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Research report

Norepinephrine inhibition in juvenile male zebra finches modulates adult song quality

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ABSTRACT

During development, male zebra finches learn a song that they eventually use in courtship and defense of nest sites. Norepinephrine (NE) is important for learning and memory in vertebrates, and this neuromodulator and its receptors are present throughout the brain regions that control song learning and production. The present study used the neurotoxin N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP4) to reduce brain levels of NE in juvenile males. This manipulation inhibited the development of quality songs, with some birds producing syllables that were unusually long and/or contained frequencies that were predominantly higher than normal. These results suggest that NE is important for the acquisition of typical song.

1. Introduction

Norepinephrine (NE) plays important roles in vertebrate learning and memory (Harley, 2004; Sara, 2009). Courtship song in passerine birds is a learned behavior. Juveniles acquire these vocalizations from adult tutors, in many cases their fathers (Slater and Mann, 1990). The timing of this process and the degree of plasticity that exists as animals mature varies among species. However, across songbirds two key phases exist – a period of template formation in which a memory of tutor song is stored and a phase of sensorimotor integration in which young animals match their developing vocalizations to the template (Marler, 1997; Nordeen and Nordeen, 1997).

Zebra finches are classic examples of 'closed-ended' or 'age-limited' learners. Memory acquisition begins at approximately 20 days post-hatching and continues to about day 60. Vocal practice overlaps with this period, beginning at about 35 days of age and completing around 3 months after hatching with the formation of crystallized song. After this point, the song remains stable throughout adulthood, in contrast to 'open-ended' learners in which songs may change in adulthood (Nordeen and Nordeen, 1997).

Song is controlled by two interconnected circuits in the forebrain. An anterior pathway including Area X and the lateral magnocellular nucleus of the anterior nidopallium (LMAN) is critical for song learning. A motor pathway important for the production of song consists of HVC (proper name; Reiner et al., 2004) projecting to the robust nucleus of the arcopallium (RA), which innervates the motoneurons of the vocal organ (syrinx; reviewed in Nordeen and Nordeen, 1997). The HVC and RA are substantially larger in males, who sing, compared to females who do not; Area X is not visible in females (Wade, 2001; Wade and Arnold, 2004).

The locus coeruleus (LoC) is a prominent source of NE in the vertebrate brain. It has widespread projections in the forebrain of birds, including the song control nuclei. These brain regions contain high levels of NE, as well as α2- and β-adrenergic receptors. This pattern in adults is consistent with a role of NE in singing, and experimental studies in adults suggest roles for NE in song production and perception (all reviewed in Castelino and Schmidt, 2010). In developing zebra finches, NE function in the song nuclei declines after 25–30 days post-hatching (Harding et al., 1998; Sakaguchi and Saito, 1989). This pattern is suggestive of a role for NE in template formation. However, it is possible that activity of this neuromodulator peaks earlier, as measurements were not taken prior to day 25.

To more directly test the role of NE in song system development, we treated birds with N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP4). This drug selectively targets axon terminals originating from the LoC and causes rapid and substantial decreases in NE from this source (Jonsson et al., 1981). Our goal was to determine effects on development of both singing behavior (see above) and morphology of the forebrain song control nuclei. These brain regions first become visible around 5–10

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days after hatching (Gahr and Metzdorf, 1999; Kim et al., 2004; Kirn and DeVoogd, 1989). Maturation, which includes substantial growth of these brain regions in males compared to females, occurs through approximately day 50; sexual differentiation occurs at a high rate between days 20 and 30 (Kirn and DeVoogd, 1989). We modified the approach that was effectively used on adult male zebra finches by Waterman and Harding (Waterman and Harding, 2008) and gave a first injection on post-hatching day 10 and a second on day 18.

2. Materials and methods

Animals. Experimental zebra finches were raised in adjacent walk-in aviaries at Michigan State University, each containing roughly 5–7 breeding pairs and their offspring, with 100 or more birds present in the room at any time. Animals were exposed to a 12:12 light:dark cycle, and provided *ad libitum* access to drinking water, seed (Kaytee Finch Feed; Chilton, WI), gravel and cuttlebone. Their diets were supplemented weekly with hard-boiled eggs, bread, spinach and oranges. Genetic sex of the birds was determined by PCR (Agate et al., 2002) on DNA extracted from toe clips used as unique identifiers on the day of hatching. Only males were used in the present experiment. All procedures were approved by the Michigan State University IACUC and followed NIH guidelines.

2.1. Treatment

On post-hatching days 10 and 18, birds were administered DSP4 (25 μg/g in saline, IP, Sigma–Aldrich, St. Louis, MO) or one of two control manipulations, randomly assigned across nests and aviaries. Because DSP4 can also be taken up by serotonergic cells, the selective reuptake blocker zimelidene dihydrochloride was injected one hour prior to prevent degradation of serotonerigic neurons ((Barclay et al., 1996; Waterman and Harding, 2008), 10 μg/g, IP, Sigma) in DSP4-treated animals. Controls consisted of injections on day 10 and 18 of either zimelidine followed by saline one hour later, or two injections of saline an hour apart.

2.2. Behavior

Birds were left to reach sexual maturity in their home aviaries, until 94–110 days of age. To acclimate subjects prior to behavioral testing, each male was then housed in an individual cage in the same room as the colony aviaries for two days. They were able to see and hear other birds within the room. The animals were then taken to a sound-proof room and placed into an individual cage containing a sexually mature female that they had not previously encountered. Sound was recorded for 1 h using a Sennheiser ME 62 onmidirectional microphone, Sound Devices USBPre2 amplifier, and Adobe Audition software at a 44.1 kHz sampling rate, with 16-bit resolution. Because audio but not video was recorded we cannot be sure, but these conditions most likely resulted in the collection of female directed songs.

To obtain a rough estimate of relative levels of song learning, vocalizations of experimental males were compared to those of their fathers using Sound Analysis Pro 2011 software (Tchernichovski et al., 2000) by an observer blind to treatment group. Fathers were unambiguously determined by repeated observations of individuals who shared the nest containing each hatchling. For father-son pairs, a representative motif from each bird was compared. To assess song quality, a spectrogram of a bout of song for each animal was analyzed based on the work of Simpson and Vicario (Simpson and Vicario, 1991). This method of selection was used because a few birds sang a single bout, and because within birds that sang multiple bouts, they were very similar in content and structure (accuracy scores for each pairwise comparison for across motifs within a bout from one randomly selected bird per group ranged from 86.0 to 97.1, average = 91.4; accuracy scores across two full bouts = 83.9–92.3, average = 88.7). Specifically, we determined whether unusual aspects of the song existed based on definitions of Simpson and Vicario, including whether the bout contained fewer than three distinct song elements (syllables), and syllables longer than 300 ms or predominantly higher than 1.5 kHz. In addition, the total number of syllables in the bout was counted. Finally, syllable duration and inter-syllable interval were determined for the bout using Adobe Audition software. Average values were calculated for each individual on these measures.

2.3. Tissue collection and processing

If the male sang, it was rapidly decapitated within 30 min of the end of the recording, and its brain was removed and flash frozen in methyl butane. If the male did not sing, it was returned in its individual cage to the colony room and recorded again two days later. This process was repeated for a maximum of 5 trials. Any birds that did not sing were euthanized at the end of the fifth recording session. Brains were stored at −80 °C until further tissue processing. The song of the father of each of the subjects was also recorded using the same protocol. Two of the fathers did not sing and were excluded from analysis along with their sons, leaving a total of 22 subjects on which behavioral and brain data were analyzed: 7 treated with DSP4 and zimelidine hydrochloride (hereafter identified as the 'DSP4' group), 7 with zimelidine hydrocholoride plus saline ('zimelidine'), and 8 with two injections of saline ('saline').

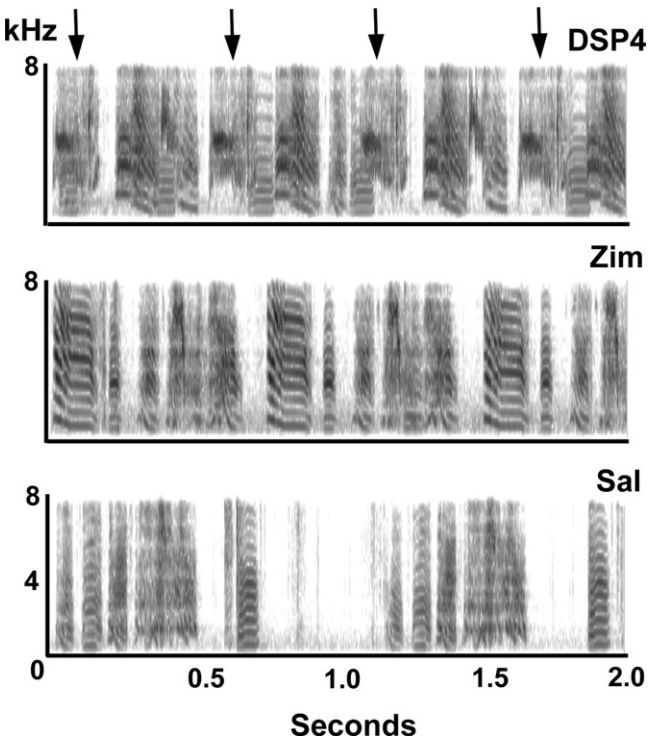

Fig. 1. Representative spectrograms from each treatment group. Images depict two seconds of song from animals administered DSP4 (top), zimelidine (middle), and saline only (bottom). In the saline-treated bird, this clip contains 2 clearly identifiable motifs. In the one administered zimelidine, roughly 2.5 motifs are included. The pattern of notes in the DSP4 was less consistent, and the long notes (>300 ms) combined with relatively limited breaks made it somewhat difficult to define discrete motifs. At least 4 notes in this clip (arrows) had elements that were predominantly higher than 1.5 kHz.

Brains were coronally sectioned at 20 μm and thaw-mounted in 6 series onto SuperFrost Plus slides (Fisher Scientific, Hampton, NH). Tissue was stored at −80 °C with dessicant until processing.

One series of slides from each animal was stained with thionin to facilitate identification of brain regions and allow analysis of song system morphology. Another set was used for dopamine beta-hydroxylase (DBH) immunohistochemistry to identify potential NE-containing cell bodies and fibers. DBH is the rate limiting enzyme for the conversion of dopamine to NE. All tissue was reacted simultaneously. After warming to room temperature, it was rinsed in 0.1 M phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 15 min, and washed 3 times in PBS (5 min each). Slides were treated with 0.9% H_2O_2/methanol for 30 min and incubated for 1 h in 10% normal goat serum in PBS with 0.3% Triton X-100. The tissue was then incubated in a DBH rabbit polyclonal antibody (0.2 μl/ml; Cat# 22806, ImmunoStar, Hudson, WI) in 0.1 M PBS containing 0.3% Triton X-100, and 10% NGS overnight at 4 °C. This primary antibody was validated for use in songbirds (Castelino et al., 2007; Sockman and Salvante, 2008). A biotin-conjugated goat anti-rabbit secondary antibody (0.5 μg/ml; Vector Labs, Burlingame, CA) was then used for 1.5 h at room temperature, followed by treatment with *Elite* ABC reagents (Vector Labs) and diaminobenzidine (DAB) with 0.0024% hydrogen peroxide to produce a brown reaction product. Slides were then rinsed in PBS to be sure the reaction was terminated, dehydrated, and coverslipped with DPX (Sigma–Aldrich, St. Louis, MO).

2.4. Quantification of neural features

Analysis of brain tissue was completed by an observer blind to treatment group. Cross-sectional areas of HVC, RA and Area X were determined in each thionin-stained section in which it was visible using Image J software (National Institutes of Health). Brain region volume was calculated by summing the areas and multiplying by the sampling interval. Measurements were taken on both sides of the brain, and an average was calculated, except in the few cases in which one side of the tissue was damaged. In those instances, only the intact side was used.

DBH labeling was also quantified using Image J as a marker for relative NE exposure at the time behavior and morphology were evaluated. A 0.05 mm^2 box was placed over the center of the LoC. Three or four locations were randomly selected from the rostro-caudal extent of the nucleus and across the two sides of