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DEVELOPMENTAL CHANGES IN BDNF PROTEIN IN THE SONG CONTROL NUCLEI OF ZEBRA FINCHES

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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 a crucial role 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 the 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 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 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

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Abbreviations: ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; E2, estradiol; LMAN, lateral magnocellular nucleus of the anterior nidopallium; mBDNF, mature BDNF; NGS, normal goat serum; p75NTR, p75 pan-neurotrophin receptor; PBS, phosphate-buffered saline; PVDF, polyvinyl difluoride; RA, robust nucleus of the arcopallium; TrkB, tyrosine kinase receptors.

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neurotrophic factor (BDNF). BDNF is a member of nerve growth factor family which plays a crucial role in the 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 20–35 (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 the 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% H_2O_2/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, USA) 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* Avidin–Biotin Complex (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, USA). We verified the specificity of the