabbit IgG at the same concentration as the trkB antibody. The characteristic labeling of somata and neuropil (described below) was eliminated. However, as in tissue incubated with primary antibody, the background was slightly elevated in particularly cell-dense regions, such as the lamina of the optic tectum. Second, alternate sections from every brain were incubated with a primary antibody to trkA, the high affinity receptor for nerve growth factor (Santa Cruz Biotechnology sc-118). This alternate primary antibody is a valuable control, because like the trkB primary, it was raised in rabbit and used at 0.2 μg/ml. No labeling above background was detected in the song control nuclei using the trkA antibody. In some cases, the remaining set of alternate brain sections was stained with thionin (following several rinses in PBS to clear the cryoprotectant). However, the consistency of anti-
trkB labeling and the ability to discern appropriate landmarks in the tissue reacted immunohistochemically made it unnecessary to rely on the Nissl stain to determine anatomical localization.

The presence of immunoreactivity in the song control nuclei was determined by observation of the tissue sections through an Olympus BX60 microscope. While the primary goal was to document when and where the trkB antibody produced labeling, judgements about the pattern and consistency were checked as follows. In all brains greater than 3 days of age that were cut a 30 \( \mu \text{m} \) (chosen because: (1) song control nuclei are not present until after day 3; (2) there were more brains cut at 30 \( \mu \text{m} \) than at 20 \( \mu \text{m} \); and (3) the labeling was more intense compared to background in the thicker sections), HVC and RA were analyzed using NIH Image analysis software on a power Macintosh computer as follows. At a magnification of 450 \( \times \), the software was used to draw a 200 \( \mu \text{m} \) wide by 50 \( \mu \text{m} \) high box within HVC (dimensions chosen to accommodate the limiting size and shape of the female HVC). The number of immunopositive cells within the box was counted manually, and the mean optical density was determined using Image. As a control, the box was then placed over the neighboring hippocampus in the same brain section, and the same measurements taken. Values considered for each section were: (1) the number of cells within HVC minus those counted in the hippocampus control; and (2) the optical density within HVC divided by that of the same-section control (optical density ratio). These values were obtained in three sections for each individual, and then an average was computed. The process was repeated for RA, with the control being the region immediately ventral to the nucleus. Because labeling was not present at some ages in one sex or the other, the counts and measurements were taken in the HVC of all animals in which the borders of the nucleus could be identified. For RA, it was possible to obtain values even in animals in which RA was not specifically labeled, because the location is so readily determined by surrounding landmarks in animals greater than approximately 1 week of age.

The optical density ratios are not large, but they reflect the nature of the labeling. That is, only a portion of the cells in HVC and RA is immunopositive, and labeling is absent and sometimes lighter than background in nuclei (see below) while quite intense in other compartments of the cell. Obtaining an average optical density in immunoreactive regions thus leads to a value only moderately higher than the homogenous light brown background in the control portions of the brain sections. However, in comparison to judgements made through the microscope about whether an area was labeled or not, there was complete agreement. That is, optical density values were greater than 1.00 for regions that were considered labeled, and ranged from 0.94 to 1.00 for regions not considered to be labeled upon visual inspection.

### 3. Results

When present in the song system, labeling consistently occurred in the somata and neuropil, but was absent in cell nuclei. The most distinct labeling was present in HVC (Fig. 1). In all males, immunoreactive cells were detected in that brain region from days 30 to 60 (measured OD ratios: 30 days = 1.12; 45 days = 1.25, 1.26; 60 days = 1.18, 1.20). HVC was labeled in all 45 and 60 day old females (45 days = 1.04, 1.13; 60 days = 1.17, 1.22). Labeled somata were scattered throughout the brain region at a similar density in males and females of all ages (average cells per 10 000 \( \mu \text{m}^2 \), males: 16.0–26.3, females 15.0–26.3). Because HVC is substantially larger in males than in females (approximately six times larger in volume at 60 days of age, for example; Grisham and Arnold (1995)), it is reasonable to conclude that the total number of trkB-like immunoreactive cells in HVC is greater in males compared to females.

Immunoreactive cells and neuropil were also present in the RA of all 30–60 day old males (Fig. 2A), although OD values were in very case lower in RA than in HVC (measured OD values for RA at these ages ranged from 1.02 to 1.11). In females, RA was only clearly obvious at 60 days of age and only in one out of three birds (measured OD ratio = 1.15; see Fig. 2C for example of the brain region in a 30 day old female). Labeling with the antibody to trkB appeared to define HVC and RA, such that it was present in and seemed to fill the extent of the brain regions, while labeling was absent, or greatly reduced, immediately outside the boarders (Fig. 2A,B). Scattered immunopositive neurons were present in some individuals in other song control regions, such as the lateral magnocellular nucleus of the anterior neostriatum (IMAN), but the boarders could not be identified using this marker.

In 15–60 day old animals of both sexes, a distinct arc of cells was also consistently detected just ventral to the lamina archistriatalis dorsalis (Stokes et al., 1974), at the level of RA (Fig. 2C,D). At those ages, there also appeared to be a diffuse field of trkB immunopositive cells in the telencephalon, extending from the ventral border of HVC to the dorsal edge of RA. A consistent pattern of labeling in the telencephalon of 3 day old animals was not detected, although scattered patches of neurons were seen in all individuals. It may be that the rate of maturation so close to hatching in these altricial birds is more variable than at later ages, in which case the expression of neurotrophin receptors might be inconsistent. Alternatively, it may be that the inconsistency was due to variations antigen–antibody reactions.
Fig. 1. TrkB-like immunoreactivity in the zebra finch HVC. The right side of each photograph is medial. (A) A 30 day old male. The immunoreactivity is localized in somata and neuropil, but is essentially absent from cell nuclei (panel B, higher magnification of approximately the middle third of A). (C) A 45 day old male. Note the increased density of darkly-labeled cells in the HVC, compared to the hippocampus immediately dorsal to it (panel D, higher magnification near dorsomedial edge of C). (E) A 60 day old male; (F) A 60 day old female. Scale bar = 200 μm for A, C, E, F; 20 μm for B; 50 μm for D.

4. Discussion

The results document the presence of trkB-like immunoreactivity in the song system of zebra finches. TrkB is the high affinity receptor for BDNF and neurotrophin-4/5 (NT-4/5). This immunoreactivity exists, particularly in HVC, in both males and females during the latter half of morphological development of the song control nuclei. Sex differences in the size of the brain regions have been reported as early as day 12 (Bottjer et al., 1985), but the sexual differentiation process continues through approximately day 60 (Nixdorf-Bergweiler, 1996). The ontogeny of labeling in the motor pathway for song production roughly coincides with, or slightly precedes, the onset of singing behavior. From approximately 20–60 days of age, males form a template of song produced by tutors. They begin producing song-like vocalizations at about 35 days post-hatching, and the song stabilizes by 80–90 days of age (Nordeen and Nordeen, 1997). The time course of potential trkB expression in the song control regions thus suggests that BDNF or NT-4/5 may be involved in phases of song learning (template formation and/or
The presence of trkB-like immunoreactivity in the song control regions of females as well as males is consistent with that hypothesis. While they do not produce song, females do need to learn something about the quality of the vocalizations, as they use them to identify appropriate mates. HVC plays a critical role in the process of song perception in female birds (Brenowitz, 1991). Perhaps, then, trkB receptors in HVC are important in individuals of both sexes for the storage of appropriate song templates. In males, the learning process involves an integration of the sensory perception of song with the motor production of the behavior. In parallel, connections in the motor pathway (HVC to RA) are enhanced in males compared to females (Konishi and Akutagawa, 1985). Axons extend from HVC to RA at approximately day 30. Perhaps the appearance of trkB-like immunoreactivity in males at that age is indicative of the receptor facilitating development of the anatomical substrate required for this motor component of song development.

The present data on the distribution of trkB-like immunoreactivity are generally consistent with previous reports. In the song control regions specifically, the localization of labeling to the somata and neuropil and the absence of labeling in neuronal nuclei is comparable to that depicted in the HVC of adult canaries using the same antibody (Rasika et al., 1999) and the RA of juvenile birds using a different antiserum to trkB (Johnson et al., 1997). The latter study reported trkB immunoreactivity in RA of 15–20 day old males (labeling in other brain regions is not discussed), and BDNF infusion was capable of maintaining the survival of deafferented neurons in that brain region. These results strongly suggest the expression of functional trkB protein in the RA of males at this young age. TrkB mRNA has also been reported in the HVC, RA and lMAN of 20–35 day old individuals of both sexes (Dittrich et al., 1999). The present data extend those results by documenting immunoreactivity to a trkB antibody in the motor pathway in older animals (30–60 day old males and 45–60 day old females). We did not detect labeling in HVC or RA of 15 day old animals, and did not investigate 20 day old individuals. However, it is certainly possible that the expression of trkB is initiated in males at approximately day 15. Had we looked at day 20, our antibody might have detected it, and by day 30 the expression in males was clear. TrkB
mRNA was also reported in lMAN in juvenile males and females (Dittrich et al., 1999; in roughly half of the RA-projecting neurons in males). Consistent with the data on lMAN, we did find scattered trkB-like immunoreactive cells in that region of the brain. However, the labeling did not define lMAN as it did the components of the motor pathway, HVC and RA (Figs. 1 and 2). The similarities detected, with both other immunohistochemistry and with in situ hybridization experiments, provide support for the idea that the labeling specific to the primary antibody used in the present study identifies brain regions containing the trkB protein. However, the possibility exists that the pattern detected reflects the presence of another protein. If the labeling does depict the protein of interest, then the present data on immunoreactivity in the female HVC and RA suggest that the trkB protein is expressed later than the mRNA (Dittrich et al., 1999). It may be that the mRNA detected in HVC and RA is not efficiently translated into protein in females until later ages or that the protein is degraded differentially in young males and females. Generation of a species-specific antibody could begin to address these issues in the future.

The ligand for the trkB receptor, BDNF, has also been identified in song control regions. Labeling in zebra finches was detected by in situ hybridization or immunohistochemistry in HVC and lMAN at various ages. For example, mRNA for BDNF was detected in males, but not females, at 30–35 days posthatching (Dittrich et al., 1999). The cytoplasm and processes of cells were labeled by immunohistochemistry in the HVC of 20 day old males (Akutagawa and Konishi, 1998), although mRNA was not present at that age (Dittrich et al., 1999). This immunoreactivity increased in intensity by day 45, and also appeared outside of cell bodies in RA at that age. The fact that immunoreactivity to a trkB antibody was also detected in HVC at day 45 is consistent with the hypothesis that autocrine or paracrine action of BDNF in the male HVC may be important at this time. By day 65, the extracellular matrix was also labeled in lMAN. Little immunoreactivity was detected in adult zebra finches (Akutagawa and Konishi, 1998), although labeling with a BDNF antibody was present in the HVC of adult male canaries (Rasika et al., 1999). Akutagawa and Konishi (1998) did not replicate BDNF immunoreactivity previously reported in 15–20 day old birds (Johnson et al., 1997), but both papers describe BDNF labeling in an arc of cells just ventral to the lamina archistriatalis dorsalis like what we detected in the present study with the trkB antibody, suggesting that autocrine or paracrine action might be important in that region as well.

Despite a few inconsistencies that exist across studies (see above), when taken together the data support the idea that BDNF acts within song control nuclei to promote the survival and/or recruitment of neurons that are likely to be involved in certain aspects of song learning and perhaps motor production of song in males. Collectively, the data are consistent with the idea that the HVC is the major site of BDNF production in the motor vocal circuit in males (Dittrich et al., 1999). BDNF probably acts not only in HVC, but is likely also transported to RA, where it can act on trkB receptors. Because the female HVC seems to lack BDNF in zebra finches and canaries, the function of the trkB receptors in their song control nuclei is unknown. It is possible that that NT-4/5 could play a role related to song perception, but to date it has not been found in birds (Hallbo¨ök, 1999).

BDNF expression can be up-regulated by exogenous estradiol administration in juvenile zebra finches (Dittrich et al., 1999) and by testosterone in adult canaries (Rasika et al., 1999). Further, BDNF infusion can prevent neuronal death in juvenile zebra finches (Johnson et al., 1997) and increase the number of new neurons in adult canaries (Rasika et al., 1999). This relationship between the neurotrophin and gonadal steroids suggests that the hormones might facilitate masculinization in either natural (e.g. seasonal changes in the adult male canary) or pharmacological (e.g. estradiol administration in hatchling female zebra finches) situations at least in part by increasing BDNF availability. As in the development of male, the data in female zebra finches are consistent with the idea that BDNF’s role in masculinization does not occur in the earliest phases of morphological differentiation. Instead, early estradiol treatment promotes a delayed increase in BDNF that coincides with the endogenous elevation in males at approximately 1 month of age (Dittrich et al., 1999).

In summary, a growing body of results is consistent with the hypothesis that BDNF and its high-affinity receptor, trkB, are important in the cascade of events that regulates masculinization of the zebra finch song system. TrkB receptors may also facilitate the learning required for accurate perception and evaluation of song in females. However, the initial trigger(s) for the sexual differentiation process still must be discovered, and it will be important to learn how they and other non-steroidal factors interact with neurotrophins.

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References


