



Evidence that dopamine within motivation and song control brain regions regulates birdsong context-dependently

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ARTICLE INFO

Article history:

Received 18 February 2008

Received in revised form 28 April 2008

Accepted 10 June 2008

Keywords:

Tyrosine hydroxylase

Dopamine beta-hydroxylase

Context

Area X

Medial preoptic nucleus

Ventral tegmental area

Songbird, European starling, *Sturnus vulgaris*

ABSTRACT

Vocal communication is critical for successful social interactions among conspecifics, but little is known about how the brain regulates context-appropriate communication. The neurotransmitter dopamine (DA) is involved in modulating highly motivated, goal-directed behaviors (including sexually motivated singing behavior), and emerging data suggest that the role of DA in vocal communication may differ depending on the context in which it occurs. To address this possibility, relationships between immunolabeled tyrosine hydroxylase (TH, the rate-limiting enzyme in catecholamine synthesis) and song produced within versus outside of a breeding context were explored in male European starlings (*Sturnus vulgaris*). Immunocytochemistry for dopamine beta-hydroxylase (DBH; the enzyme that converts DA to norepinephrine) was also performed to provide insight into whether relationships between song and TH immunoreactivity reflected dopaminergic or noradrenergic neurotransmission. Measures of TH and DBH were quantified in song control regions (HVC, Area X, robust nucleus of the arcopallium) and regions implicated in motivation (medial preoptic nucleus (POM), ventral tegmental area (VTA), and midbrain central gray). In Area X, POM, and VTA measures of TH correlated with song produced within, but not outside of a breeding context. DBH in these regions did not correlate with song in either context. Together, these data suggest DA in both song control and motivation brain regions may be more tightly linked to the regulation of highly goal-directed, sexually motivated vocal behavior.

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1. Introduction

Across vertebrate species, vocal communication is critically involved in coordinating social interactions among conspecifics. Animals communicate in a variety of contexts and display high levels of vocal behavior in response to many different social, physiological, and environmental cues. Successful social interactions often require an animal to adjust vocal behavior so that it occurs in an appropriate context in response to appropriate stimuli. To date, the neuroendocrine mechanisms that regulate context-appropriate vocal communication are not clear.

Male European starlings (*Sturnus vulgaris*) provide a powerful model in which to examine the neural control of context-appropriate vocal communication. Male starlings sing year-round but the function of song and the social and physiological cues that stimulate song production differ depending upon the context in which it occurs [1]. Within a breeding context (i.e., during the spring breeding season) circulating levels of testosterone (T) are elevated [2,3] and singing behavior is critical for mate attraction. Males increase their rate of

song production in response to females [4], and high rates of song may be reinforced through copulation [5–9]. In contrast, within a non-breeding context (i.e. fall and winter) circulating levels of T are basal [2,3]. Males do not respond to females with an increase in song production [4] and song appears unrelated to mate attraction [1]. At this time, starlings form flocks [10] and song is thought to maintain flock cohesion and dominance hierarchies within the flock [11,12]. Overall, males within and outside of a breeding context sing at similar rates [4,13], but on average breeding context song is slightly longer than non-breeding context song [4]. Taken together, breeding context song can be described as highly sexually motivated and goal directed, whereas non-breeding context song does not appear to be directed towards a particular individual or immediate goal but to be motivated by affiliation with a social group (i.e. what motivates singing behavior differs ‘context-dependently’).

Little is known about the neurotransmitter systems involved in regulating context-appropriate vocal communication. The neurotransmitter dopamine (DA) has a long history of being associated with reinforcement and reward [14]. DA has also been implicated in highly motivated, goal-directed or anticipatory responses to stimuli associated with reward, including sexual behavior, feeding, and the use of drugs of abuse [15–22]. We have found that within a breeding context, peripheral injections of DA agonists stimulate whereas DA antagonists inhibit song produced by male starlings in response to the introduction of a female

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[23]. In male zebra finches, DA antagonists also inhibit courtship singing [24] and *in vivo* microdialysis reveals that levels of DA in the avian basal ganglia (Area X) are elevated during song that is directed towards a conspecific [25]. A role for DA in the regulation of goal-directed vocal behavior extends beyond songbird model systems. For example, DA has also been found to stimulate ultrasonic vocalizations associated with the anticipation of social reward in rats [26,27].

Overall, it appears that DA may play an important role in modulating vocal behavior associated with social or sexual reinforcement in birds and mammals, but the role of DA in the regulation of vocal communication in other contexts remains unclear. The effects of peripheral DA manipulations on non-breeding context song in male starlings have not been explored; however, dopaminergic drugs have been shown to influence multiple types of vocalizations in domestic chicks (i.e. trills, twitters, and warbles), none of which appear to be directed towards an individual [28]. Furthermore, microdialysis demonstrates that, relative to directed song, DA levels in Area X of zebra finches are significantly lower during undirected song (i.e. the bird is either oriented away from a conspecific or alone) [25]. Together these data suggest that dopaminergic neurotransmission may differentially modulate vocal behavior depending upon the context in which it occurs.

In songbirds, Area X, HVC (used as a proper name), and the robust nucleus of the arcopallium (RA) are components of the song control system, a series of interconnected nuclei that regulate song production, perception, and learning [29–32]. These song control nuclei receive dopaminergic input. DA [33–35] and the DA synthetic enzyme tyrosine hydroxylase [36,37] are found throughout the song control system and the boundaries of Area X can be defined based on a high density of DAD1 receptors in a manner that is consistent with Nissl-defined boundaries [38]. As indicated above, DA within Area X appears to be involved differentially in modulating directed versus undirected singing behavior in male zebra finches [25]. The role of DA in other song control regions remains unknown; however, several lines of evidence demonstrate that Area X, HVC, and RA regulate song context-dependently [13,39–42].

Tract-tracing studies have identified the ventral tegmental area (VTA) and midbrain central gray (GCT) as the primary sources of dopaminergic inputs to Area X, HVC, and RA, (Fig. 1) [43–45]. VTA and GCT are also reciprocally connected to the medial preoptic nucleus (POM) [46] (Fig. 1), a region implicated in sexually motivated behaviors, including song (detailed below). As in mammals, in birds DA-related proteins and receptors are dense within POM, VTA, and GCT [34,35,47–49]. These dopaminergic systems are considered central to the neural regulation of motivation (including sexual motivation) and reward [16–18,20,50,51].

Taken together, dopaminergic neural circuits including the POM, VTA, and GCT are ideally positioned to influence the song control system and regulate socially appropriate vocal behavior in songbirds.

Consistent with this idea are data that demonstrate that POM and VTA regulate singing behavior context-dependently. In starlings, lesions to the POM suppress sexually motivated song, but stimulate song in other contexts [52]. Additionally in starlings, the numbers of immediate early gene (IEG)-labeled cells in POM and VTA are positively correlated with breeding, but not non-breeding, context song [13]. Similarly, in house sparrows the numbers of IEG-labeled cells in POM correlate with vocalizations produced on, but not away from, a nest site [40]. Electrophysiological data in zebra finches also support a role for VTA in the regulation of female-directed song [53], and research in zebra finches and quail has shown elevated activity of DA neurons in VTA, GCT, and the preoptic area of males displaying sexual behavior, including courtship song [47,54]. Although these regions are DA-rich, the possibility that DA in POM, VTA and GCT might play a context-dependent role in song has yet to be explored.

Several lines of evidence suggest that DA systems are sensitive to changing levels of circulating steroid hormones. Gonadal steroid hormone receptors and receptor mRNA are present at high concentrations in POM, VTA, GCT and the song control system [55–57]. Furthermore, steroid hormone treatment alters various aspects of dopaminergic neurotransmission in the song system and hypothalamus [35,58]. The link between steroid hormones and DA further suggests that DA may differentially regulate song within (when T is high) versus outside (when T is low) a breeding context.

To test the hypothesis that DA within motivation and song control brain regions regulates singing behavior in male starlings context-dependently, we examined relationships between tyrosine hydroxylase (TH; the rate-limiting enzyme in catecholamine synthesis) immunolabeling and singing behavior in breeding context and non-breeding context male starlings. We also used immunocytochemistry for dopamine β -hydroxylase (DBH; the enzyme that converts DA to norepinephrine (NE)) to provide insight into whether TH immunoreactivity reflects dopaminergic or noradrenergic neurotransmission.

2. Methods

2.1. Capture and housing

Twenty-seven adult male and eight adult female European starlings were captured from December 2005 through February 2006 on a

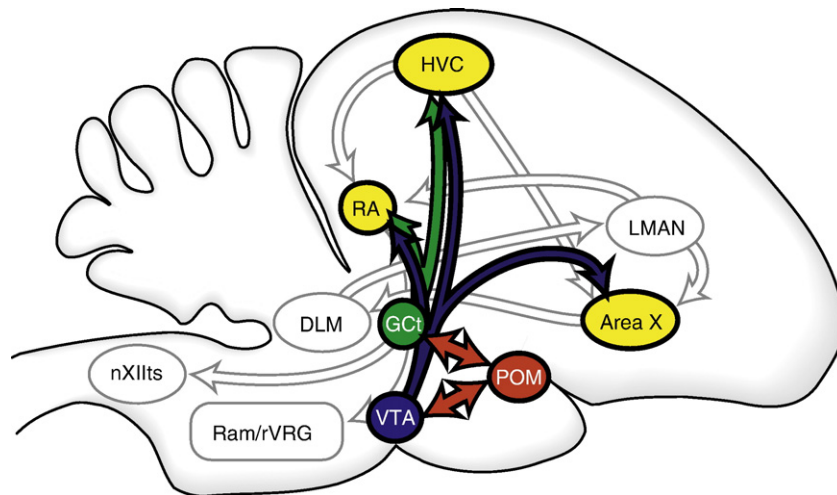


Fig. 1. Sagittal schematic showing projections from the medial preoptic nucleus (POM) to the ventral tegmental area (VTA) and mesencephalic central gray (GCT) and from VTA and GCT to Area X, HVC, and robust nucleus of the arcopallium (RA). Other abbreviations: LMAN, lateral portion of the magnocellular nucleus of the anterior midopallium; DLM, medial portion of the dorsolateral nucleus of the anterior thalamus; Ram/rVRG, nucleus retroambigialis/rostral ventral respiratory group; nXIIIts, tracheosyringial portion of the hypoglossal nucleus.

single farm northwest of Madison, Wisconsin using fly-in traps. After capture, birds were housed indoors in the University of Wisconsin-Madison Department of Zoology animal facilities in single sex cages (91 cm×47 cm×47 cm) on photoperiods matching the outdoor light cycle. After three months, birds were shifted from a natural photoperiod to 18L:6D for six weeks and then to 6L:18D for six weeks. This photoperiod manipulation is routinely used in our lab to induce photosensitivity; a condition in which a bird will respond to an increase in photoperiod (>11 L:13D) and/or hormone treatment with sexual behavior. Photosensitive birds were randomly assigned to either breeding context or non-breeding context social groups and photoperiod/hormone manipulated (see below) to induce context-appropriate endocrine states and associated behaviors. Breeding context males ($n=17$) were given two subcutaneous implants of testosterone (T) and breeding context stimulus females ($n=4$) were given two subcutaneous implants of 17 β -estradiol (see below) and moved to a photoperiod of 11L:13D. Non-breeding context males ($n=10$) and non-breeding context stimulus females ($n=4$) were given blank implants and remained on a photoperiod of 6L:18D. Protocols used for bird acquisition, surgery, and behavioral testing were in adherence to guidelines approved by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (DHEW Publication 80-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205) as well as the University of Wisconsin-Madison Institutional Animal Care and Use Committee.

2.2. Hormone implants

Thirteen days prior to the first day of behavioral testing, all birds were lightly anesthetized using isoflurane gas anesthesia and a small incision was made in the skin over the breast muscle. Breeding context males received two, 14 mm lengths of silastic tubing (i.d., 1.47 mm; o.d. 1.96 mm; Dow Corning, Midland, MI) packed for 10 mm with crystalline testosterone propionate (Sigma-Aldrich, St. Louis, MO) and breeding context stimulus females received two, 17 mm lengths of silastic tubing packed for 13 mm with 17 β -estradiol (Sigma-Aldrich). Implants were sealed with silastic glue. Non-breeding context males and non-breeding context stimulus females received empty implants. Implants were placed under the skin, the incision was sutured and birds were allowed to recover on a heated pad.

2.3. Behavioral observations

Three days after implant surgery one social group of non-breeding context males ($n=10$) and two social groups of breeding context males ($n=10$ and $n=7$) were introduced into similar indoor observation aviaries. Birds were behaviorally tested in social groups because we were most interested in examining song behavior as it occurs within a dynamic social system (i.e. a flock). A greater number of breeding context males were used because past data from our lab has shown that within a breeding context, nestbox-ownership influences song production (i.e. males with nestboxes sing significantly more than males without nestboxes [4,13]) and we wanted to ensure that a sufficient number of nestbox-owners were included in the study. Social groups were visually and acoustically isolated from one another. Aviaries contained five nestboxes and branches for perching. Food and water were provided *ad libitum*. Birds were housed in the observation aviaries throughout the duration of behavioral observations. The breeding context social groups were housed on 11L:13D and the non-breeding context social group was housed on 6L:18D. Behavioral observations began after a ten day habituation period.

On four consecutive days, social groups were observed for 45 min after the introduction of a stimulus female. Breeding context stimulus females were presented to breeding context males to elicit sexually motivated singing behavior. Non-breeding context males do not alter singing behavior in response to the introduction of stimulus females

[e.g. 4]. However, to control for the effect of a novel opposite sex conspecific being introduced to the observation aviary, non-breeding context males were presented with non-breeding context stimulus females. We selected a non-breeding context female in order to maintain ecological validity (i.e. in the wild, a non-breeding context male would never encounter a breeding context female).

On each test day, a novel female was presented to the social groups in a randomized order. To characterize male singing behavior, a point sampling technique [5] was used to estimate the proportion of time every member of the social group spent singing. During the forty-five minute observation period a weak beep was emitted from a timer at one minute intervals and at every beep it was noted whether each member of the social group was or was not singing. The number of times males entered and exited nestboxes was noted throughout the forty-five minute period.

2.4. Tissue processing

Immediately after the last forty-five minute observation period, all males in a social group were sacrificed via rapid decapitation. Brains were removed, fixed in 5% acrolein solution overnight, placed in cryoprotectant, and frozen at -80°C until sectioning. Using a cryostat, brains were sectioned in three, 40 μm series and stored in anti-freeze until processing. Series one was used for TH immunocytochemistry and series two was used for DBH immunocytochemistry. Series three was used for an immunocytochemistry not discussed here.

2.5. Immunocytochemistry

Immunocytochemistry was run separately for TH and DBH, however all males ($n=27$) were run in a single batch for each marker. Sections were rinsed in phosphate buffered saline (PBS) for 30 min, incubated in 0.5% sodium borohydride solution for 15 min, rinsed in PBS for 20 min, incubated in 0.5% hydrogen peroxide solution for 10 min, rinsed in PBS for 20 min, incubated in 5% normal goat serum (NGS (made in PBS with 0.2% triton (PBS-T))) solution for 1 h, and then incubated in 2% NGS (made in PBS-T) primary solution overnight at room temperature (for TH, mouse anti-TH at 1:10,000 and for DBH, rabbit anti-DBH (ImmunoStar, Inc., Hudson, WI.; catalog #s 22941 and 22806 respectively) at 1:5,000). Sections were then rinsed in PBS-T for 30 min and incubated in 2% NGS (made in PBS-T) biotinylated secondary solution for 90 min at room temperature (for TH, goat anti-mouse at 1:500 and for DBH, goat anti-rabbit at 1:250 (Vector Laboratories, Burlingame, CA)). Then, sections were rinsed in PBS-T for 30 min, incubated in AB solution (Vector ABC kit) for 1 h, rinsed in PBS-T for 30 min, and the avidin-biotin complex was visualized using Vector SG (Vector). Sections were float mounted on gel-coated slides, dehydrated, and cover slipped.

2.6. Quantification

TH and DBH immunoreactivity were quantified within POM, VTA, GCt, Area X, HVC, and RA using MetaVue software (Fryer Company, Inc., Huntley, IL). A Spot camera connected a Nikon Eclipse E600 microscope to a PC computer and images were acquired at 20X magnification. In MetaVue, a unique threshold for each label in each region was set. Within a region, the same threshold was used for every individual. On each acquired image, the optical density of immunolabeling and the pixel area covered by labeled fibers were quantified using MetaVue Integrated Morphometry Analysis. We used both optical density and pixel area because each measure provides different information about the nature of labeling in a given area. Optical density (the sum of gray value means) provides information about how concentrated label is, whereas pixel area provides information about the relative area covered by labeled fibers. TH and DBH optical density and pixel area were quantified in all areas on three serial sections bilaterally. When

present, the numbers of cell bodies immunopositive for TH and DBH were hand-counted. In cases of tissue damage or lost sections, measures were made on a fourth section. If tissue damage was extensive, that individual was dropped from quantification for that brain area. In Area X, HVC, and RA TH and DBH were measured within an area of $0.58 \text{ mm} \times 0.44 \text{ mm}$; in POM and VTA, $0.37 \text{ mm} \times 0.43 \text{ mm}$; in GCt, $0.51 \text{ mm} \times 0.33 \text{ mm}$ (see Fig. 2 for region placement). The quantification of TH and DBH occurred well within the boundary of each nucleus based on past studies in which the placement and size of these regions were confirmed using alternate Nissl stained sections [13]. For every individual, mean optical density, mean pixel area, and mean number of cell bodies were calculated separately for TH and DBH and used for statistical analysis.

2.7. Statistics

Data were analyzed using Statistica 6.0 software (Stat Soft Inc., Tulsa, OK). The mean number of point samples with singing behavior across the four days of behavioral testing was calculated for each male. If a male was observed entering and exiting a nestbox multiple times on more than one day, he was designated a nestbox-owner. Because our goal was to understand dynamic social interactions, males were observed singing within social groups. Thus the behavior of each individual likely influenced other members of its social group. Due to this lack of independence of data points, non-parametric statistics were used. Spearman rank order correlation analyses were used to determine the extent to which TH and DBH optical density, pixel area,

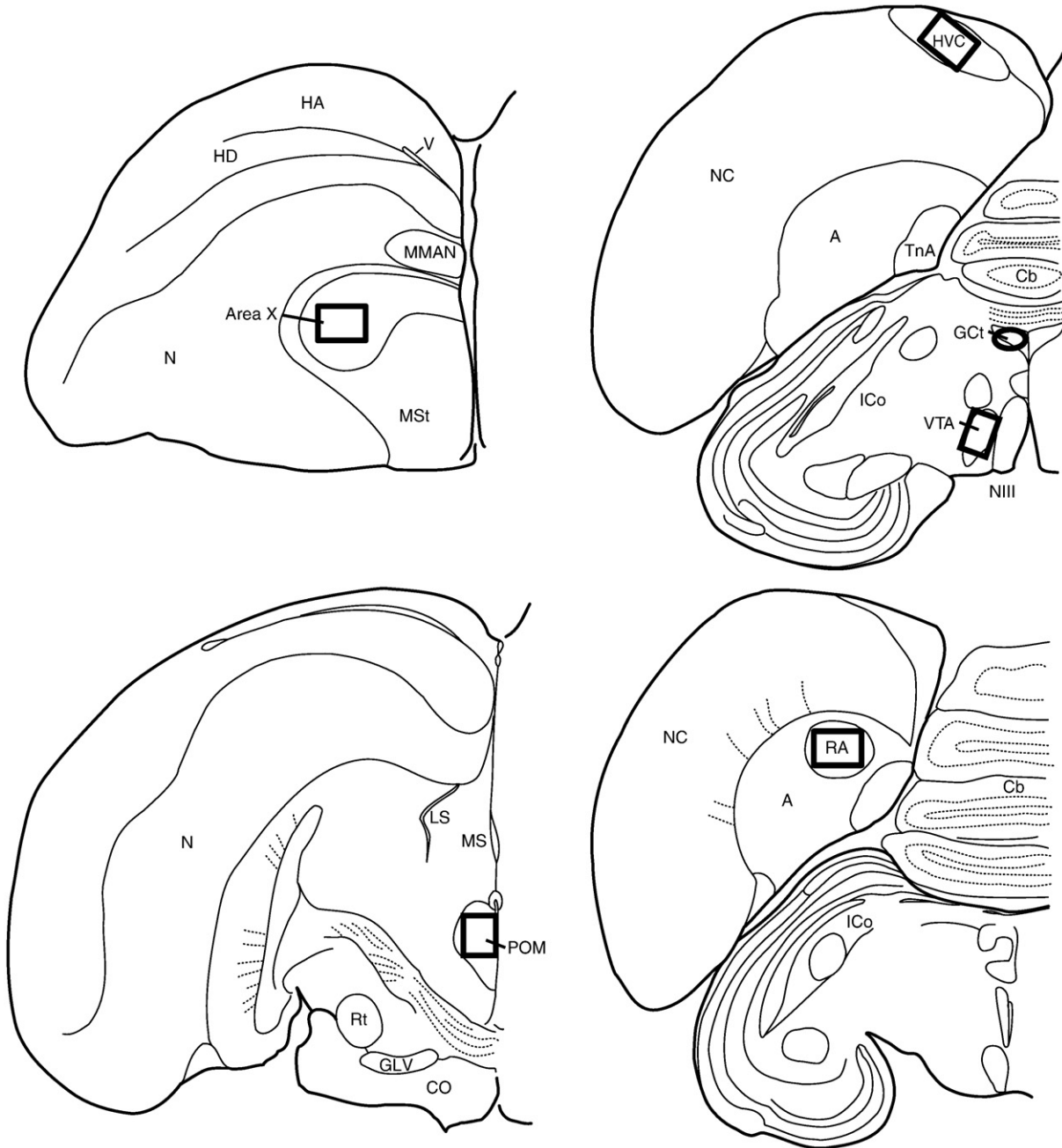


Fig. 2. Boxes/circle indicate approximate areas in which TH and DBH were measured. Abbreviations: A, arcopallium; Cb, cerebellum; CO, optic chiasm; GCt, mesencephalic central gray; GLV, nucleus geniculatus lateralis, pars ventralis; HA, apical part of the hyperpallium; HD, densocellular part of the hyperpallium; ICo, nucleus intercollicularis; LS, lateral septum; MMAN, medial magnocellular nucleus of the anterior nidopallium; MS, medial septum; MSt, magnocellular part of the medial striatum; NIII, third cranial nerve; N, nidopallium; NC, caudal nidopallium; POM, medial preoptic nucleus; RA, robust nucleus of the arcopallium; Rt, nucleus rotundus; TnA, nucleus taeniae of the amygdala; V, ventricle; VTA, ventral tegmental area.

and numbers of immunopositive cell bodies in POM, VTA, GCt, Area X, HVC, and RA correlated with singing behaviors in each context. Mann–Whitney *U* tests were used to compare singing behavior and indices of TH and DBH between breeding context and non-breeding context males as well as between breeding context males with nestboxes and breeding context males without nestboxes.

3. Results

Individuals within the breeding context social groups displayed behaviors that are only observed in wild birds during the breeding season. They occupied/defended nestboxes, collected nest material, wing-waved (another courtship behavior) and responded to the introduction of an opposite sex conspecific with an increase in singing behavior. No non-breeding context males were observed displaying these behaviors. Taken together this indicates the hormone/photoperiod manipulations were effective in placing birds in context-appropriate behavioral states.

The song behavior and measures of TH and DBH in the two breeding context social groups were compared statistically using Mann–Whitney *U* tests and did not significantly differ. Furthermore, results of correlations between song and immunolabeling for the two breeding con-

text social groups were similar for each brain region. Therefore the two breeding context social groups were combined for analysis.

In Area X, TH optical density was significantly negatively correlated with breeding context, but not non-breeding context, singing behavior (Figs. 3A, 4A: non-breeding context: $r_s=0.50$, $p=0.21$; breeding context: $r_s=-0.671$, $p=0.01$). In POM, TH pixel area was significantly negatively correlated with only breeding context song (Figs. 3B, 4B: non-breeding context: $r_s=-0.24$, $p=0.51$; breeding context: $r_s=-0.62$, $p=0.02$). In VTA, TH optical density was significantly positively correlated with only breeding context song (Figs. 3C, 4C: non-breeding context: $r_s=0.38$, $p=0.28$; breeding context: $r_s=0.60$, $p=0.02$). In VTA, there were two non-breeding context males and two breeding context males whose TH optical density values were noticeably higher than all other individuals (Fig. 3C). Removal of these individuals did not change the significance of the correlations (non-breeding context: $r_s=0.67$, $p=0.07$; breeding context: $r_s=0.59$, $p=0.03$). In RA, there was a trend for TH pixel area to relate positively to breeding context song but the relationship failed to reach significance (non-breeding context: $r_s=0.04$, $p=0.91$; breeding context: $r_s=0.49$, $p=0.07$).

No other measures of TH (optical density, pixel area, or numbers of immunopositive cell bodies) were significantly correlated with song in either context ($p>0.16$ in all cases). Measures of DBH in Area X, POM and VTA did not significantly correlate with singing behavior in either context ($p>0.39$ in all cases). However it was not the case that song did not relate to DBH in any brain region. For example, while beyond the scope of the present paper, DBH pixel area in the ventromedial nucleus of the hypothalamus significantly correlated with breeding context song and DBH optical density in medial bed nucleus of the stria terminalis significantly correlated with non-breeding context song (Heimovics and Riters, *unpublished data*).

Mann–Whitney *U* tests revealed no significant differences between breeding context and non-breeding context males in either the mean proportion of time males in either group spent singing or any index of either TH or DBH (optical density, pixel area, or numbers of immunopositive cell bodies) in any of the brain regions examined. Mann–Whitney *U* tests also revealed no significant differences between breeding context males with nestboxes and breeding context males without nestboxes in song behavior or measures of TH or DBH.

4. Discussion

The immunocytochemical data presented here support the hypothesis that DA plays a context-dependent role in the regulation of vocal communication in songbirds. Significant relationships between singing behavior and measures of TH immunoreactivity (THir) in POM, VTA, and Area X were observed for song produced within, but not outside of, a breeding context. No relationship between DBH in these regions and breeding context song was observed suggesting that indices of THir predominantly reflect relationships between song and dopaminergic, rather than noradrenergic, neurotransmission. Although the present data do not preclude a role for DA in non-breeding context song, they do suggest that DA synthesis in POM, VTA, and Area X may be more tightly linked with sexually motivated vocal communication in male starlings.

4.1. TH and Breeding context song

4.1.1. VTA

Across vertebrate taxa, DA producing neurons in VTA that project to the forebrain have been shown to mediate reward and/or motivation associated with numerous goal-directed behaviors, including sexual behavior, feeding and the use of drugs of abuse [16,19,50,59,60]. VTA also projects to the song control system [43–45] and POM [46] in songbirds. In the present study, the relationship between breeding context singing behavior and measures of THir in VTA was positive.

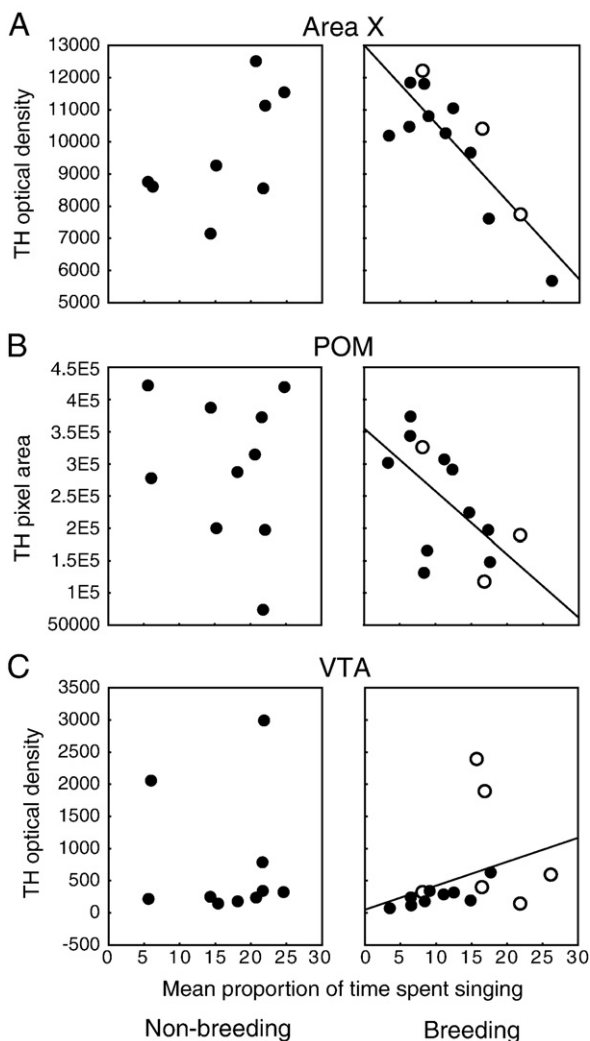


Fig. 3. Plots showing the relationship between singing behavior and (A) Area X TH optical density, (B) POM TH pixel area, and (C) VTA TH optical density. Left panels are non-breeding context males and right panels are breeding context males. Each point represents one individual and open circles represent males with nestboxes. Presence of regression line indicates significant ($p<0.05$) relationships.

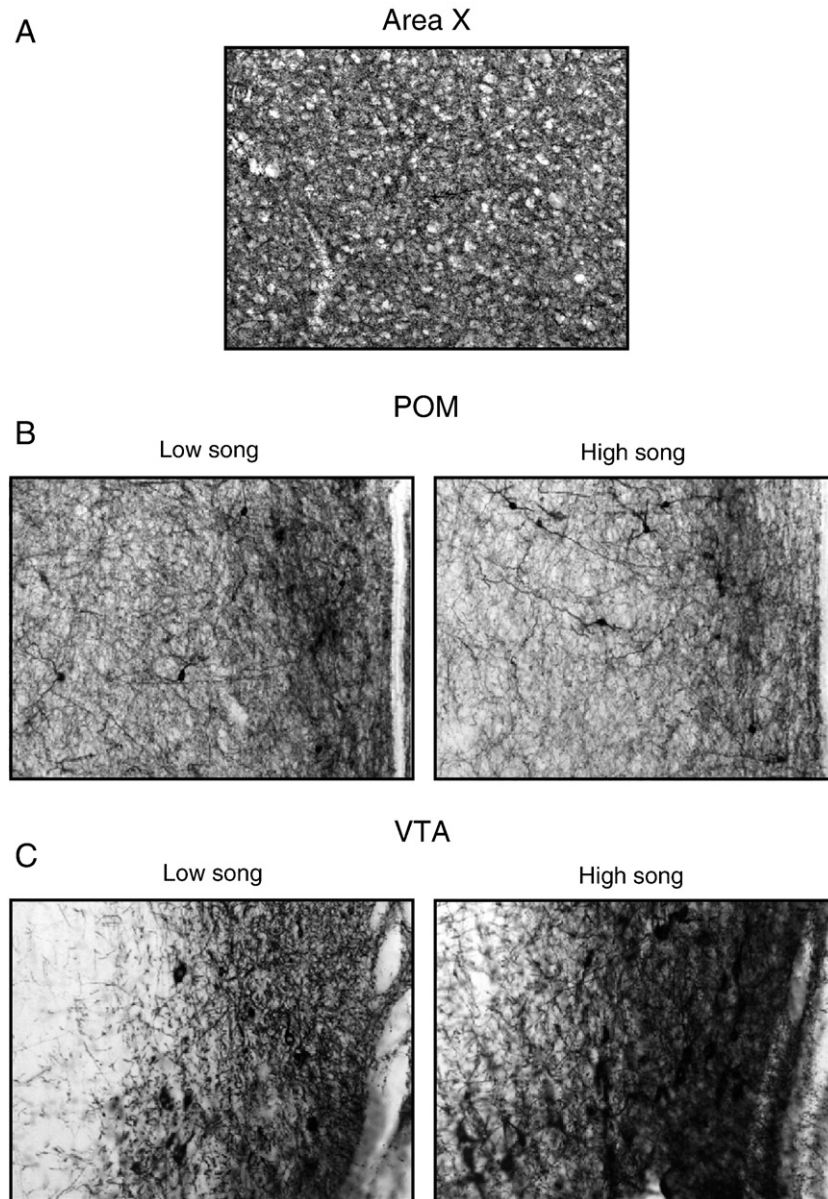


Fig. 4. Representative photomicrographs of TH labeling in (A) the center of Area X in an intermediate singer, and in low and high singers within (B) POM, and (C) VTA of breeding context males. For POM the lateral ventricle is visible in the right side of the image. For VTA the 3rd cranial nerve is visible in the right side of the image. TH labeling in Area X was so dense that the contrast between low and high singers is difficult to discern with the naked eye (i.e. requires the use of computer-aided analysis).

Given that VTA is one of the primary sources of dopaminergic input to much of the brain, our findings suggest that VTA synthesizes and sends high levels of DA to terminal fields in males singing high levels of breeding context song. Our data also suggest that VTA sends less DA to terminal fields in males singing low levels of breeding context song. This relationship was not observed within a non-breeding context, suggesting that DA in VTA may regulate singing behavior context-dependently. This pattern is consistent with past data showing immediate early genes in VTA correlate with breeding, but not non-breeding context song in starlings [13].

4.1.2. Area X and POM

Also consistent with past data, the present results highlight context-dependent participation of both POM [13,52] and Area X [25,41,42,61] in the regulation of singing behavior. However, in contrast to VTA our data revealed a negative relationship between breeding context song and indices of THir in Area X and POM. As mentioned above, VTA sends dopaminergic projections to the song control system, including Area X

[43–45]. VTA is also a source of DA inputs to the preoptic area [62]. Microdialysis studies have shown that DA release is elevated in Area X of male zebra finches singing song that is directed towards a conspecific [25]. Similarly, DA levels are elevated in the medial preoptic area of male rodents after exposure to cues associated with mating (i.e. visual and olfactory stimuli) [17,63]. Past data from our lab show that peripheral DA agonists stimulate breeding context singing behavior in male starlings [23] and preliminary findings from our lab demonstrate that site-specific injections of DA agonists into POM also stimulate starling breeding context song (Pawlisch and Riters, unpublished data). Based on this literature, we believe the negative relationship between measures of THir in Area X and POM and breeding context song probably reflects negative feedback on TH [see 64 for review]. Specifically, within a breeding context DA release may be high in Area X and POM of males singing high levels of sexually motivated song. This high dopaminergic tone could lead to end-product inhibition lowering TH in these individuals. In contrast, dopaminergic tone may be low in Area X and POM of males singing low levels of breeding context song which could reduce

end-product inhibition leading to higher TH. The negative correlation between breeding context song and TH in POM and Area X could also be due to the fact that DA is being synthesized at high rates in the highest singers thus depleting stores of TH.

An alternative interpretation of these findings is that high indices of THir in Area X and POM reflect elevated DA synthesis and release. This would mean that males producing high levels of breeding context song have lower levels of DA in Area X and POM, suggesting that high DA in Area X and POM inhibits sexually motivated singing behavior. Although the present data do not rule out this possibility, given that studies performed to date suggest that sexually motivated behaviors, including courtship song, are associated with elevated DA in Area X and POM (as reviewed above) we believe this interpretation to be unlikely.

It is important to note that changes in density measures of TH do not necessarily indicate changes in DA exocytosis and it is changes in extracellular levels of DA that are most important to the behavioral response (i.e. song) being examined. Thus, we are limited in our ability to interpret the functional relationships between high versus low TH in a given brain area and actual DA release in that region or its terminal field. Future studies that combine immunocytochemistry for TH and microdialysis for extracellular DA would help to further interpret the patterns observed in the present study. However, in spite of these limitations, we find it noteworthy that TH in Area X, POM and VTA significantly correlates with breeding but not non-breeding context singing behavior. Thus, the present findings highlight a potential context-dependent role for DA within Area X, POM, and VTA in the regulation of singing behavior in male European starlings.

4.2. Differences in indices of immunolabeling

In the present analysis we quantified both the optical density of TH immunolabeling and the pixel area covered by labeled fibers. Interestingly, in VTA and Area X, TH optical density (but not pixel area) correlated with breeding context song. In contrast, in POM, TH pixel area (but not optical density) correlated with breeding context song. It is possible that an increase in the area of POM that is innervated by DA (as reflected in pixel area) relates to recruitment of new postsynaptic targets; whereas changes in levels of TH activation within a localized area (as reflected in optical density) of Area X and VTA relates to changes in DA levels within an area already targeted. Taken together, the present study highlights the importance of quantifying multiple indices of immunolabeling to provide a more complete picture of the nature and distribution of labeled cells and fibers.

4.3. TH and endocrine state

Unlike previous work in songbirds showing that DA systems are modulated by exogenous steroid hormones [34,35,58], here we found no significant difference between breeding (T-implanted) and non-breeding (blank-implanted) context males in indices of THir in any of the regions we examined. The effect of androgens on TH in birds and mammals has been shown to vary in a complex time and region specific manner [58,65–71]. Our results suggest that after seventeen days of T treatment, THir does not differ within the regions examined in this study. Additional studies are needed to elucidate the role of androgens on DA and context-dependent song.

4.4. Conclusions

Past studies show that peripheral DA agonists stimulate and antagonists inhibit breeding context singing behavior in starlings [23]. Additional data from pharmacological manipulations in zebra finches support a role for DA in stimulating directed vocal communication [24]. Because these drugs were administered systemically, it is impossible to discern where in the brain altered dopaminergic function influences sexually motivated vocal communication. Past data have highlighted

Area X as a site where DA regulates song behavior context-dependently [25]. The present data further support a context-dependent role for Area X and also identify POM and VTA as potential sites where dopaminergic drugs may act to modulate sexually motivated vocal behavior in songbirds.

The present data taken together with past work expand upon a growing body of data implicating brain regions outside of the song control system in the regulation of singing behavior in songbirds [4,13,40,72–77]. Specifically, the present results support the idea that interactions between POM and VTA may be critically involved in adjusting vocal behavior so that it occurs in the appropriate context in response to appropriate stimuli. Furthermore, the data presented here clearly implicate DA as an important modulator of context-appropriate vocal communication in songbirds.

4.5. Broader implications

The incentive salience hypothesis of DA function posits that DA may be more critically involved in regulating goal-directed, motivated responses to rewarding stimuli or the “wanting” of a particular stimulus (as opposed to underlying the reward itself) [15]. Other neurochemicals, such as opioid neuropeptides, have been implicated in reward or reinforcement components of behavior, or the “liking” of a particular stimulus [15,78]. Consistent with this framework, in the present study we found THir to be linked exclusively to song produced within a context in which it is highly sexually motivated, goal-directed and associated with the wanting of a female (i.e. a breeding context) whereas in past work we have found that immunolabeling for the opioid neuropeptide met-enkephalin in POM to be more tightly linked to song produced within a context in which it does not appear to be goal-directed and has no obvious form of immediate external reinforcement (i.e. a non-breeding context [79]). Taken together, these data suggest the intriguing possibility that the context-dependent roles of dopamine and opioids in the regulation of song may relate to their differential involvement in “wanting” versus “liking” associated with song behavior. Importantly, the present study examined one exemplar of goal-directed vocal communication (i.e. sexually motivated, female-directed song) and future studies must examine other forms of goal-directed vocal behavior to verify this hypothesis.

Acknowledgements

The data presented in this paper are based upon work supported by a grant from NIMH (R01MH080225) to LVR and a graduate research fellowship from NSF to SAH. We gratefully acknowledge Kate Skogen, Jeff Alexander, Chris Elliot, and John Irwin for help with starling capture and animal care; Ben Pawlisch, Sarah Alger, Eric Lyngaas, Stephen Bowman, Charles Dulberger, Lara Peschke, Laura Klein, Laura Knepel, and Lea Gardiner for their help with tissue processing; and Bill Feeny for assistance with illustrations.

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