Brief Communications

Social Context-Dependent Singing-Regulated Dopamine

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Like the mammalian striatum, the songbird striatum receives dense dopaminergic input from the midbrain ventral tegmental area–substantia nigra pars compacta complex. The songbird striatum also contains a unique vocal nucleus, Area X, which has been implicated in song learning and social context-dependent song production. Area X shows increased neural firing and activity-dependent gene expression when birds sing, and the level of activation is higher and more variable during undirected singing relative to directed singing to other birds. Here we show in the first report of *in vivo* microdialysis in awake, behaving songbirds that singing is associated with increased dopamine levels in Area X. Dopamine levels are significantly higher with directed relative to undirected singing. This social context-dependent difference in dopamine levels requires the dopamine transporter, because local *in vivo* blockade of the transporter caused dopamine levels for undirected singing to increase to levels similar to that for directed singing, eliminating the social context-dependent difference. The increase in dopamine is presumably depolarization and vesicular release dependent, because adding of high K^+ increased and removal of Ca^{2+} increased and decreased extracellular DA levels. Our findings implicate DA and molecules that control DA kinetics in singing behavior and social context-dependent brain function.

Key words: **basal ganglia; zebra finch; nomifensine; DAT; birdsong; egr-1 (ZENK)**

Introduction

Zebra finches sing two song types, "directed" toward another bird and "undirected" when not oriented toward another bird (Zann, 1996). These songs are produced as repetitive stereotyped motif sequences of four to seven syllables (Zann, 1996). This social context-dependent difference in singing behavior is associated with dramatic changes in singing-related neural firing and activity-dependent gene expression in Area X of the striatum; the neural firing and gene expression levels are higher and more variable during undirected relative to directed song (Jarvis et al., 1998; Hessler and Doupe, 1999). Area X is part of a specialized pallial–basal ganglia–thalamic loop (see Fig. 1*A*) necessary for song learning, and it is similar to basal ganglia systems of mammals (Scharff and Nottebohm, 1991; Doupe et al., 2005). Like the mammalian striatum, Area X receives dense dopaminergic input from midbrain dopamine (DA) neurons (Lewis et al., 1981; Reiner et al., 2004). Furthermore, DA modulates the strength of excitatory synaptic inputs to Area X spiny neurons (Ding and Perkel, 2002, 2004; Ding et al., 2003). Control of DA level kinetics by the DA reuptake transporter (DAT) on DA axons is strikingly similar to DAT function in rodent and primate striatum (Gale and Perkel, 2005). Because DA plays an important role in the function of the basal ganglia in motor control and learning, this led us to hypothesize that DA release in Area X is also differentially regulated by social context-dependent singing (Jarvis et al., 1998). To test this hypothesis, we measured DA levels in Area X of singing birds via microdialysis. We found that singing is associated with high DA levels in Area X in a social context-dependent manner that requires the DAT.

Materials and Methods

Animals. We used 22 adult male zebra finches (>90 d old) bred at Duke University. Several weeks before surgery, birds were housed individually in sound-attenuation boxes to acclimate them to the novel environment. Daily singing behavior was recorded using the Sound Analysis Live program (Tchernichovski et al., 2000), and strong singers were selected (birds that sang for at least 30 min/d). Experiments were approved by the Duke University Animal Care and Use Committee.

Microdialysis probe and guide cannula implantation. To maximize singing behavior without gliosis around the probe, a guide cannula (CMA-7; CMA Microdialysis, Solna, Sweden) was implanted above Area X. Birds were allowed to acclimate to the cannula for at least 1 week or until recovery of strong singing behavior. Thereafter, a microdialysis probe (1 mm membrane length, 0.24 mm outer diameter, Cuprophane, 6 kD cutoff, CMA-7; CMA Microdialysis) was inserted into Area X. Weak singers received a microdialysis probe into Area X without a guide cannula implantation because we wanted to record DA levels without singing. To perform surgery, birds were anesthetized with 30–40 μl of intramuscular ketamine–xylazine (40 and 8 mg/kg, respectively) and placed in a stereotaxic apparatus. A marking pipette was set at interaural zero and moved 0.3 mm caudal to the bifurcation point of the midsagittal sinus by rotating the head angle in the stereotaxic apparatus. This set the head angle to 45°. Then the head angle was moved an additional 30°, making the total angle 75°. The pipette was moved 1.8 mm rostral and 1.3 mm lateral. The skull was then marked, which points vertically to the center of Area X, passing rostral to and preventing lesions to the lateral magnocellular nucleus of anterior nidopallium (LMAN). Then the guide cannula or probe was inserted. After recovery from surgery, the birds were returned to their sound-attenuation boxes.

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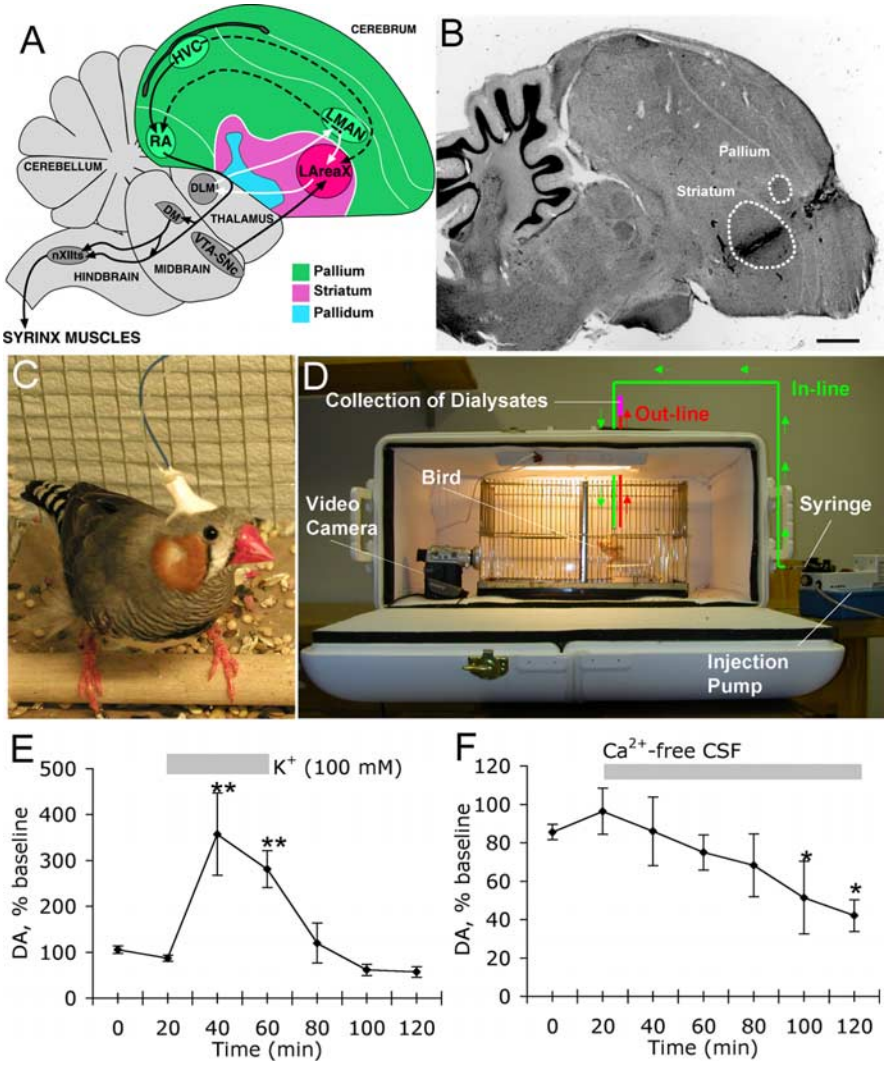

Figure 1. Microdialysis setup and K^+/Ca^{2+} regulation of DA levels in Area X. ***A***, Vocal pathways showing DA input from VTA–SNc to Area X. White lines, Anterior vocal pathway involved in vocal learning and social context production; black lines, posterior vocal pathway involved in production of learned vocalizations; dashed lines, connections between the two pathways. Abbreviations are according to new nomenclature (Jarvis et al., 2005): DLM, dorsal lateral nucleus of mediodorsal thalamus; DM, dorsal medial nucleus of midbrain; HVC, nucleus HVC; LAreaX, lateral Area X; nXIIts, tracheosyringeal part of XII motor nucleus; RA, robust nucleus of arcopallium. ***B***, Representative sagittal histological photograph of probe track in Area X. Dashed lines, Boundaries of Area X and LMAN. Scale bar, 1 mm. ***C***, Microdialysis probe implanted in a freely moving songbird. The perch and seed were on a clean floor to prevent tubing from being tangled. ***D***, Microdialysis setup showing sound attenuating chamber, video camera, pump, and tubing. ***E***, Effect on DA levels from infusion of high K^+ through the dialysis probe in Area X ($n = 3$ birds). ***F***, Effect on DA levels from infusion of Ca^{2+} -free artificial media into Area X ($n = 3$ birds). *$p < 0.05$, **$p < 0.01$ relative to the 0 time point (repeated-measures ANOVA). Error bars indicate SEM.

Microdialysis procedure. After restoration of singing behavior, for birds with cannulas, a microdialysis probe was inserted through the cannula into Area X under inhalant isoflurane anesthesia, 24 h before the start of the experiment (Fig. 1*B*,*C*). At 2–3 h before microdialysis collection, the incoming probe tubing was connected to FEP tubing with adaptors (CMA Microdialysis), which was passed through a ball bearing (BMNM-8; Small Parts, Miami, FL) and a swivel (375/22P; Instech, Solomon, PA) to a syringe pump (Fig. 1*D*). The outgoing probe tubing (20 cm) was connected to 43 cm of FEP tubing, adaptors, and then to a 250 μl collection vial; outgoing and incoming probe tubing were taped together inside of the box. The swivel with manual turning by the experimenter outside of the box (where the bird could not hear or see the experimenter) allowed the bird freedom of movement during dialysate collection. The probe was then perfused with artificial CSF (CMA Microdialysis) at a rate of 1 μl/min for 2–3 h. The birds were in the dark to eliminate possible effects of singing and other behaviors on DA levels. To determine basal DA levels using the quantitative "low-perfusion-rate" technique (Justice, 1993; Gainetdinov et al., 2003), the lights were turned on and three samples were collected at a flow rate of 56 nl/min, 120 min each, into the vial containing 2.5 μl of 0.5 M perchloric acid, and the vial was placed immediately on ice and then stored at −80°C. For the K^+ and Ca^{2+} experiments, CSF with added K^+ (100 mM) or without Ca^{2+} were used (Westerink, 1995), and dialysate samples were collected at a normal flow rate (1 μl/min) every 20 min. For determining singing-associated DA levels, we performed experiments in a similar manner with normal CSF, except that dialysate samples were collected every 10 min before, during, and after singing or other behaviors. The birds' behaviors were monitored on a television screen and video recorded.

Singing experiments consisted of two phases. In the first phase, male birds were either presented with a female bird in a separate cage for 30 min for directed singing or remained alone for 30 min for undirected singing, followed by seclusion for 90 min in darkness. After this 90 min period, the second phase began, in which each male bird was counterbalanced with respect to its first phase, in that birds presented with the receptive female before darkness during the first phase remained alone for 30 min for undirected singing in the second phase, and vice versa. The order of directed and undirected singing for each bird in the first phase was chosen randomly. If a bird did not sing, it was assigned to the light-only condition, or we repeated the experiment the following day. Detection of DA deteriorated after 4–5 d of dialysate collection, likely as a result of gliosis around the dialysis probe (Westerink, 1995); thus, data shown are from dialysates collected 1–3 d after probe insertion.

Infusion of DAT blocker. Throughout the experiment, the probe was perfused with the DA reuptake inhibitor nomifensine (Sigma, St. Louis, MO), which was dissolved in artificial CSF at a concentration of 10 μM (Mazei et al., 2002; Robinson and Wightman, 2004).

HPLC with electrochemical detection. DA was assayed using HPLC-electrochemical detection. DA was separated from 5 μl of each dialysate sample on a microbore Unijet C18 RP column (5 μm, 1 × 150 mm; BAS, West Lafayette, IN) with a mobile phase in 0.03 M citrate-phosphate buffer containing 2.1 mM octyl sodium sulfate, 0.1 mM EDTA, 10 mM NaCl, and 17% methanol, pH 3.6, at a flow rate of 90 μl/min, and detected by a 3 mm glassy carbon electrode (Unijet; BAS) set at +0.8 V. Sensitivity permitted detection of ~3 fmol of DA. DA concentration was quantified by comparing peak heights from samples to external standards (Gainetdinov et al., 2003).

Histology. At the end of the experiments, birds were overdosed with pentobarbital, and brains were fixed by intracardiac infusion of 0.9% saline, followed by 4% paraformaldehyde in saline. Sagittal sections (60 μm) were cut and stained with cresyl violet to verify probe placement (Fig. 1*B*).

Data analysis. For analysis of DA levels, data were lag-shifted by one time bin (10 min) to compensate for the delay between DA release in the brain and dialysate collection in the vial (total length of probe, tubing,

and connectors between bird and collection vial was ~65 cm; minimum internal tube diameter of 0.12 mm). Before performing our main experiments, we established that a flow rate of 1 μl/min results in a ~10 min delay between the microdialysis probe and collection vial (delay volume, ~10 μl), consistent with factory-specified volumes. The three 10 min microdialysis values for each bird during singing or light exposure was divided by the average of the three previous baseline values and then averaged across the three time bins (except for one bird in which the technical problems precluded reliable detection of the last singing sample, and therefore data were averaged for two time bins) and multiplied by 100 to generate a percentage of change relative to baseline. To measure statistical differences in DA levels relative to baseline, we used the Wilcoxon signed rank test. To measure differences relative to each social context, we used *t* test or one-way ANOVAs followed by Fisher's *post hoc* PLSD tests.

Results

Extracellular DA levels are reflective of depolarization-induced exocytosis

We first tested, as in mammals, whether in the brain of awake songbirds dialysate DA levels reflect physiological release from DA terminals. Using the quantitative "low perfusion rate" microdialysis technique, we found that during average daily activity (eating, drinking, singing, and moving), extracellular DA levels in Area X were 23.0 ± 3.7 nM (SEM; $n = 6$). These concentrations are similar to those found in the mammalian striatum when using microdialysis (Justice, 1993; Gainetdinov et al., 2003; Watson et al., 2006). To determine whether DA levels in Area X are activity dependent, we infused high K^+-containing artificial CSF through the dialysis probe; K^+-induced depolarization is known to activate Ca^{2+} channels of presynaptic terminals causing vesicle fusion and neurotransmitter release (Westerink, 1995). K^+-induced depolarization evoked a 350% increase in extracellular DA levels (Fig. 1*E*). After removal of high K^+ concentrations, it took ~40 min for DA to return to baseline levels. As a corollary of this finding, removal of Ca^{2+} by infusion of Ca^{2+}-free artificial media to prevent presynaptic vesicle fusion and neurotransmitter release significantly reduced extracellular DA levels (Fig. 1*F*). Thus, consistent with findings in brain slices (Gale and Perkel, 2005), these data indicate that DA levels in songbird Area X reflect depolarization-dependent vesicular exocytosis.

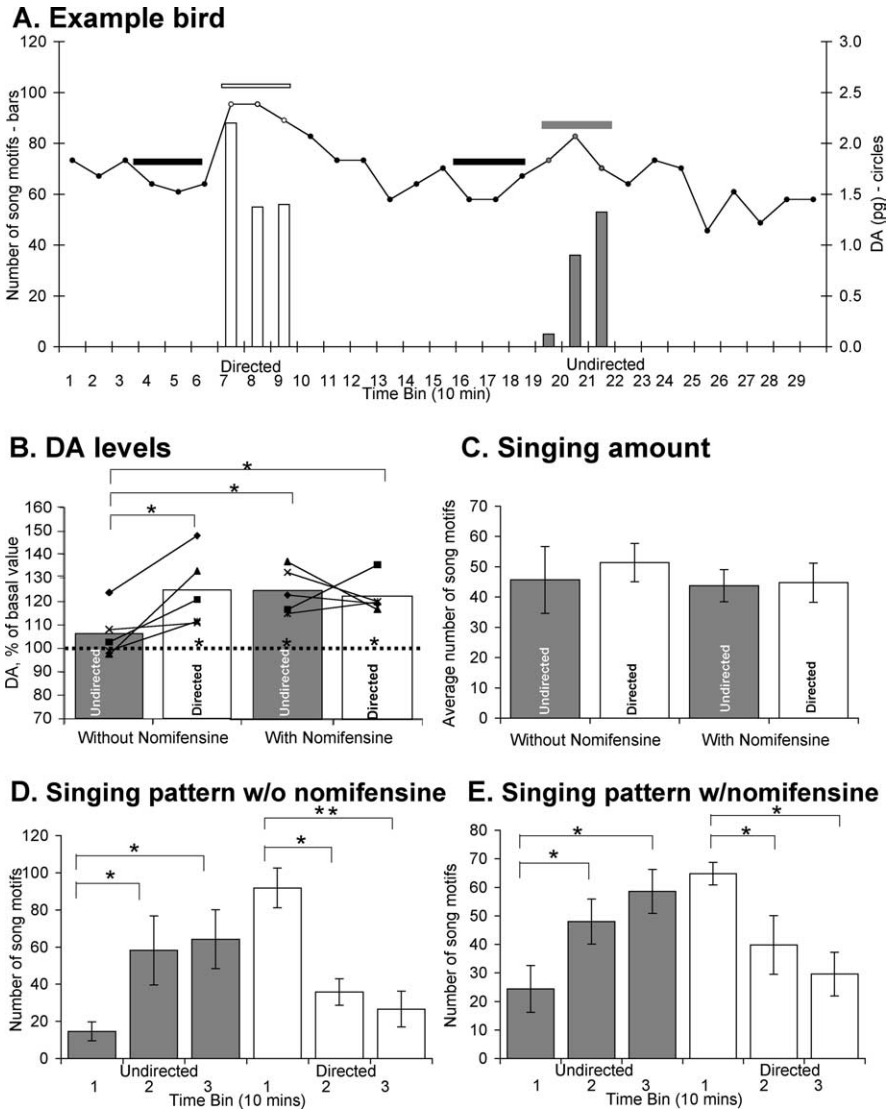

Figure 2. Singing behavior and DA levels in Area X. ***A***, Number of songs and microdialysate DA levels in Area X of an example bird. Vertical bars, Singing amounts; lines, DA levels at every 10 min for 290 min; horizontal bars, the three 10 min bins of baseline (black) and singing (white and gray) DA values used to quantify relative DA levels in the graphs in ***B***. ***B***, Extracellular DA levels in Area X during undirected and directed singing with or without nomifensine (n = same 5 birds for each condition). Dashed line, Average nonsinging baseline DA levels normalized to 100%; inside bars, $^*p < 0.05$ (Wilcoxon signed rank test), singing relative to baseline; above bars, $^*p < 0.05$ and $^{**}p < 0.01$ (repeated-measures ANOVA), between social contexts. Data are averages of three 10 min bins. Error bars indicate SEM. ***C***, Average number of song motifs produced in each 30 min singing session during the periods of DA measurement in ***B***. ***D***, Pattern of singing across the 10 min bins when the birds did not have localized unilateral nomifensine perfusion into Area X. ***E***, Pattern of singing across the 10 min bins when the birds had localized nomifensine perfusion. The statistical test for symbols above bars in ***C–E*** are the same as in ***B***.

Singing and social context-dependent regulation of DA levels

We next tested whether DA levels in Area X are modulated during singing and in different social contexts. To measure baseline DA levels, we turned the lights off before and after singing to prevent the birds from singing. Figure 2*A* shows one example of dialysate DA levels from Area X of a singing bird across an entire 1 d session, and Figure 2*B* shows the average DA levels of all birds in 30 min of singing relative to the immediately preceding, nonsinging baseline levels. Relative to nonsinging baseline conditions, undirected singing did not result in a significant increase in DA levels in Area X, whereas directed singing resulted in a significant increase (Fig. 2*B*, left two bars). Relative to social context, DA levels in Area X were significantly higher during directed relative to undirected singing (Fig. 2*B*). Exposure to light cannot explain these differences, because the birds were exposed to light in each context (also see below). After singing, it took 30–40 min for DA to return to baseline levels (Fig. 2*A*). There was no significant difference between baseline DA levels in the 30 min (three time bins) before each singing session ($p > 0.05$, paired *t* test; preundirected vs predirected).

The difference in DA levels in Area X between undirected and

directed singing was not attributable to a difference in the number of songs produced, because the average number of song motifs was not significantly different between the two social contexts (Fig. 2*C*, left two bars). Furthermore, we did not find a significant correlation between DA levels and the amount of song produced per 10 min bin in each context ($r^2 = 0.051$, $p = 0.419$ for undirected singing; $r^2 = 0.157$, $p = 0.144$ for directed singing). However, the pattern of singing across time differed between social contexts (Fig. 2*D*). During undirected singing, birds gradually increased the number of songs produced over the 30 min period, producing the greatest number in the last 10 min. In contrast, during directed singing, birds produced the greatest number of songs in the first 10 min and gradually decreased their number of songs during the remaining 20 min. We do not believe that this difference in the singing pattern can account for differences in DA levels, as revealed below for the DAT blocking experiments.

Social context-dependent differences in DA require the DAT

We examined the role of DA reuptake in Area X to determine whether there were differences between social contexts. Birds with probes in Area X in the previous experiment were retested on the following day using the DAT blocker nomifensine (Robinson and Wightman, 2004). Local infusion of nomifensine through the microdialysis probe eliminated the difference in DA levels between undirected and directed singing, because of a significant increase in the DA levels during undirected singing that was similar to the levels observed during directed singing (Fig. 2*B*, right two bars). As in the previous experiment, this effect in the presence of nomifensine was not a result of a difference in singing amount, because the average number of song motifs produced in each context did not differ (Fig. 2*C*, right two bars). Furthermore, in the presence of nomifensine, the singing patterns were similar to those described without nomifensine (Fig. 2*E*), consistent with the expectation that nomifensine only affected DA transmission in the immediate vicinity of the probe. This suggests that the difference in the singing pattern is not responsible for the differences in DA levels, but rather other factors of social context are responsible.

The DA increase in Area X is specific to singing

Finally, we wondered whether turning on the lights could have had some effect on DA levels in Area X, so we examined DA levels with the lights turned on, but without singing, in the presence of nomifensine to maximize DA detection. We found that in birds exposed to light only, the mean DA levels in Area X were slightly above baseline (108.70 ± 3.91% SEM; $n = 5$), but this difference was not significant ($p = 0.08$, Wilcoxon signed rank test). More importantly, compared with these birds exposed to light only, and that were also actively moving and eating, DA levels in Area X were still significantly higher in singing birds (directed plus undirected mean was 122.96 ± 2.59% SEM of baseline; $n = 10$; same birds as in Fig. 2*B*; unpaired *t* test, $p = 0.008$ between light only vs singing). We caution that these experiments were done in the presence of nomifensine, but note if light alone was able to induce high DA levels in Area X, we should have seen the effect in the undirected singing group without nomifensine, which was not the case (Fig. 2*B*). Thus, neither exposure to light nor nonsinging movement behavior can explain the increased DA levels in Area X seen during singing. The main factors appear to be singing and social context.

Discussion

This report is the first to show modulation of DA levels during singing behavior. We demonstrate that in Area X, in the presence of the DAT blocker, DA levels are higher during singing than during nonsinging behaviors. Furthermore, in the absence of the DAT blocker, directed singing is associated with higher DA levels relative to undirected singing, and thus DA is differentially modulated depending on the social context of singing behavior. To explain these findings, we offer three alternative interpretations.

One possible interpretation is that during directed singing, there is more DA release in Area X from terminals that project from the ventral tegmental area–substantia nigra pars compacta (VTA–SNc) of the midbrain. This is consistent with the hypothesis that DA input from the midbrain may modulate social context differences in the basal ganglia song nucleus, Area X (Jarvis et al., 1998). Alternatively, our findings with the DAT inhibitor suggest that similar amounts of DA may be released into Area X during undirected and directed singing but that during directed singing, the DAT reuptake rate is less. In mammals, reuptake rates can differ for different parts of the striatum (Venton et al., 2003; Wightman, 2006), whereas here we suggest the rates may differ in the same striatal area for different social contexts. Rapid changes in the reuptake rates on the time scale of singing behavior we observed here can be controlled by signaling molecules, phosphorylation, and rapid recycling of DAT (Mortensen and Amara, 2003). A third alternative is that both mechanisms could work in concert, coordinated differential DA release, and DAT reuptake in Area X. In mammalian VTA neurons, phasic firing-induced DA release is associated with rapid DA reuptake through the DAT that is difficult to detect by microdialysis (possible with undirected singing), whereas tonic firing-induced DA release is associated with slow DA reuptake through the DAT that is easier to detect by microdialysis (possible with directed singing) (Floresco et al., 2003; Venton et al., 2003). It is likely that more temporally refined measurement of extracellular DA, such as in voltammetry (Phillips et al., 2003; Gale and Perkel, 2005; Wightman, 2006), will lead to a more precise determination of DA kinetics in each social context, because microdialysis can only detect accumulation of DA that has escaped reuptake and diffused some distance from release sites (Westerink et al., 1987). We also do not yet know whether the higher DA levels seen during directed singing occurs only in Area X or in Area X and the surrounding striatum, because we did not measure DA outside of Area X.

Our results have implications for understanding the mechanism of differential gene expression and neural activity in Area X after directed and undirected singing. During directed singing, expression of the activity-dependent transcription factor *egr-1* and neural activity increases moderately, whereas during undirected singing, they increase considerably (Jarvis et al., 1998; Hessler and Doupe, 1999). One possible mechanism by which *egr-1* expression and neural activity may be differentially regulated is through a neuromodulator such as DA. In both mammalian and avian striatal spiny neurons, DA binding to D_1 and D_2 receptors enhances and inhibits, respectively, evoked firing (Ding and Perkel, 2002) and *egr-1* gene expression (tested in mammals only) (Gerfen et al., 1995); Area X spiny neurons have colocalization of both receptor types (Ding and Perkel, 2002).

Finally, our findings implicate DA and the DAT in singing behavior and social context-dependent brain function. DAT kinetics are similar in songbirds and mammals (Gale and Perkel, 2005). In mammals, DAT protein is localized perisynaptically, where it quickly removes high levels of DA released from midbrain DA neurons when they fire (Robinson and Wightman, 2004; Wightman, 2006). This function of DAT is thought to sharpen the DA signal in the striatum during learning, by error detection (Schultz, 2002). Area X is necessary for vocal learning

(Scharff and Nottebohm, 1991) and is part of a pathway proposed to be involved in error detection (Brainard and Doupe, 2000). Furthermore, high *egr-1* expression in Area X (Liu and Nottebohm, 2005) and variable neural activity in one of its inputs, LMAN (Kao et al., 2005), is associated with more variable song output during undirected singing (or dawn singing) (Liu and Nottebohm, 2005) relative to directed singing (or day singing). Therefore, it may be that during undirected singing, DAT rapidly takes up high DA levels associated with error detection, whereas during directed singing, DAT function is minimized, leaving DA levels high, perhaps to dampen the circuit for plasticity. In conclusion, our findings open a window to assess the role of DA in a learned social context-dependent behavior that has behavioral similarities with human speech.

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