Dopamine Physiology in the Basal Ganglia of Male Zebra Finches During Social Stimulation

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Abstract

Accumulating evidence suggests that dopamine (DA) is involved in altering neural activity and gene expression in a zebra finch cortical-basal ganglia circuit specialized for singing, upon the shift between solitary singing and singing as a part of courtship. Our objective here was to sample changes in the extracellular concentrations of DA in Area X of adult and juvenile birds, to test the hypothesis that DA levels would change similarly during presentation of a socially salient stimulus in both age groups. We used microdialysis to sample the extracellular milieu of Area X in awake, behaving adult and juvenile male zebra finches, and analyzed the dialysate using high performance liquid chromatography coupled with electrochemical detection (HPLC-ED). The extracellular levels of DA in Area X increased significantly during both female presentation to adult males and tutor presentation to juvenile males. DA levels were not correlated with the time spent singing. We also reverse-dialyzed Area X with pharmacologic agents that act either on DA systems directly or on norepinephrine (NE), and found that all of these agents significantly increased DA levels (3- to 10-fold) in Area X. These findings suggest that changes in extracellular DA levels can be stimulated similarly by very different social contexts (courtship and interaction with tutor), and influenced potently by dopaminergic and noradrenergic drugs. These results raise the possibility that the arousal level or attentional state of the subject (rather than singing behavior) is the common feature eliciting changes in extracellular DA concentration.
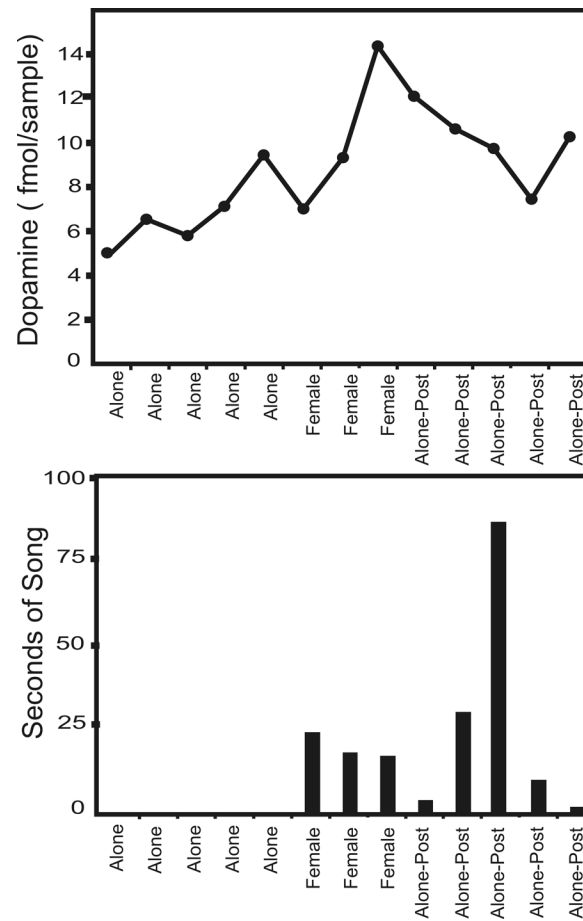
Graphical abstract

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We used microdialysis to sample the extracellular milieu of Area X in awake, behaving adult and juvenile male zebra finches. The results of a representative experiment with an adult are shown. We found that the mean extracellular levels of DA in Area X increased significantly during both female presentation to adult males and tutor presentation to juvenile males. DA levels were not correlated with the amount of singing in either case. These findings suggest that increases in extracellular DA concentration can be stimulated similarly by different social contexts.

INTRODUCTION

In songbirds, both song learning and adult song production are powerfully modulated by social factors and attentional cues (Tchernichovski & Nottebohm, 1998; Kojima & Doupe, 2011; Hessler & Doupe, 1999; Adar *et al*., 2008; Hauber *et al.*, 2007; Lehongre *et al*., 2009; Cousillas, *et al*., 2006). Numerous recent studies of song learning reveal the influences of social cues: for example, adolescent male zebra finch song performance decreases markedly in variability and increases in stereotypy in the presence of an adult female (Kojima & Doupe, 2011), and sound processing in a starling central auditory nucleus depends on the social interactions occurring during development (Cousillas *et al*., 2006). In addition to sensory and motor areas that process and produce song, the male zebra finch brain contains a cortico-basal ganglia circuit specialized for song that is affected by social cues. This rostral

circuit known as the anterior forebrain pathway (AFP; Figure 1a) is composed of the lateral magnocellular nucleus of the anterior nidopallium (LMAN, a cortex-like output structure), the medial dorsolateral nucleus of the thalamus (DLM, a thalamic nucleus), and Area X (a striatopallidal nucleus). Neural activity (Hessler & Doupe, 1999; Kao *et al.*, 2008) and immediate early gene (IEG) expression (Jarvis *et al.*, 1998) in LMAN and Area X are higher in male birds singing alone than in males singing to females. The importance of social cues beyond courtship has not been investigated as extensively (e.g., Nordeen *et al.*, 2009).

Much evidence suggests that both DA (Sasaki *et al*, 2006; Heimovics & Riters, 2008; Huang & Hessler, 2008; Leblois *et al*., 2010) and NE (Barclay *et al.*, 1996; Riters & Pawlisch, 2007; Castelino *et al*., 2007) are involved in courtship-mediated changes in basal ganglia activity. Area X receives dopaminergic projections from the substantia nigra (SNc) and ventral tegmental area (VTA), as well as NE from locus coeruleus (Lewis *et al*., 1981; Bottjer *et al*., 1989; Soha *et al*.,1996; Castelino *et al*., 2007; Person *et al*., 2008). Depletion of either NE (Castelino & Ball, 2005) or DA (Hara *et al*., 2007) results in altered IEG expression in the song system. DA levels in Area X increase significantly when males sing song directed at females but not when they sing alone (Sasaki *et al*., 2006). D1 receptor antagonist chronically delivered to Area X prevents the usual decrease in song variability seen during directed (courtship) singing (Leblois *et al*., 2010). It remains unclear, however, whether artificially raising DA levels with direct agonist treatment would reproduce levels seen in response to social cues.

The goal of the present study was to determine whether changes in extracellular DA concentration in Area X differed during two different social interactions, courtship and tutoring. We monitored fluctuations in extracellular DA levels using microdialysis. We also assessed how behaviorally-induced changes in DA concentration compared to those induced by pharmacological manipulation. Here, we report that similar changes in DA levels are associated with different types of social interactions, and that these changes are much smaller than those induced by pharmacologic agents.

METHODS

Subject Animals

Male zebra finches (*Taeniopygia guttata*) were born and raised in our colony, and kept on a 14 hour light: 10 hour dark cycle with access to seed, grit, mash, and water *ad libitum*. Adult males were all > 180 days old, while juvenile males ranged in age from 42 to 81 days old (average age was 61.7 days old) at the time of the behavior experiments. All procedures were performed in accordance with protocols approved by the University of California, San Francisco Institutional Animal Care and Use Committee. Experiments were conducted on a total of 22 birds. For behavior experiments, 13 zebra finches were used (2 birds' data were excluded due to incorrect probe placement). These 13 birds and an additional 9 adult birds were also used for pharmacology experiments; pharmacology experiments were conducted several days after the behavior experiments.

Chemicals

All salts for artificial cerebrospinal fluid (ACSF) except $CaCl_2$ were obtained from Fluka (Devon, England). $CaCl_2$ and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Surgical Procedures

Birds were prepared for surgery by pre-anesthetizing them with a mixture of ketamine (40 mg/kg) and midazolam (1.5 mg/kg) injected intramuscularly. Under isofluorane anesthesia (0.5 – 2%) a guide cannula (CMA 7, CMA Microdialysis, Stockholm, Sweden) was stereotaxically implanted just above Area X and 45 degrees from vertical (to avoid LMAN) several days prior to experiments (with coordinates relative to the posterior bifurcation of the saggital sinus: AP = 5.1–5.2 mm, ML = 4.0 mm, at a depth of 2 mm from the dorsal surface of the brain). For experiments that involved baclofen infusion into the VTA a second guide cannula was implanted; this VTA-directed cannula was implanted in a similar fashion but with VTA-specific coordinates: AP = 3.65 mm, ML = 0.45 mm, depth = 6 mm. Guide cannulae were secured to the top of the skull with epoxy.

Behavior Protocol

Each bird was isolated for several days prior to the experiment by housing it alone in a sound attenuating chamber (Acoustic Systems, Austin, TX). Approximately 15–18 hrs before the beginning of the experiment a microdialysis probe (CMA 7, active membrane length = 1 mm) was prepared by flushing the membrane with 70% EtOH, and then HPLC-grade water, before attaching it to the liquid swivel (375/D/22QM, Instech, Plymouth Meeting, PA) of our microdialysis apparatus. The other component of the microdialysis apparatus was a CMA syringe pump (model 100), housed outside the sound attenuating chamber. ACSF (145 mM NaCL, 2.7 mM KCl, $CaCl_2$ 2.4 mM, 1.0 $MgCl_2$, 2.0 mM Na_2HPO_4, 5 mM glucose) was pumped through the inlet channel of the swivel, through the probe, out through the outflow channel, and collected outside the sound attenuating chamber in small vials (SciPro, Sanborn, NY). Once attached to the swivel, the microdialysis probe was inserted into Area X through the guide cannula and allowed to equilibrate with ACSF overnight (at a pump rate of 0.3 ul/min) while the dialysate was discarded. Following probe equilibration and while the subject was awake and alone in the light, baseline samples were collected at 10- or 15-minute intervals at a pump rate of 1.5 ul/min. After baseline samples were collected a "stimulus" zebra finch (a female for the adult males or the tutor for the juveniles) was presented to the subject (by placing the stimulus individual's cage along side the subject's cage in the sound attenuating chamber), and samples continued to be collected. Following removal of the stimulus bird, additional samples ("post-baseline" or "recovery") were collected; for adults, this recovery period was sampled for 60–75 minutes and for juveniles, up to 130 minutes. Samples were collected on ice, and then stored at −80°C until analyzed.

All experiments were digitally recorded to capture songs and behavior for review and analysis following microdialysis sample collection. Amount of singing was quantified as number of seconds elapsed from the first call note to the end of the song bout. Pauses within the song were not included in the quantification; for example, if a bout lasted 5 seconds, but

within the bout there was a 0.5 second pause, the amount of singing was quantified as 4.5 seconds. Subject behavior was observed and recorded in general descriptive terms (preening, courtship, approach, aggression, etc.) during each collection interval.

Pharmacology Protocol

The effects of a GABA-ergic (baclofen), a dopaminergic (amphetamine), and noradrenergic (nomifensine, reboxetine, and norepinephrine) pharmacologic agents on neurotransmitter release were tested using reverse microdialysis. Baclofen is an agonist at $GABA_B$ receptors; it decreases the firing rate of neurons possessing these receptors. VTA neurons possess $GABA_B$ receptors, and at moderate-high doses of baclofen the effect on DA projection neurons predominates, and decreases DA release from the VTA (Cruz *et al.*, 2004). Amphetamine reverses the action of the dopamine transporter (DA-T) and causes release of DA from neurons that express DA-T (Sulzer *et al.*, 2005). Nomifensine inhibits the function of the norepinephrine/dopamine transporter (NE/DA-T) and theoretically results in an increase in the extracellular levels of both NE and DA (Gianutsos *et al.*, 1982), although in birds it has been used as a DA reuptake inhibitor (Sasaki *et al.*, 2006). In mammals reboxetine blocks reuptake of NE by inhibiting the action of the synaptic NE transporter (NE-T; Kasper *et al.*, 2000) and results in an increase in the extracellular levels of NE.

The protocol for this type of experiment was as described above, with the following exceptions: 1) the subject remained isolated throughout the experiment; and 2) after baseline samples were collected while ACSF was pumped through the probe, the dialysate was changed to one containing the pharmacologic agent (in an ACSF vehicle). Given the pump rate and tubing length from pump to probe, the lag time for the drug to reach the probe was approximately 15 minutes; the effects of the drug were apparent within 15 additional minutes, the lag time from the probe to the collection vials. For each drug, 3 subjects were tested (i.e., N = 3 for each pharmacology experiment) and each subject was drug-naïve for each such experiment (i.e., no subject was exposed to more than 1 drug nor was used more than once for any pharmacology experiment).

For the purpose of graphically representing group mean changes within experiments, we determined change from baseline using the following calculation: the microdialysis value obtained for each collection interval during and after behavioral or pharmacologic stimulation was divided by the average of the 3 – 5 previous baseline values and the averaged across the three experimental time bins (baseline, during, after) and multiplied by 100 to generate a percentage of change relative to baseline.

High Performance Liquid Chromatography and Electrochemical Detection

All dialysate samples from behavioral stimulation experiments, as well as the reboxetine and norepinephrine pharmacology experiments were analyzed for neurotransmitter content using HPLC-ED as described in Cremers *et al.*, 2004, and detailed below. The effects of baclofen, amphetamine, and nomifensine were analyzed using the HPLC-ED method described in Becker & Cha, 1989.

Dopamine and Norepinephrine Detection

Separation—Microdialysis aliquots (20 μl) were injected onto the analytical column (BDS Hypersil C18 column, 150×2.1 mm, 3 μm particle size, Thermo Scientific, USA) by a refrigerated microsampler system, consisting of a syringe pump (Gilson, model 402, France) and a multi-column injector (Gilson, model 233 XL, France). The column was held at a constant temperature of 32.5° Celsius by a column oven (Intro, Antec Leyden, the Netherlands). Chromatographic separation was isocratic at a flow rate of 0.35 ml/min by an HPLC pump (Shimadzu, model LC-10AD vp, Japan). The mobile phase consisted of a NaAc buffer (4.1 g/l) with MeOH (2.5 % v/v), EDTA (150 mg/l), OSA (150 mg/l), and TMA (150 mg/l) and adjusted with glacial acetic acid to pH = 4.1 (Cremers *et al.*, 2007).

Electrochemical detection—DA and NE were detected electrochemically using a potentiostat (VT-03 flow cell, Antec Leyden, The Netherlands) fitted with a glassy carbon electrode set at +500 mV vs. Ag/AgCl reference electrode. This potential was selected to maximize sensitivity while minimizing noise.

Data were analyzed by Chromatography Data System (Shimadzu, class-vp, Japan) software. Concentrations of NE and DA were quantified by the external standard method. The average retention time for NE was 4.1 min and the detection limit was 0.7 fmol/sample [signal to noise ratio (S/N) >5]. The average retention time for DA was 16.2 min with a detection limit of 1 fmol/sample (S/N>5).

Histology

At the conclusion of all experiments, subjects' brains were harvested to determine the location of the microdialysis probe (Figure 1b). Following anesthesia with isofluorane, subjects were perfused transcardially with filtered, room temperature PBS followed by perfusion with room temperature 3.7% formalin. Brains were cryoprotected in a 30% sucrose solution, and then sectioned (40 micron thickness) on a microtome in the coronal plane. Subjects whose probes were not located within Area X (N = 2) were excluded from analysis.

Statistical Analyses

To compare mean extracellular DA levels in adult male finches in the presence of a female to those in the absence of a female, we created a three-level categorical variable for experimental condition. Baseline measurements (prior to introduction of female) served as the reference category for both "stimulus" measurements (during the time that the female was present) and "recovery" measurements (immediately after the removal of the female). Similar comparisons were made in the juvenile group, with tutor substituting for female in the stimulus condition. Changes in extracellular DA concentration in response to pharmacologic manipulation were also examined for the pharmacologic agents baclofen, amphetamine, nomifensine, reboxetine, and NE, with similar comparisons made between referent baseline levels and both stimulus (pharmacologic agents) and recovery periods.

All analyses were conducted using random effects models, a type of repeated measures analysis. Because, during the course of one experiment, repeated measurements were taken

on the same subjects over extended periods of time (i.e., hours), they were likely correlated. To accommodate this correlation, mixed model regression (specifically, a random effects model) was used (SAS Proc MIXED, SAS Ver 9.2, SAS Institute, Cary NC). Unlike repeated measures ANOVA, mixed model regression controls for repeated measures (multiple samples comprising a single condition) as well as repeated trials within subjects (pre- and post- stimulation conditions), while also allowing for unbalanced data, use of all available data when some are missing, and for incorporation of predictors that change from one time to the next. In particular, this model was also able to assess whether any amount of change was accounted for by time (given the long duration of the experiments) by modeling time as a potential predictor of DA level. Potential correlations between amount of singing and DA content during the different time periods were examined using non-parametric Spearman correlations.

RESULTS

Extracellular Dopamine Levels in Area X Increase in Response to Different Social Contexts

We collected dialysate from Area X of adult males zebra finches using a technique similar to one described previously (Sasaki *et al.*, 2006), except that we did not manipulate lighting conditions. For all subjects extracellular DA levels increased in the presence of a female (see Figure 2a, top panel, for a representative adult experiment of behavioral manipulation of DA levels). Compared to baseline (set to be zero), DA levels during time spent with a female were increased by 5.4 fmol/sample ($p < 0.0001$, N = 5; Table 1). All males sang directed song and engaged in courtship displays in the presence of a female. Some males sang exclusively during female presentation, while others sang as much during time alone as with a female (see Figure 2a, bottom panel, for a representative bar graph quantifying singing). The amount of singing for each 15-minute interval, as measured by seconds of singing, was not correlated with the DA content of the sample collected during that interval, using Spearman (non-parametric) correlation, $r = -0.29$, $p=0.15$, consistent with the results of Sasaki *et al.* (2006). The effect of this social stimulation, namely a 26.5±8.4% increase in the normalized group mean DA content (Figure 2b), partially reversed during the time period immediately following the stimulus; compared to baseline, DA levels after the female was removed remained increased but only by 2.5 fmol/sample ($p = 0.003$, N = 5, Table 1).

We then extended this study to examine the effect of social interaction in juvenile male zebra finches. We measured extracellular DA levels in dialysate samples collected at 10-minute intervals in the presence of the juvenile birds' tutors (fathers) (see Figure 2c, top panel, for a representative juvenile experiment of behavioral manipulation of DA levels). We found a significant increase in Area X DA concentration in juvenile males during tutor presentation that was almost as large as that seen for the social stimulus of courtship in adult males. Compared to baseline, DA levels during tutor presentation were increased by 3.3 fmol/sample ($p < 0.0001$, N = 6, Table 2). Unlike adults, juveniles' singing behavior varied widely in the presence of the stimulus (their tutor); some sang in response to tutor song while others decreased their song output, quieting in the presence of their tutor when they previously had been singing frequently while alone (see Figure 2c, bottom panel, for a representative bar graph quantifying singing both by the juvenile, black bars, and the tutor,

gray bars). All juveniles changed their behavioral repertoire from solitary activities (eating and preening, for example) to interactive activities upon presentation of the tutor (alerting to, approaching, and occasionally even sparring with the tutor). All juveniles displayed these behaviors at some point during the presentation of the tutor, even when the tutor did not sing.

Strikingly, unlike the situation with adults, in juveniles recovery of DA levels was not evident within the first hour following removal of the tutor; in fact, DA continued to increase following removal of the tutor (5.0 fmol/sample increase in DA over baseline; $p < 0.0001$, N = 6). Whereas the tutor presentation was associated with a 21.4±7.3% increase in the normalized group mean DA content compared to baseline, removal of the tutor was associated with a 24.1±8.1% increase in the normalized group mean DA content compared to baseline (Figure 2d). Recovery only became evident in later samples, 90–130 minutes following removal of the tutor, but samples from the first hour post-stimulation were still used in the analysis, to be consistent with the parameters established in the experiments with adult subjects. In this experimental condition there was a very small effect of time; for each additional minute of the experiment, the DA level increased by 0.02 fmol/sample. As with adults, the amount of DA increase did not appear to be correlated with the amount of singing observed in subjects. Statistical correlation analysis could not be used because, for multiple subjects, a similar change in DA was seen with very different changes in behavior. For example, the three subjects with mid-range changes in DA (increases of 15–27%) had no change in song frequency, either because they did not sing at all throughout the experiment or because the number of songs did not differ between baseline and stimulation conditions.

The maximum amount of change from baseline in DA concentration in response to physiologic (behavioral) manipulation was 49% in the adult experiments, and 35% in the juvenile experiments. The passage of experimental time was found to have an effect only in juveniles, and even then only accounted for < 1% of the change in DA levels from baseline. Given this effect of behavioral stimulation we then undertook to determine the degree to which DA levels could change in Area X by pharmacologically manipulating changes in extracellular DA concentration.

Extracellular Dopamine Levels in Area X Can Be Rapidly Modulated by a Number of Pharmacologic Agents

Baclofen—Since Area X is known to be innervated by VTA, we directly infused a small volume of a high-dose baclofen solution (0.1 ul of a 0.1 ug/ul solution) into the midbrain, targeting the VTA, and measured the resultant change in [DA] in Area X from baseline. Compared to baseline, DA levels decreased by 1.69 pg/sample during baclofen infusion ($p = 0.002$, N = 3, Table 3, Figure 3a), and subsequently recovered to levels not significantly different from baseline ($p = 0.51$).

Amphetamine—We used reverse dialysis to deliver 10 uM amphetamine to Area X and measured the change in [DA] from baseline. Compared to baseline, levels of DA increased by 45.3 pg/sample during amphetamine ($p = 0.002$, N = 3, Table 3, Figure 3a), a level 10-fold higher than the increases triggered physiologically.

Nomifensine—We used reverse dialysis to deliver 10 uM nomifensine (half of the dose used in Bert *et al.* (2002)) to achieve a 30-fold increase in rat striatal DA efflux) to Area X and measured the change in [DA] from baseline. Compared to baseline, DA levels increased by 0.55 pg/sample during nomifensine ($p<0.0001$, N = 3, Table 3, Figure 3a). Because this subset of experiments was analyzed using an HPLC system adapted to detect only DA (Becker & Cha, 1989) we could not examine the effect of nomifensine on NE levels.

Reboxetine—We used reverse dialysis to deliver 250 nM reboxetine (M. van der Hart, personal communication; ~0.1× the dose used in rodent studies; Linnér *et al.*, 2001) into Area X. We found that reboxetine changed the extracellular concentration of NE to a much lesser extent (44.3±12.7%) than for DA (775.2±290%); see the inset for Figure 3a. In three experiments, the [DA] level in the presence of 250 nM reboxetine was increased by 113.9 fmol/sample ($p<0.0001$, N = 3, Table 3, Figure 3a), although the magnitude of the increase varied between birds (from 2- to 10-fold).

Norepinephrine—To determine whether the effect of reboxetine on DA levels was direct (acting on the DA-T in a promiscuous fashion) or the down-stream result of increased NE levels (which then modulate DA levels through an as yet undetermined mechanism), we reverse-dialyzed a supraphysiologic concentration of NE (500 nM; M. van der Hart, personal communication) into Area X and measured the change in [DA] from baseline. This approach allowed us to monitor the onset of the [DA] change in relation to the time at which NE became detectable in the dialysate (Figure 3b). Early in the experiment, during the period of the greatest change in [NE], the extracellular [DA] began to rise. After 40 minutes, when the [NE] had plateaued at a mean level of 2116 fmol/sample, the [DA] began to decrease. After another 40 minutes, the extracellular [DA] also plateaued, but to a slightly lower level than baseline (late in the experiment). Compared to baseline, DA levels increased by 2.3 fmol/sample during NE ($p=0.002$ vs. baseline, N = 3, Table 3, Figure 3c), and decreased to 1.4 fmol/sample in the late phase, back to a level not different from baseline ($p=0.052$). Thus, supraphysiologic levels of NE transiently increase [DA] in Area X.

As in the adult behavioral experiments, time was not a significant predictor of DA level in the pharmacology experiments.

DISCUSSION

Using *in vivo* microdialysis we have determined that the content of extracellular DA in Area X increases during different social interactions. Not only were the social stimuli very different (courtship and exposure to a tutor), but subjects' behavioral responses were also divergent (directed singing and courtship displays in adults, in contrast to quieting or aggression in juveniles, respectively). The end result in terms of DA levels was similar, however, suggesting that a salient social signal likely brought about changes in extracellular DA in both cases. These findings also suggest that similar brain circuits are involved, whether an adult male responds to a female or a juvenile male responds to a tutor. Our study deliberately focused on only two conditions of social stimulation, to investigate the predictions that arousal may be the contextual cue in the neural changes seen in directed

singing (Hessler & Doupe, 1999) and that increased VTA activity in response to tutor song may be associated with increased DA release in Area X (Nordeen *et al.*, 2009). Hessler & Doupe (1999) performed the control experiment of using a male to elicit directed singing and found that neural activity recorded during directed song in the presence of a male companion was not different from directed singing produced as part of the courtship display (both conditions resulted in a decrease in neural activity in the song circuit). Although courtship behavior has been demonstrated previously to result in increases in extracellular DA concentration in Area X (Sasaki *et al.*, 2006), the finding that interaction with a tutor similarly influences extracellular neurotransmitter fluctuations in this key nucleus of the juvenile AFP is a novel result.

The likely source of the DA we measured in Area X is VTA/SNc. VTA (area A10) neurons of adult males, which have robust projections to the AFP, show both enhanced IEG induction and increased firing in the presence of a female (Heimovics & Riters, 2005; Yanagihara & Hessler, 2006; Hara *et al.*, 2007; Huang & Hessler, 2008; Riters, 2011). Similarly, Nordeen *et al.* (2009) reported that presentation of the bird's tutor increased the density of IEG expression in the VTA and SNc (area A9) of juvenile male zebra finches relative to unfamiliar controls, again suggesting tutor-driven VTA neuronal activation. Such activation could reflect the social salience of the signal or a possible function of the VTA in song memorization or evaluation during learning.

Our finding that singing is neither necessary nor sufficient for causing change in extracellular DA in Area X is consistent with the results of Hessler and colleagues (Yanagihara & Hessler, 2006; Huang & Hessler, 2008), who observed VTA activation in the presence of a female regardless of whether the adult male bird sang or not, and with the finding of Sasaki *et al.*, (2006) that undirected singing did not significantly increase extracellular DA levels. It remains unclear what aspects of the social stimuli mediate the increase in extracellular DA concentration or what suite of behaviors DA may regulate. In our experiments, both adults and juveniles engaged in behaviors that reflected changes in attention and activity level. The findings reported in our study suggest that changes in DA levels in Area X can result from components of behavior that are associated with, but are not the same as, vocalization: even juvenile subjects who did not sing in the presence of their tutors, but whose behavioral arousal levels changed markedly when the tutor appeared, had elevated DA levels, and these elevations persisted for longer than those in adults in response to females. This conclusion extends those reached by Leblois *et al.*, (2010), who focused primarily on the role of DA in shaping motor stereotypy, by decoupling DA from song and other behaviors associated with adult singing. Our study also suggests that the phenomenon of juvenile song performance enhancement in the presence of an adult female (Kojima & Doupe, 2011) could result from the female-triggered elevation of DA in Area X, in a manner similar to the social cues provided by the presence of a tutor; this increased DA could then modulate singing-related neuronal activity. Our work, along with many previous studies (e.g., Gale *et al.*, 2008), lends further support for the idea that dopaminergic mechanisms involved in reward and motivation and/or salience influence the function of the AFP.

In our experiments here, microdialysis coupled with sensitive HPLC-ED allowed us to detect fluctuations in [DA] in response to social cues that, averaged over a small number of

subjects, revealed statistically significant increases from baseline. The same was not true, however, for 5-HT or NE. It is possible that 5-HT could not be measured reliably because the 5-HT content of the extracellular milieu in Area X is below the detection limit for the HPLC-ED system we used. Alternatively, this nucleus may have a dearth of serotonergic and noradrenergic input. Previous reports documenting the total neurotransmitter content of Area X have found lower levels of 5-HT and NE, relative to DA, in canaries (Barclay & Harding, 1988) and zebra finches (Gale & Perkel, 2005); however, total tissue content of neurotransmitter is not equivalent to extracellular concentrations (the latter is likely to be less than the former).

In addition to using microdialysis to assess physiologic changes in DA levels after behavioral stimuli, we also used it to simultaneously deliver pharmacologic agents to Area X and measure their effects on extracellular DA levels. This approach allowed us to better understand the capacity of Area X for changes in extracellular DA content. Our pharmacologic manipulation of Area X with dopaminergic agonists revealed that changes in extracellular DA concentration could be much greater than those occurring during behavioral manipulations, consistent with what has been described for natural rewards (Bassareo *et al.*, 2011). These results suggest that the capacity of the system to modulate DA is quite large, even though the behavioral manipulations, as we applied them, did not elicit such extensive increases in extracellular DA concentration. We also expected to be able to detect pharmacologically–induced changes in extracellular NE levels, given the results of the potential role of NE in Area X function (Barclay & Harding, 1988; Barclay *et al.*, 1996), as well as the reports of cross-talk between catecholamines (Cornil *et al.*, 2008; Ball & Balthazart, 2010). While reboxetine did increase the NE concentration of the extracellular fluid this change was considered to be negligible compared to the substantial increase in DA concentration it caused.

The finding that extracellular DA content in Area X could be modulated by a pharmacologic agent (reboxetine) that blocks reuptake of NE via NE-T's (Wong *et al.*, 2000), was surprising, albeit consistent with findings that locally applied reboxetine causes release of DA in the nucleus accumbens of awake, behaving rats (Linnér *et al.*, 2001; Valentini *et al.*, 2004). Our finding that supraphysiologic levels of NE could transiently increase [DA] in Area X underscores the point that avian NE-T's may be like mammalian NE-T's in their capacity for transporting DA. Although our results cannot provide insight into monoamine transporter function or cross-talk in Area X, more characterization of the monoamine system is needed to understand the differences and similarities of avian monoamine transporters compared to those in mammalian basal ganglia.

Our findings suggest directions for future research. It will be useful to determine the effect of other social interactions (juvenile male with adult female, juvenile male with juvenile male, dummy/video female [stimulus] presented to an adult male [subject]) to attempt to determine which social stimuli are salient and rewarding. Closer observation of animal behavior (both of the subject and stimulus birds) during the experiment would also be warranted, to more deliberately evaluate arousal, attention, and orienting behaviors. Although microdialysis in birds has been focused on the AFP, much could be learned from sampling from other structures, particularly those associated with motivated behaviors (e.g.,

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POA, nucleus accumbens) or those with projections to the midbrain. Determining what triggers the changes in extracellular levels of DA as we have measured it can give insight into the circuitry of the song system (and beyond). It remains unclear whether VTA activity alone is sufficient to increase DA levels in Area X, or whether VTA activity must be coordinated with another nucleus (e.g., amygdala), song circuit structure, or cortical input into Area X. Finally, it would be a logical extension of our pharmacologic findings to attempt to influence song learning by administering dopaminergic agents to juveniles during both the sensory and sensorimotor periods; we hypothesize that learning (as measured by recognition of tutor song during the sensory period) might be accelerated by the administration of dopaminergic agents.

Song is a complex behavior learned during a critical period in the development of male zebra finches, with parallels to the development of human speech (Doupe & Kuhl, 1999). In both species, vocal learning and adult vocal production are modulated by social context. In humans, learning depends on social (and affective) influences (see Vinogradov *et al*., 2012, for a review). Small animal models provide additional insight into the mechanisms underlying these influences, and, hence, potentially how to target such mechanisms in more complex animals (see Acheson *et al.*, 2013). The actions of conspecifics must be perceived, interpreted, and further processed by the individual observer before it can respond in an appropriate way. Tractable animal models such as songbirds allow us to probe the fundamental neurobiological mechanisms of behavioral modulation in response to social context. With such models we may eventually shed light on the pathophysiology of disorders such as autism and schizophrenia, neuropsychiatric syndromes that have a strong locus in frontal-striatal circuits and that are particularly devastating because of their impact on social functioning.

ACKNOWLEDGEMENTS

ABBREVIATIONS

ACSF	artificial cerebrospinal fluid
AFP	anterior forebrain pathway
CI	confidence interval
DA	dopamine
DA-T	dopamine transporter
HPLC-ED	high performance liquid chromatography with electrochemical detection
IEG	immediate early gene

LMAN	lateral magnocellular nucleus of the anterior nidopallium
NE	norepinephrine
NE-T	norepinephrine transporter
SNc	substantia nigra, pars compacta
VTA	ventral tegmental area

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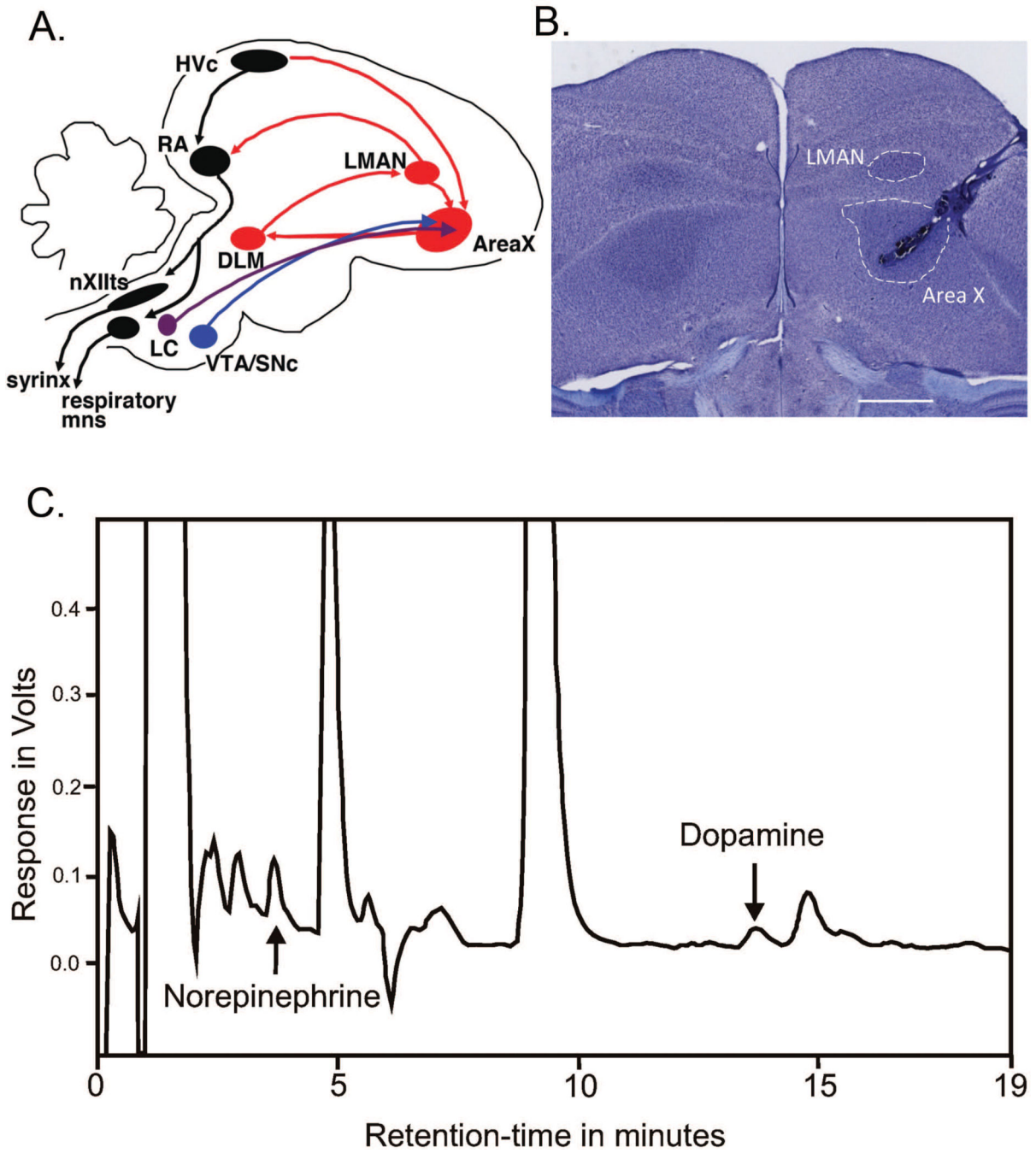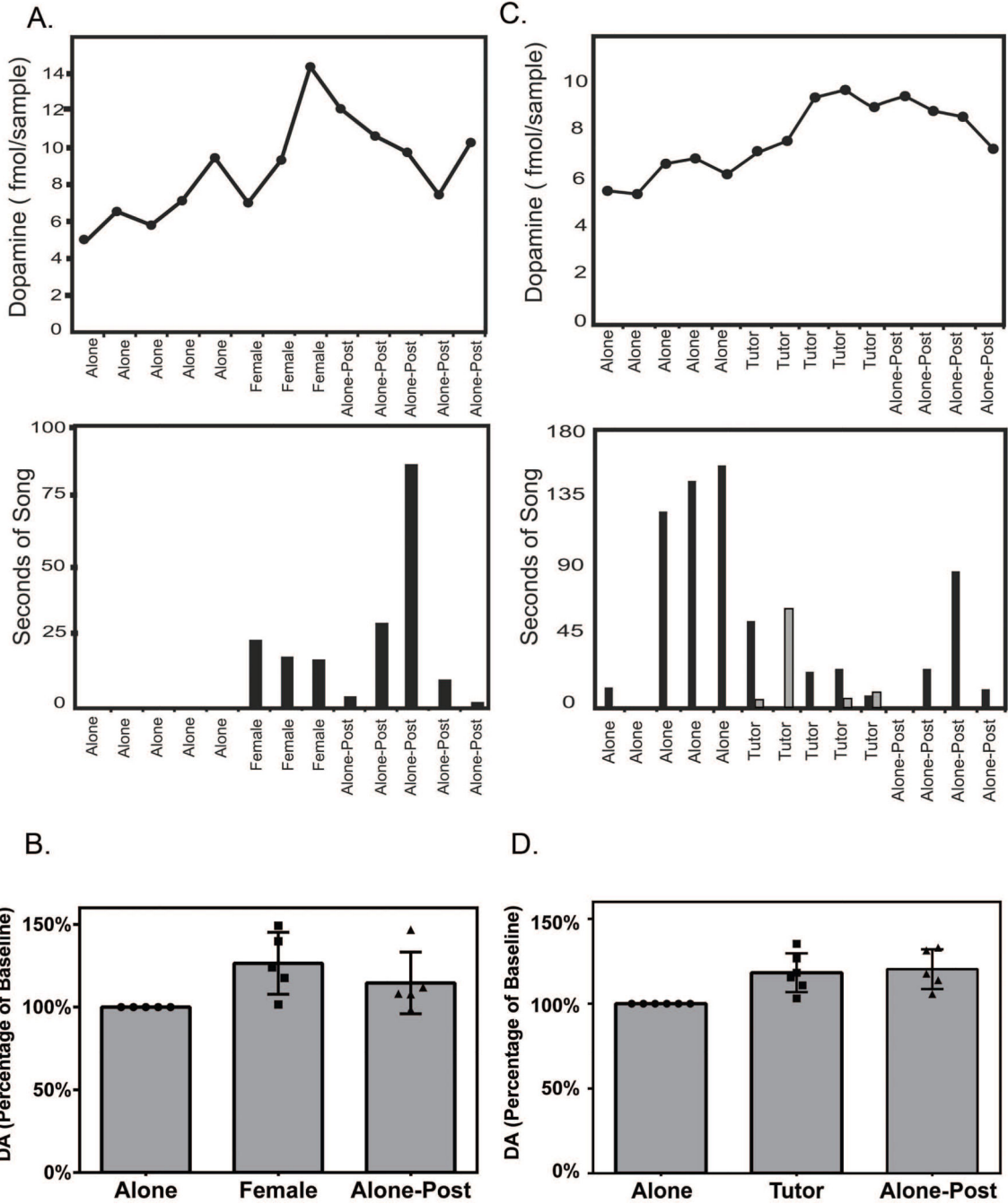
Figure 1.
A. Schematic representation of important song-related circuits in the zebra finch brain, including catecholaminergic projections (in purple and blue) to the anterior forebrain pathway (AFP). HVC, letter-based proper name; RA, robust nucleus of the arcopallium; nXIIts, cranial nerve XII; LC, locus coeruleus; VTA/SNc, ventral tegmental area/substantia nigra pars compacta; DLM, dorsolateral nucleus of the medial thalamus; LMAN, lateral magnocellular nucleus of the anterior nidopallium.

B. Photomicrograph of representative coronal section of male zebra finch brain, illustrating location of microdialysis probe. Note that placement of probe avoids damage to LMAN. Image obtained on Nikon Eclipse E800 microscope, with 2x objective and 10x ocular, captured with Zeiss AxioCamMRc5 and Axiovision 4.4 software. Scale bar = 1mm.

C. Representative chromatogram for a single microdialysis sample collected from Area X of an adult zebra finch. Signal amplitude (in volts) is plotted on the Y-axis and retention time (in minutes) is plotted on the X-axis. NE and DA peaks are designated with arrows. A clear separation from interfering peaks is apparent for both NE and DA.

Figure 2. Social interaction is associated with increases in extracellular dopamine levels
A. Representative experimental time course (top panel) for an individual adult male zebra finch, illustrating extracellular DA levels (on the Y-axis) at 15-minute intervals throughout the course of one experiment, with histogram (bottom panel) illustrating amount of singing (in seconds, on the Y-axis) produced during the same time intervals. Different experimental conditions [baseline state ('alone'), social stimulation ('female'), and post-stimulation ('alone-post')] are indicated on the X-axis.
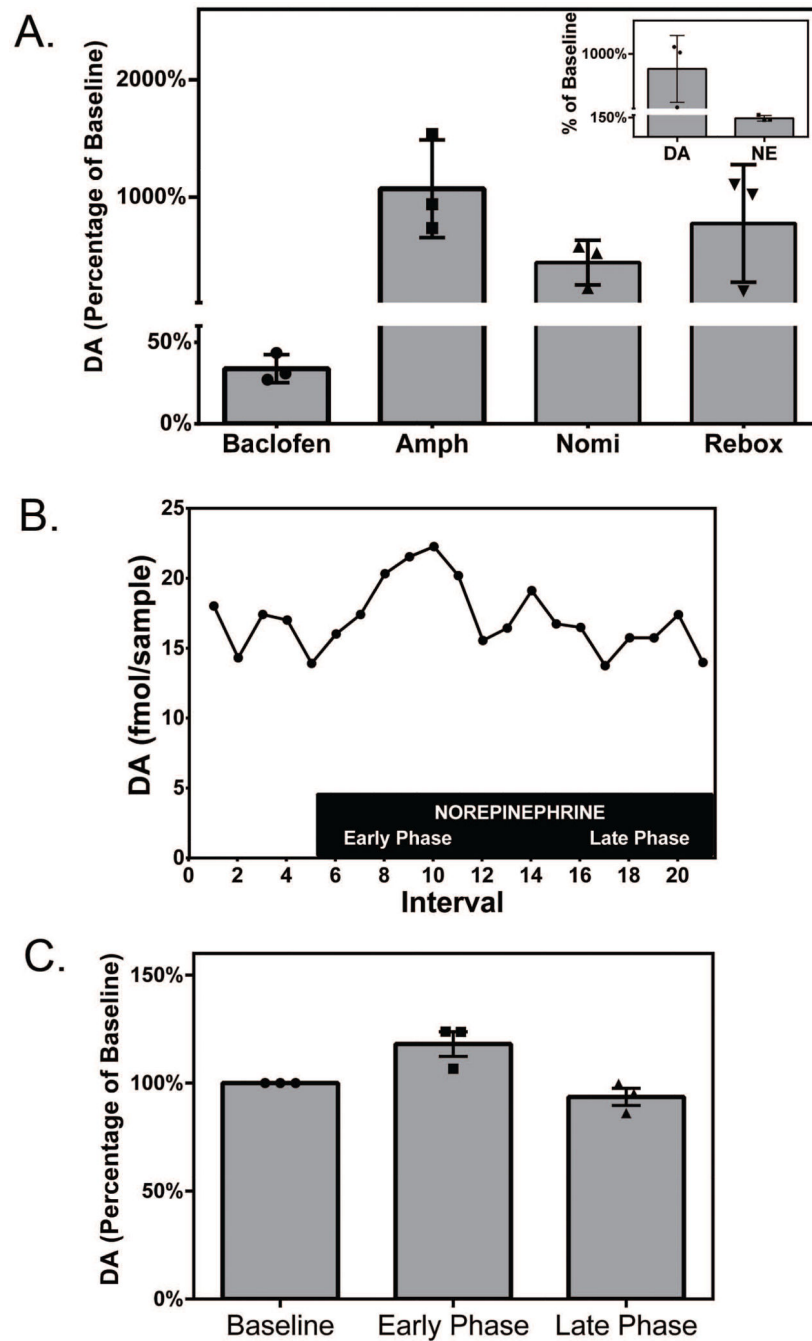
B. Bar graph illustrating change in extracellular DA levels in adult male zebra finch Area X during courtship behavior. Group (N=5) mean DA levels are expressed as normalized values on the Y-axis, and the experimental conditions (as described in A) are indicated on the X-axis. The individual values of the five data points composing the group means are plotted within the bars for each condition. Error bars indicate SEM.

C. Representative experimental time course for an individual juvenile male zebra finch, illustrating extracellular DA levels at 10-minute intervals throughout the course of one experiment, with histogram illustrating amount of singing (in seconds) produced by both the juvenile (black bars) and the adult tutor (gray bars) zebra finch. Different experimental conditions [baseline state ('alone'), social stimulation ('tutor'), and post-stimulation ('alone-post')] are indicated on the X-axis. Because of the duration of the experiment, every other interval is plotted.

D. Bar graph illustrating change in extracellular DA levels in juvenile male zebra finch Area X during observation of tutor. Group (N=6) mean DA levels are expressed as normalized values on the Y-axis, and the experimental conditions are indicated on the X-axis. The individual values of the six data points composing the group means are plotted within the bars for each condition. Error bars indicate SEM.

Figure 3. Extracellular DA levels can be pharmacologically manipulated by drugs that target dopaminergic and noradrenergic synapses

A. Bar graph illustrating the effects of pharmacologic agents on Area X DA levels. The GABA-ergic agent baclofen (0.1 μl of a 0.1 μg/μl solution) was infused into VTA, and the dopaminergic [amphetamine ('Amph,' 10 μM) and nomifensine ('Nomi,'10 μM)] and noradrenergic [reboxetine ('Rebox,' 250 nM)] agents were reverse-dialyzed into Area X. Bars represent normalized group mean DA values, and error bars indicate SEM. N = 3 for each pharmacologic agent. The individual values of the three data points composing the group means are plotted within the bars for each condition. The effect of reboxetine on NE

is demonstrated with the inset, where bars represent normalized group mean DA values on the left and normalized group mean NE values on the right. Error bars indicate SEM (N = 3).

B. Representative experimental time course for an individual zebra finch, illustrating extracellular DA levels at 10-minute intervals throughout the course of one experiment as exogenous norepinephrine (500 nM, indicated by the black box) was reversed dialyzed into Area X.

C. Bar graph illustrating the mean change in extracellular DA in Area X that occurred in the presence of norepinephrine (N = 3). Bars represent normalized group mean DA levels (Y-axis) at baseline, in the first 50 minutes ('early phase'), and last 50 minutes ('late phase') of NE reverse dialysis, as indicated on the X-axis. Error bars indicate SEM. The individual values of the three data points composing the group means are plotted within the bars for each condition.

Table 1

Comparison of behavioral conditions, using a random effects model

Solution for Fixed Effects, Adults (N = 5)

	Characteristic	Estimate (DA, [fmol]/sample)	95% Confidence Interval (C I)		P value
Condition					
	Baseline	0	n/a	n/a	n/a
	Stimulus[*]	+5.4003	3.8753	6.9253	$p < 0.0001$
	Recovery	+2.5013	0.8786	4.1239	$p < 0.003$
Time		0.02807	−0.00476	0.06090	$p = 0.0926$

[*] Presence of female

Table 2

Comparison of behavioral conditions, using a random effects model

Solution for Fixed Effects, Juveniles (N = 6)

	Characteristic	Estimate (DA, [fmol]/sample)	95% C I		P value
Condition					
	Baseline	0	n/a	n/a	n/a
	Stimulus*	+3.2774	1.8679	4.6869	p < 0.0001
	Recovery	+5.0826	3.5859	6.5793	p < 0.0001
Time		0.02057	0.0004123	0.03703	p = 0.0146

*
Presence of tutor

Table 3

Analysis of effects of Pharmacologic agents, using a random effects model

Solution for Fixed Effects, Pharmacologic Agents (N = 3 subjects for each drug tested)

	Stimulus			Recovery		
	Estimate[*]	95% C I	P value	Estimate[*]	95% C I	P value
Baclofen	−1.69	−2.48, −0.90	0.0002	−0.26	−1.08, 0.56	0.51
Amphetamine	+45.33	19.19, 71.47	0.0024	n/a	n/a	n/a
Nomifensine	0.55	0.42, 0.68	<0.0001	n/a	n/a	n/a
Reboxetine	+113.88	65.34, 162.42	<0.0001	n/a	n/a	n/a
Norepinephrine	+2.28	0.90, 3.65	0.0018	−1.36	−2.74, 0.009	0.05

[*] unit change in [DA] per sample, relative to baseline