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Patterns of phosphorylated tyrosine hydroxylase vary with song production in female starlings

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Abstract

Vocal signal production in male songbirds is well studied, but the neural correlates of female song production are poorly understood. In European starlings, females sing to defend nesting resources, and song can be considered agonistically motivated. Across vertebrates catecholamines strongly influence motivated, agonistic social behaviors. The present study was designed to provide insight into a possible role for catecholamine activity in territorial song in female starlings. We presented females that were defending nest-cavities with an unfamiliar female and assessed song production. We then measured immunolabeling for phosphorylated tyrosine hydroxylase (pTH-ir), a rate-limiting enzyme for catecholamine synthesis, in brain regions in which catecholamines stimulate agonistic behavior. Females that sang had higher pTH-ir in the caudomedial ventral tegmental area and the lateral septum than females that did not sing. Furthermore, the number of songs produced correlated positively with pTH-ir in the medial preoptic nucleus. Phosphorylation of TH is thought to occur after catecholamine release, so these results link increased catecholamine activity in several brain regions governing agonistic behavior to territorial song production in females.

Keywords

norepinephrine; dopamine; motivation; communication; social behavior; female song

1. Introduction

Across vertebrates, vocal signals are used to coordinate social interactions (e.g. Bradbury and Vehrencamp, 2011). The information provided in vocal signals covers a wide spectrum, ranging from readiness to mate to agonistic intent to fear, alarm and hunger, among others (Blumstein et al., 2004; Catchpole and Slater, 2008; Ellis et al., 2009; Manser, 2001; Sánchez, 2003). Songbirds have proven a useful model system for examining neural correlates of communication (see multiple references in Ziegler and Marler, 2008), but most

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of this work has thus far focused on males, in particular on their production of song that functions to attract mates. In many species, however, both sexes sing, and males and females may be motivated to vocalize for different reasons.

In European starlings (*Sturnus vulgaris*), both sexes sing (Pavlova et al., 2007a; Pavlova et al., 2007b; Sandell and Smith, 1997). In the non-breeding season the sexes sing in similar social contexts, but under breeding conditions females sing after claiming a nest cavity, to signal their occupancy to other females (Pavlova et al., 2005; Pavlova et al., 2007a; Pavlova et al., 2007b; Sandell and Smith, 1997; Smith and Sandell, 2005). Although male song may function to deter male rivals, males sing primarily to attract mates to nest cavities (Eens et al., 1989; Eens et al., 1990; Eens et al., 1991). Given the social motivation and signal function of song in females, the underlying brain areas that regulate song control regions would be expected to reflect the agonistic context in which females sing.

The catecholamine dopamine (DA) is associated with motivation and reward, while the catecholamine norepinephrine (NE) is associated with attention and arousal. Both underlie psychological drives critical for responding appropriately to a territorial intrusion by a conspecific, especially when territories and nest sites are limited (Aston-Jones and Bloom, 1981; Berridge, 2008; Castelino and Schmidt, 2010; Wise, 2002; Wise, 2004). Brain regions rich in these neurotransmitters have been closely linked to territorial behavior. These in particular include the lateral septum (LS), the medial preoptic area (mPOA), the periaqueductal gray (PAG), and the ventral tegmental area (VTA) (Goodson et al., 2012; Haller et al., 2006; Sewall et al., 2010; Siegel et al., 1999; Silverin et al., 2004). In birds, all of these regions show high levels of catecholamines, their receptors and their synthetic enzymes (e.g. Appeltants et al., 2001; Bailhache and Balthazart, 1993; Balthazart and Absil, 1997; Bottjer, 1993; Durstewitz et al., 1998; Heimovics et al., 2009; Mello et al., 1998). Several of these regions appear to influence song produced when defending territories and nest sites. In song sparrows (*Melospiza melodia*), VTA and PAG show higher c-fos labeling in males that sing more territory defense songs (Maney and Ball, 2003). In male starlings, D1-dopamine receptors in the PAG and LS relate to song produced in the presence of other males by males defending nest boxes (Heimovics et al., 2009). In field sparrows, lesions of LS increase multipurpose song and aggression against same-sex conspecifics (Goodson et al., 1999).

Given the agonistic context in which female starlings sing, we predicted that females singing to defend nest boxes would show higher levels of catecholamine activity in these regions. To test this, we presented females claiming nest boxes with an unfamiliar female as a competitor, and assessed the presence of phosphorylated tyrosine hydroxylase (pTH) via immunohistochemistry. Tyrosine hydroxylase (TH) is a rate-limiting enzyme in the synthesis of dopamine, and phosphorylation of TH occurs during short-term regulation of this enzyme and triggers synthesis of catecholamines (Dunkley et al., 2004; Kumer and Vrana, 1996; Lew et al., 1999). It is generally accepted that such up-regulation of DA synthesis occurs after the release of either DA or NE (Dunkley et al., 2004; Gordon et al., 2008; Wakade et al., 1988). Examining pTH immunolabeling shortly after behavioral observations provides insight into which brain regions are likely actively synthesizing new

catecholamines in association with recent behavior. Because dopamine is a precursor to NE, pTH can indicate the production and probable release of either catecholamine.

2. Results

Singers and non-singers did not differ in pTH-labeled area in either the rostral or caudolateral VTA (rostral: $t_{11} = 0.18$, $p = 0.9$; caudolateral: $t_{11} = 1.3$, $p = 0.20$, $n = 13$). However, the small cells of the caudomedial VTA showed higher labeling in singers compared with non-singers ($t_{11} = 3.05$, $p = 0.011$, $n = 13$; Figures 2, 3). No significant correlations were found between labeled cells in any of the subdivisions of VTA and specific measures of singing behavior.

The number of labeled cells in the PAG did not differ significantly between singers and non-singers ($t_{10} = 1.5$, $p = 0.14$, $n = 12$; Figure 3). Furthermore, no significant correlations were found between labeled cells in PAG and specific measures of singing behavior.

In the LS, the area covered by pTH-labeled fibers was greater in singers than in non-singers ($t_9 = 2.7$, $p = 0.026$, $n = 11$; Figures 2, 3). However, no significant correlations were found between labeled cells in LS and specific measures of singing behavior.

There was a trend for singers to have higher numbers of pTH-labeled cells in the mPOA than non-singers ($t_{10} = 2.0$, $p = 0.073$, $n = 12$; Figures 2, 3). Additionally, there was a significant positive linear relationship between number of cells labeled and both number of songs and total time spent singing (Figure 4; number of songs: $F_{1,10} = 7.4$, $p = 0.021$, $n = 12$; total time singing: $F_{1,10} = 16$, $p = 0.0025$, $n = 12$).

Correlation analyses were also run to examine possible links between pTH-ir and non-specific behaviors (feeding, drinking, and preening). In PAG, pTH-ir correlated negatively with the natural log of drinking behavior ($R^2 = 0.31$, $y = 2.1 \times -5$, $F_{1,10} = 6.1$, $p = 0.032$). mPOA pTH-ir also correlated negatively with the natural log of drinking behavior ($R^2 = 0.32$, $y = 1.3 - 0.6 \times$, $F_{1,10} = 5.2$, $p = 0.033$). No other non-specific behaviors correlated with pTH-ir in any region.

3. Discussion

The catecholamines DA and NE play a central role in social interactions with conspecifics through their modification of arousal and reward states (Berridge and Waterhouse, 2003; Berridge, 2003; Berridge et al., 2009). Here, we examined a possible role for catecholamines in production of song by female starlings encountering a same-sex individual that represented a possible competitor for a claimed nest site. pTH-ir in VTA, LS, and mPOA, all regions in which catecholamine activity is known to modify agonistic behavior (Filipenko et al., 2001; Korzan et al., 2000; Razzoli et al., 2011; Scotti et al., 2011; Siegel et al., 1997; Siegel et al., 1999), was associated with the production of female song in this context. This result suggests a role for catecholamines in vocal production used in territory or resource defense.

Our results implicating the VTA in singing to defend nesting sites are consistent with data from song sparrows, in which males that sang more territory defense songs showed higher induction of the immediate early gene (IEG) fos in VTA (Maney and Ball, 2003). The VTA, specifically catecholamine activity in VTA, has also been linked to sexually-motivated male song used to attract mates. For example, TH in the rostral VTA is associated with increased time spent singing in breeding condition male starlings, as is labeling for the immediate early gene (IEG) fos, an indirect marker of neural activity (Heimovics and Ritters, 2006; Heimovics and Ritters, 2008). In male zebra finches, fos activity in TH neurons in VTA also correlated positively with courtship song (Goodson et al., 2009), and the expression of the IEG ZENK in the VTA as a whole was higher in singers than non-singers (Lynch et al., 2008). Given the role of midbrain limbic dopamine in motivation (Berridge, 2007; Richard and Berridge, 2011; van Furth et al., 1995; Wise, 2004) and the fact that catecholamines in VTA show relationships to song in both mate attraction and nest defense contexts, it may be that dopamine activity in VTA is important for motivated singing behavior in any context (Goodson et al., 2009; Hara et al., 2007; Heimovics and Ritters, 2005; Heimovics and Ritters, 2008; Huang and Hessler, 2008; Lynch et al., 2008; Nordeen et al., 2009; Yanagihara and Hessler, 2006).

Differences in VTA pTH labeling in singers and non-singers were restricted to the caudomedial VTA. The cmVTA in birds is morphologically distinct from the rostral and caudolateral parts of the avian VTA, tending to have much smaller cells (Goodson et al., 2009). In both birds and mammals the rostral and caudal VTA may play different roles in behavior (Goodson et al., 2009; Shabat-Simon et al., 2008). In zebra finches TH labeling in the caudal VTA related to singing and courtship behavior, while in the rostral VTA it related to social behaviors and observing courtship (Alger et al., 2011). Additionally TH-fos colocalization was higher in male zebra finches singing directed courtship songs (Alger et al., 2011). Data thus support functionally distinct regional subdivisions of VTA in singing behavior. The above studies have examined what we term the clVTA and the rVTA, and have not examined the cmVTA. Our findings suggest that the cmVTA may differ functionally from the clVTA.

Our data linking LS to female song production are consistent with past studies demonstrating a role for LS in responding to territorial intrusion in male songbirds (Goodson et al., 1999; Goodson and Evans, 2004; Goodson et al., 2005; Goodson et al., 2012; Kelly et al., 2011). While catecholaminergic activity in the LS during territorial and agonistic behavior has not been widely investigated in birds, a study in male starlings linked D1-dopamine receptors densities in LS to song thought to function to deter male rivals (Heimovics et al., 2009). Furthermore, in mice, catecholamines in the LS modulate agonistic defense behaviors (Clarke and File, 1982); more specifically, NE in LS plays a role in generating postpartum maternal defense of offspring (Scotti et al., 2011). These findings suggest that the pTH-ir we saw in response to an unfamiliar competitor may indicate the release of NE or DA.

Converging research shows that the mPOA plays a central, integrative role in song produced in multiple social contexts (Alger et al., 2009), but that its role differs depending upon the context in which a bird sings (Alger and Ritters, 2006). In starlings catecholamines in mPOA

have been linked to sexually-motivated (Heimovics and Ritters, 2008) and affiliative male song (Heimovics et al., 2009), but the present data are the first to link catecholamine activity in mPOA to song in an agonistic context. Here, singers tended to have more pTH labeled cells in mPOA than non-singers, and the number of pTH-labeled cells related linearly to the number of songs produced and total time spent singing. Although links between catecholamine activity in the mPOA and territorial singing have not been well studied, multiple studies suggest mPOA activity relates to agonistic behavior during territoriality in studies of mammals (Bhatt et al., 2003; Gregg and Siegel, 2001; Siegel et al., 1999; Sweidan et al., 1991), and a link has been found between mPOA and territorial song in songbirds (Silverin et al., 2004). In chickadees, fos IEG labeling in this region increased as chickadees gave more and longer *chick-a-dee* mobbing calls in response to a predator (Ellis and Ritters, 2012). Resident male prairie voles presented with a male intruder showed increased numbers of cells double labeled for fos + TH in the mPOA (Gobrogge et al., 2007; Gobrogge and Wang, 2011). Furthermore, DA in the medial preoptic-anterior hypothalamus in cats stimulated defensive behavior (Sweidan et al., 1991), suggesting that the pTH label in the present study may reflect DA synthesis. These results are consistent with the role of the preoptic area in defensive behaviors and support the hypothesis that catecholamine activity in mPOA plays a role in agonistic social behavior in birds (Siegel et al., 1997; Siegel et al., 1999; Xie et al., 2010; Xie et al., 2011). The negative relationship of pTH to drinking in the mPOA is difficult to explain, but could be explained by reduced time spent drinking as motivated behaviors like singing increased.

The PAG has been strongly implicated in agonistic behavior (Adams, 2006; Mobbs et al., 2007; Oliveira et al., 2004), including territorial singing behavior in males (Maney and Ball, 2003). Past work in male starlings also linked D1 DA receptors in PAG to song produced by males occupying nest sites in the presence of other males (a context in which song is presumed to function to deter rivals) (Heimovics et al., 2009). In contrast, our study showed no obvious differences in pTH-ir in singers compared to non-singers. Future studies are needed to evaluate the role of catecholamines in this region in territorial song.

We found no correlations between pTH and singing in the VMH. VMH is well known for its role in control of female sexual behaviors (Anderson et al., 2001; Flanagan-Cato, 2011; Leedy and Hart, 1985). However, nest defense, while important for successful breeding, is not a sexual behavior per se, but rather an agonistic one. While VMH is involved in other social behaviors aside from female sexual behavior, the agonistic context may explain why pTH was not linked to singing in this region (Pan et al., 2010; Sweidan et al., 1991).

Overall, these results provide support for the hypothesis that catecholamines may be important during song production during territory or resource defense, and suggest that the LS, mPOA and VTA in particular are involved. Future work is necessary to determine whether DA, NE or both are linked to production of song, and how exactly catecholamines influence male vocal production during agonistic encounters.

4. Experimental Procedures

4.1 Subjects, Experimental Setup, and Design

Wild starlings (25 females) were captured in Madison, WI and housed on a photoperiod of 18 hours light (L):6 hours dark (D) for 6 weeks, and then switched to 6L:18D for six weeks to induce photosensitivity. This puts subjects in a physiological state in which long day lengths stimulate hormonal and behavioral changes associated with breeding (Dawson, 1983; Ritters and Pawlisch, 2007). Photosensitive starlings were implanted using standard techniques (Heimovics and Ritters, 2006; Ritters and Pawlisch, 2007) with silastic implants containing 17β -estradiol (two, 17 mm in length of i. d., 1.47 mm; o. d. 1.96 mm; Dow Corning, Midland, MI USA, packed with 13-mm 17β -estradiol, Sigma Aldrich, St. Louis, MO, USA) to facilitate breeding season typical endocrine conditions necessary to promote singing behavior. After hormone implantation, individuals were placed in same-sex groups of six on 11L:13D in indoor aviaries with four nest boxes. Females were provided with nest material (dry grass) to stimulate interest in nest boxes. All subjects were collected, housed and sacrificed in accordance with the University of Wisconsin Institutional Animal Care and Use Committee and all procedures adhered to methods approved by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

4.2 Observations

Behavioral observations were performed from behind one-way glass at least two days after two individuals in a room began singing, entering and occupying nest boxes, and carrying nest material to boxes. Each group of six individuals was exposed to an unfamiliar female starling for 30 minutes, during which the number of and length in seconds of all songs were recorded to the nearest second. These values were used to calculate rate of song production (song rate) and the mean duration of each song (song bout length). While all groups were audio-recorded with a Marantz 661 PMD recorder and a Sennheiser ME67 microphone, because only two individuals ever sang simultaneously, the observer easily notated song starts and ends. The number of eating, drinking, bill-wiping and preening events were recorded for each individual, as were the number of wing-waves, a visual display produced while singing, the number of nest box entries (to any nest box) and the number of times a subject picked up nest material. After 30 minutes, the female stimulus was removed; different stimulus females were used for each group. Thirty minutes after stimulus removal two individuals were sacrificed. Individuals were selected based on nest box occupancy, defined by entering nest boxes and carrying nesting material; a female was defined as occupying a specific nest box if she carried nest material to that box repeatedly without being displaced, if she entered a specific nest box repeatedly without being displaced, or if she sang most of her songs from a nest box perch (but not roof). Subjects were sacrificed by rapid decapitation and their brains submersion fixed over four days in 5% acrolein, cryoprotected in 30% sucrose, flash frozen, and stored at -80°C until processing. In all, 16 females were sacrificed. In three cases, the individuals remaining in the room were supplemented with additional individuals to form new groups of six. In these cases, after one week the new group was again subjected to the same observation regime. Most females only went through the paradigm once, and none more than twice.

4.3 Immunohistochemistry and Data Analysis

Brain tissue was sectioned at 40 microns and stored in antifreeze (PBS, polyvinylpyrrolidone, sucrose and ethylene glycol mixture) at -20°C . Every 3rd section was immunolabeled via standard protocols (Ritters et al., 2007) with 1:1000 anti-pTH antibody made in rabbit (GeneTex, Irvine CA, #GTX16557) and 1:1000 biotinylated anti-rabbit secondary (Vector Labs), and visualized with diaminobenzadine. Control tissue run with primary omitted showed no label, and previous work has validated the identical antibody in preadsorption tests in songbirds (Matragrano et al., 2011). All individuals were run in a single batch to eliminate batch variability. Prior studies have demonstrated that labeling patterns using pTH antibodies and antibodies against TH (not specific to its phosphorylated form) on consecutive sections show markedly different labeling patterns within the same regions in which TH is found (Ritters et al., 2007), indicating that the pTH antibody is not labeling unphosphorylated TH. pTH-immunoreactive label (pTHir) was quantified on a Nikon microscope with a Spot camera (Diagnostics Instruments, Inc.) and MetaVue software (Universal Imaging Corp) in standardized boxes and ovals placed on digital photomicrographs within the boundaries of the following regions (Figure 1): PAG, LS, VMH, mPOA, and VTA. We used two boxes for each section of PAG due to its triangular shape when cut coronally, combining cell counts in these areas. VTA was further divided into rostral (rVTA), caudolateral (clVTA) and caudomedial (cmVTA) sections as in Goodson et al. (2009). VMH and LS were measured at the level of the anterior commissure (CoA). Labeling in mPOA was measured just rostral to the CoA. The MetaVue autoscale function was used to calculate the correct exposure of each image as a percentage of the total range of light, helping to reduce the variation in background among individuals, although background labeling was fairly uniform across individuals. Each region was thresholded separately because of the difference in labeling patterns from region to region. The threshold was set such that it automatically recorded and displayed the area of labeled cells and fibers. Two independent observers checked the thresholds for each region to agree that each setting was marking obviously labeled tissue but not extraneous background regions. A single threshold was used for each region across all subjects, but each measurement was visually checked to confirm that extraneous objects were not included in the measure. A researcher blind to the behavioral output of the subject then counted the number of discrete cell bodies within the standardized area that the program had marked as above the threshold, yielding a cell count for each side of each section. Cell counts were made for PAG, mPOA and VMH. In LS, only fibers were labeled, so the total area marked above threshold was used as a measure. In all subregions of VTA, labeled cells were too dense to count and fibers were heavily labeled, so the total area computed to be above the threshold was used.

4.4 Statistics

For all variables that did not meet the requirements of parametric statistics, we transformed data with the natural log plus one to meet those requirements. Three sections were analyzed per subject, on both sides of the midline, and these six counts were averaged over subject. To assess whether singing at all was associated with pTH measures in the brain, we compared subjects that sang to those that did not sing at all using unpaired t-tests. We also examined linear correlations between pTH measures and both number of songs produced

and the total time spent singing. Because rostral and caudal VTA have been shown to have different relationships with catecholaminergic measures in other studies, we separated these regions, and further examined the medial and lateral areas of the caudal VTA separately, given that TH positive cells show different morphologies in the two regions (Goodson et al., 2009). We used a sequential Bonferroni correction to adjust the p-values for VTA (adj. $p = 0.017$).

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Highlights

- We examined pTH in female starlings exposed to an unfamiliar nest box competitor
- Singers showed higher pTH-ir in lateral septum and ventral tegmental area
- In medial preoptic area, pTH-ir correlated positively with number of songs produced
- There were no differences between groups in the ventromedial hypothalamus

Figure 1.

Brain regions examined via immunohistochemistry for pTH-ir. Heavy boxes and circles indicate measurement regions. Large text indicates brain regions examined. BNST: bed nucleus of the stria terminalis; CoA: anterior commissure; Cb: cerebellum; CO: optic chiasm; DM: dorsomedial part of the nucleus intercollicularis; HP: hippocampus; HVC: used as a proper name; ICc: nucleus intercollicularis; LHv: lateral hypothalamus; LS: lateral septum (0.10 mm^2); mPOA: medial preoptic nucleus (0.092 mm^2); MS: medial septum; NIII: oculomotor nerve; PAG: periaqueductal gray (0.124 mm^2); PVN: paraventricular nucleus; Rt: nucleus rotundus; TnA: nucleus taeniae of the amygdala; VMH: ventromedial

nucleus of the hypothalamus (0.117 mm^2); VTA: ventral tegmental area; caudolateral: clVTA (0.138 mm^2); rostral: rVTA(0.138 mm^2); caudomedial: cmVTA (0.088 mm^2)

Figure 2.

Representative photomicrographs of areas in which song production significantly related to pTH-ir with birds that sang did not sing on the left and birds that sang at least one song on the right. In the caudomedial ventral tegmental area (cmVTA) and the medial preoptic area (mPOA), the midline runs down the center of each photo. Horizontal bar in mPOA is 100 μ m, all photos same scale.

Figure 3. pTH-ir in brain regions as a function of singer status, comparing individuals that sang at least one song with those that did not sing. Y-axis shows density of pTH-ir in the measurement area or the mean number of cells/mm² that were pTH-positive averaged over three consecutive sections and both left and right sides. Asterisks indicate significant differences at $\alpha = 0.05$.

Figure 4.

pTH-ir in the mPOA showed a significant relationship with a) number of songs produced in the 30 min. observation period, as well as b) with the amount of time spent singing during that period.