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ScienceDirect

Behavioural Brain Research 176 (2007) 333–343

BEHAVIOURAL BRAIN RESEARCH

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Research report

ZENK labeling within social behavior brain regions reveals breeding context-dependent patterns of neural activity associated with song in male European starlings (*Sturnus vulgaris*)

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Received 28 June 2006; received in revised form 20 October 2006; accepted 24 October 2006

Abstract

In songbirds, song learning and production are regulated by the song control system. How the rest of the brain interacts with song nuclei to ensure that song is produced in an appropriate context is not yet clear. In male European starlings (*Sturnus vulgaris*), breeding context song is sexually motivated, whereas, non-breeding context song is more broadly socially motivated. Brain regions involved in regulating social behavior might differentially regulate starling song depending upon the context in which it is produced. Here, we compared the number of ZENK-labeled cells in song and social behavior nuclei in starlings singing in either a breeding or a non-breeding context. Numbers of ZENK-labeled cells in HVC related positively to song produced in both contexts. Interestingly, numbers of ZENK-labeled cells in one subdivision of the lateral septum (LS) related *negatively* to breeding context song but *positively* to non-breeding context song. In a subdivision of the medial bed nucleus of the stria terminalis (BSTm) ZENK labeling only related positively to non-breeding context song, whereas, in the ventromedial nucleus of the hypothalamus (VMH) ZENK labeling showed a tighter positive relationship with breeding context song. Together, these findings indicate that social behavior brain regions outside of the song control system regulate singing behavior differently depending upon whether song is sexually or more broadly socially motivated. Breeding context-dependent regulation of song by LS, BSTm, and VMH suggests that these nuclei may be central to adjusting song production so that it occurs in response to appropriate social and environmental stimuli.

Keywords: Songbird; Season; Social context; ZENK; High vocal center; Lateral septum; Bed nucleus of the stria terminalis; Ventromedial nucleus of hypothalamus

1. Introduction

Vocal communication plays an important role in mediating successful social interactions between individuals in a variety of contexts. The neural circuitry involved in vocal signal production and perception has been identified in some model systems, including songbirds [see 32]. However, what motivates an animal to communicate can depend upon many factors including presence or absence of conspecifics, dominance status, and season. Often, vocal behavior must be adjusted so that communication occurs in response to appropriate social and environmental stimuli. To date, how the rest of the brain interacts with vocal signal production circuits to ensure that vocal communication occurs in the appropriate context is not yet clear.

Much is already known about how the songbird brain regulates singing behavior. Song learning and production are controlled by a series of cytoarchitecturally distinct, interconnected nuclei including Area X, HVC (used as a proper name), robust nucleus of the arcopallium (RA), and lateral magnocellular nucleus of the anterior nidopallium (lMAN) [e.g. 11,46,52,66]. Collectively, these four regions (and several others) are known as the song control system, and a growing body of literature indicates that activity within song control nuclei is context-dependent. The relationship between singing behavior and activity in HVC, RA, Area X and lMAN can depend upon whether song is sexually or socially motivated [34,42,58] and whether song is directed towards or away from a conspecific [35,37]. Taken together it appears that song nuclei differentially regulate singing behavior depending upon what is motivating a male

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doi:10.1016/j.bbr.2006.10.023

to sing. However, no research currently implicates the song control system in regulating motivational aspects of singing behavior. Lesions to song nuclei result in deficits in song production, but lesioned birds continue to display motor patterns associated with singing behavior and will assume singing postures, suggesting an intact motivation to communicate [10,51,52,63]. Thus, context-dependent activation of the song control system may be a reflection of context-dependent inputs to song nuclei from areas of the brain regulating the motivation to sing.

In support of this hypothesis are data showing that brain regions outside of the song control system, well known for regulating sexual motivation and social behavior, appear to regulate singing behavior in songbirds. Specifically, neuronal activity within the medial preoptic nucleus (POM), lateral septum (LS), medial bed nucleus of the stria terminalis (BSTm), anterior portions of the hypothalamus (AH), ventromedial nucleus of the hypothalamus (VMH), ventral tegmental area (VTA), and midbrain central gray (GCt) correlates with singing behavior [33,34,44], and lesions to POM and the septum have been shown to modulate song production [1,25,55]. However, the role of these nuclei in the regulation of vocal behavior in songbirds appears to be context-dependent and is influenced by whether song is sexually motivated [33,34,58], sung in response to territorial intrusion [23,44], or observed in gregarious or territorial species [25]). Together, these studies suggest that these seven brain regions are part of a neural circuit that influences the song control system to ensure that singing behavior occurs in an appropriate context in response to appropriate social and/or environmental stimuli.

European starlings (*Sturnus vulgaris*) provide an excellent model for the study of context-dependent regulation of vocal behavior. Male starlings sing throughout the year, but the social and environmental factors that motivate song production differ depending upon whether it occurs within or outside of a breeding context [see 16 for review]. Outside of a breeding context, in fall and winter, circulating levels of gonadal steroids (i.e. testosterone (T)) are low [6,56], and male and female starlings are found feeding and roosting in large, mixed-sex flocks [20]. At this time, song appears to play no direct role in mate attraction [57], but rather is thought to be important for flock cohesion and establishing/maintaining social hierarchies within the flock [31,64]. Thus, starling song produced in a non-breeding context appears to be broadly socially motivated.

In contrast, breeding context song can be highly sexually motivated. In late winter and early spring, circulating levels of T become elevated [6,56]. Flocks disperse and males compete over limited numbers of appropriate nest sites [30]. Song at this time can play a critical role in mate attraction and nest site defense [e.g. 18,29]. Interestingly, nest site status profoundly influences male singing behavior within a breeding context. Males with nest sites increase their rate of song production in response to the introduction of a female conspecific, whereas, female introduction has no significant effect on the song rate observed in males without nest sites [57]. Once males with nest sites pair with females, song is restricted to periods immediately prior to copulation [13,19]. Taken together, it appears that nest site ownership significantly influences the degree to which breeding context song is sexually motivated.

In past work on starlings we found that the numbers of cells labeled for the protein products of the immediate early gene (IEG) c-fos (an indirect marker of neural activity) in HVC, RA, POM, BSTm, AH, VMH and VTA related positively only to breeding context song, whereas, numbers of Fos-labeled cells in LS related positively only to non-breeding context song [33,34]. Not all brain regions express Fos and the use of multiple indirect markers of neural activity allows for a more complete picture of brain regions regulating behavior than any one IEG alone [see 5,48,58]. Therefore, in the present study we used immunocytochemistry for the protein products of the IEG ZENK (avian homologue of *zif-268*, *egr-1*, *ngfi-a*, and *krox-34* [see 49]) to provide a more complete picture of how the brain regulates context-dependent vocal communication in male songbirds.

2. Methods

2.1. Animals

Starlings were caught on a single farm north of Madison, Wisconsin in fall 2002 and early winter 2003 using fly-in traps. These are the same birds used in a previous study examining song and numbers of Fos-labeled cells [33,34]. The natural photoperiod at the time of capture was <11 L. After capture, 42 adult male and four adult female starlings were housed indoors in single-sex cages (91 cm × 47 cm × 47 cm) in the University of Wisconsin Department of Zoology animal facilities. Photoperiod and hormone manipulations were used to put birds into either breeding or non-breeding conditions (see below).

2.1.1. Males

Eighteen male starlings were randomly assigned to the non-breeding group. After capture, these birds were housed indoors on a photoperiod matching the natural light/dark cycle. On 18 December 2002, when the outdoor photoperiod was ∼10 L, non-breeding males were placed into two aviaries (3.7 m × 2 m × 2.8 m) in social groups of 8–10 birds. Behavioral testing of non-breeding males began in January after a 1-month habituation period.

The remaining 24 males were assigned to the breeding condition group. After capture, these birds were housed on 6 L for 2 months and then 11 L for approximately 6 months. This photoperiodic regime causes birds to become photosensitive (a condition in which they respond to T with courtship and mating behavior) [14]. Two weeks prior to introduction to an outdoor aviary (identical to those used for the non-breeding groups), birds were implanted with T (see below). After implant surgery, breeding condition males were placed into outdoor aviaries in social groups of 12 birds and given 2 weeks to habituate to the outdoor aviaries. Behavioral observations for the present study took place at a time when the outdoor photoperiod was ∼13 L.

2.1.2. Stimulus females

To stimulate sexually motivated singing behavior in breeding condition males, a female was introduced to the outdoor aviary immediately prior to the start of behavioral testing. As described in Section 1, non-breeding context song plays no role in immediate mate attraction. However, to control for the possibility that the introduction of a novel conspecific might induce ZENK expression in some of the brain regions examined, a stimulus female was introduced to non-breeding condition males as well. To maintain ecological validity of the male singing behavior observed, breeding condition females were released into the aviaries containing breeding condition males and non-breeding condition females were released into aviaries containing non-breeding condition males. Breeding condition stimulus females were housed indoors on an 11 L photoperiod. Non-breeding condition females were housed indoors on a 6 L photoperiod.