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Catecholaminergic contributions to vocal communication signals

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ABSTRACT

Social context affects behavioral displays across a variety of species. For example, social context acutely influences the acoustic and temporal structure of vocal communication signals like speech and birdsong. Despite the prevalence and importance of such social influences, little is known about the neural mechanisms underlying the social modulation of communication. Catecholamines are implicated in the regulation of social behavior and motor control, but the degree to which catecholamines influence vocal communication signals remains largely unknown. Using a songbird, the Bengalese finch, we examined the extent to which the social context in which song is produced affected immediate early gene expression (EGR-1) in catecholamine-synthesizing neurons in the midbrain. Further, we assessed the degree to which administration of amphetamine, which increases catecholamine concentrations in the brain, mimicked the effect of social context on vocal signals. We found that significantly more catecholaminergic neurons in the ventral tegmental area and substantia nigra (but not the central grey, locus coeruleus, and subcoeruleus) expressed EGR-1 in birds that were exposed to females and produced courtship song than in birds that produced non-courtship song in isolation. Furthermore, we found that amphetamine administration mimicked the effects of social context and caused many aspects of non-courtship song to resemble courtship song. Specifically, amphetamine increased the stereotypy of syllable structure and sequencing, the repetition of vocal elements, and the degree of sequence completions. Taken together, these data highlight the conserved role of catecholamines in vocal communication across species, including songbirds and humans.

INTRODUCTION

The social environment and context have profound effects on the structure of behavior, in particular communicative behaviors. For example, humans alter the structure and content of verbal and non-verbal communicative signals depending on the age, familiarity, and social status of their audience (Fernald & Kuhl, 1987; Giles et al., 1987; Gudykunst & Kim, 2003). Rats alter the composition of their ultrasonic vocalizations when interacting with another conspecific (Wright et al., 2010), and primates alter the structure of their calls depending on the composition of the social group (Clark & Wrangham, 1993). Such changes by the signaler can affect the salience and interpretability of the signal (Kuhl, 2010; Seyfarth et al., 2010). Despite the prevalence and importance of such social influences, little is known about the mechanisms with which social context modulates social and communicative behaviors.

Songbirds are an excellent model system to reveal mechanisms of social influences on social and communicative behaviors (Doupe & Kuhl, 1999). Songbirds, like humans, use learned vocal signals ('songs') for communication, and social context rapidly modulates the structure and organization of birdsong (reviewed in Sakata & Vehrencamp, 2012). For example, the songs produced by male zebra and Bengalese finches during courtship interactions with females are more stereotyped in structure, faster, and preceded by more introductory elements than spontaneous songs produced in isolation (e.g., Cooper & Goller, 2006; Hampton et al., 2009; Kao & Brainard, 2006; Sakata et al., 2008; Woolley & Doupe, 2008). Additionally, male canaries produce more rapid song elements ('syllables') in the presence of a female than in isolation (Kreutzer et al., 1999). Such vocal changes function to increase the

attractiveness of the male's song (Dunning et al., 2014; Vallet & Kreutzer, 1995; Woolley & Doupe, 2008).

Catecholamines such as dopamine and norepinephrine regulate the expression of social behavior and motor control across a variety of species and could mediate social influences on vocal communication. For example, dopamine influences the expression and form of male-typical social behaviors in mammals, birds, and reptiles (Ball et al., 2003; Dominguez & Hull, 2005; Woolley et al., 2004; Vahaba et al., 2013), the sequencing of grooming behaviors in rodents (Berridge et al., 2005; Cromwell et al., 2005; Taylor et al., 2010), and repetitiveness of various motor gestures in mammals (Canales & Graybiel, 2000; Saka et al., 2004). However, little is known about the degree to which catecholamines contribute to vocal motor control. In songbirds, catecholamine-synthesizing neurons in the midbrain project to vocal control nuclei; therefore, catecholaminergic populations are well positioned to mediate the effect of social context on vocal communication signals (Castelino & Schmidt, 2010; Hara et al., 2007; Leblois et al., 2010; Sasaki et al., 2006).

Here we investigated the contribution of catecholamines to the social modulation of song in the Bengalese finch. The Bengalese finch is an excellent model system for this endeavor because social context acutely modulates spectral and temporal features of song, enabling us to examine the differential contribution of catecholamines to syllable structure, sequencing, and timing. We first examined the extent to which social context differentially affected immediate early gene expression in midbrain catecholaminergic neurons. The results from this experiment suggested that catecholamine concentrations in the brain were higher when birds produced courtship song to females than when they produced non-courtship song in isolation. Therefore, we then examined whether administration of

amphetamine, which increases catecholamine concentrations in the brain (Seiden & Sabol, 1993; Fleckenstein et al., 2007), could mimic the effects of social context and cause the structure of non-courtship song to resemble courtship song.

MATERIALS AND METHODS

Animals:

Adult male Bengalese finches (>4 months; n=36) were raised in our colony at McGill University or purchased from vendors (Exotic Wings and Things, Ontario, Canada). Birds were housed in all-male group cages outside the periods of experimentation. Beginning at least one day before experimental manipulations, birds were housed individually in sound-attenuating chambers ('soundboxes'; TRA Acoustics, Cornwall, Ontario, Canada). Birds were housed on a 14L:10D light cycle and provided food and water *ad libitum*. All procedures were approved by the McGill University Animal Care and Use Committee in accordance with the guidelines of the Canadian Council on Animal Care.

Experiment 1: Effect of social context during song production on EGR-1 expression in midbrain catecholaminergic neurons

In this experiment, we examined the degree to which the social context in which song is produced influenced EGR-1 expression in catecholamine-synthesizing neurons in the midbrain. In particular, we analyzed the degree to which EGR-1 expression differed depending on whether birds produced songs when exposed to females or in isolation (e.g., Castelino & Ball, 2005; Hara et al., 2007; Jarvis et al., 1998). Birds were individually housed in soundboxes for at least one night before the day of the experiment. On the morning of the experiment, birds were allowed to

eat and drink for 5-10 minutes after lights on. Subsequently, individual Bengalese finches were exposed to females and allowed to produce only female-directed (FD) song ('FD birds'), kept in isolation and allowed to produce only undirected (UD) song ('UD birds'), or kept silent in isolation ('silent birds'; n=8/grp). To elicit FD song, FD birds were repeatedly (1-5 min intervals) and briefly (<30 sec) exposed to female Bengalese finches housed in separate cages (e.g., Sakata et al., 2008). Female-directed songs are readily distinguishable from UD songs because they are produced after a male approaches or faces the female, accompanied by a courtship dance (e.g., pivoting body from side to side), and associated with the fluffing of the male's plumage (Morris, 1954; Zann, 1996). Males were prevented from producing UD song between presentations of females as per previous experiments by tapping on the soundbox whenever the male attempted to produce UD song (e.g., Castelino & Ball, 2005; Hara et al., 2007; Jarvis et al., 1998).

UD birds were left alone in their soundbox and allowed to produce spontaneous UD song. Bengalese finches can produce UD songs in rapid succession, and because of the temporal sensitivity of EGR-1 expression (Mello & Clayton, 1994), we encouraged UD birds to space out their songs like FD birds by occasionally tapping on the soundbox. Importantly, the amount of song produced by FD and UD birds was not significantly different: FD and UD birds produced, respectively, 116±12.6 (mean±SEM) and 124±12.4 seconds of song before brain collection (t-test: t_{14}=0.43, P=0.68). Therefore, context-dependent differences in EGR-1 expression are not due to differences in the quantity of song.

In addition to comparing EGR-1 expression between FD and UD birds, we also examined EGR-1 expression in silent birds to analyze the extent to which song production generally affected EGR-1 expression. As for FD birds, silent birds were

prevented from producing UD song by tapping on the soundbox whenever the male attempted to produce song. Additionally, silent birds were kept quiet by keeping the soundbox door slightly ajar (e.g., Castelino & Ball, 2005; Hara et al., 2007; Jarvis et al., 1998; Kimpo & Doupe, 1997).

Taken together, this experimental design allowed us to analyze the effect of social context during vocal production (i.e., FD vs UD birds), general effects of vocal production (FD & UD birds vs. silent birds), and the degree to which social context modulated the effect of vocal production on EGR-1 expression in catecholaminergic neurons (i.e., silent->FD vs. silent->UD).

Tissue collection and immunocytochemistry:
60-90 min after of the start of the experiment, lights were turned off, and within 5 minutes after lights off, birds were removed for perfusion. Birds were deeply anaesthetized with isoflurane vapor, then transcardially perfused with heparinized saline (100 IU/mL) followed by 150 mL of 4% paraformaldehyde (pH=7.4). Brains were removed, post-fixed for 4 hours at 4°C in 4% paraformaldehyde and then transferred to a 30% sucrose solution overnight at 4°C. Coronal sections were cut at 40 µm using a sliding microtome (Leica Biosystems, Wetzlar, Germany) and stored in 0.025M phosphate-buffered saline (PBS) containing 0.05% sodium azide at 4°C.

To analyze the extent to which social context and singing affected immediate early gene expression in catecholaminergic populations, we processed brains for the expression of EGR-1 and tyrosine hydroxylase (TH; rate-limiting enzyme in catecholamine synthesis) using previously published methods (e.g., Bharati & Goodson, 2006; Goodson et al., 2009; Lynch et al., 2008). Briefly, we rinsed sections for 10 min in 0.025M PBS (pH=7.4) 3X then blocked the sections for 1 h in PBS +

5.0% donkey serum + 0.3% Triton-X. Thereafter, we incubated sections for 48 h at 4°C with sheep anti-TH (NB300110, Novus Biologicals, Littleton, CO, USA) and rabbit anti-EGR-1 (SC189; Santa Cruz Biotechnology, Santa Cruz, CA, USA), each diluted 1:1000 in PBS + 2.5% donkey serum + 0.3% Triton-X + 0.05% sodium azide. This was followed by 2X 30 min rinses in PBS and a 2 h incubation in donkey anti-rabbit secondary conjugated to Alexa Fluor 594 (5 µl/ml; Life Technologies) and donkey anti-sheep conjugated to Alexa Fluor 488 (3 µl/ml; Life Technologies) in PBS + 2.5% donkey serum + 0.3% Triton-X. Sections were then rinsed for 10 min 3X in PBS and transferred to PBS+0.05% sodium azide before mounting. Sections were mounted on chrom-alum subbed slides and coverslipped with Prolong Gold Antifade with or without DAPI (Life Technologies; P36930).

Image acquisition and analysis

Across FD, UD, and silent birds, we compared the proportion of TH-immunoreactive (ir) neurons that expressed EGR-1 in five midbrain catecholaminergic cell groups implicated in social behavior and motor control: the ventral tegmental area (VTA), substantia nigra (SN), central grey (CG), locus coeruleus (LC), and subcoeruleus (SC; Figure 1). These cell groups in birds are homologous to similarly named midbrain populations in mammals (reviewed in Reiner et al., 2004) and were identified using cytoarchitectural landmarks (e.g., Appeltants et al., 2000, 2002; Bharati & Goodson, 2006; Bottjer, 1993; Goodson et al., 2009; Kabelik et al., 2010; Kingsbury et al., 2011). We subdivided the VTA into three subregions based on cytoarchitectural and functional properties: the rostral VTA (rVTA), the lateral magnocellular portion of the caudal VTA (cmVTA) and the medial parvocellular portion of the caudal VTA (cpVTA; Goodson et al., 2009; Lammel et al., 2008).

Photomicrographs were taken of each midbrain nucleus in each hemisphere at 20X using a Zeiss Axio Imager upright microscope and AxioCam MRm Zeiss camera (Carl Zeiss, Jena, Germany). For each section, separate monochrome images were obtained for EGR-1 and TH expression, which were then color-coded in the ZEN Imaging software (Carl Zeiss, Jena, Germany). We manually counted the number of TH-ir neurons and TH-ir neurons expressing EGR-1 for each image to calculate the proportion of TH-ir neurons that expressed EGR-1 (Figure 1f; e.g., Bharati & Goodson, 2006; Goodson et al., 2009; Kabelik et al., 2010). We counted EGR-1 expression in 6.1±1.7 (mean±SEM) sections per bird, and values were averaged across sections and hemispheres within an individual for data analysis. Images of focal brain areas in some birds could not be obtained due to tissue damage, but in most cases n>7 per group. Image acquisition and quantification were done blind to experimental condition. The degree to which singing and social context affected immediate early gene expression in catecholaminergic populations in our study was comparable to those previously reported in other songbirds (Figures 2 & 3; e.g., Bharati & Goodson, 2006; Goodson et al., 2009; Lynch et al., 2008; Maney & Ball, 2003).

Experiment 2: Effect of amphetamine administration on song organization

In this experiment, we investigated whether administration of amphetamine could mimic the effects of social context on song organization. Specifically, we analyzed the effect of peripheral injections of amphetamine on song using a randomized and balanced repeated-measures design. Amphetamine is known to affect the concentration of catecholamines like dopamine in the brain and the expression of social behaviors (Fleckenstein et al., 2007; Seiden & Sadol, 1993). Adult Bengalese finch males (n=15) were individually housed in a sound-attenuating chamber overnight, then administered either a 0, 1, or 2 mg/kg dose of amphetamine

(dissolved in 30 µl sterile saline) on the following morning. We administered amphetamine subcutaneously in the inguinal fold at approximately the same time each morning (e.g., Kabelik et al., 2010; Schroeder & Riters, 2006), and the order of treatment within a bird was randomized and balanced across individuals. We recorded the vocalizations of experimental birds following the injections and observed the birds remotely via video camera to ensure there were no adverse reactions. General observations of birds did not reveal substantial variation in the activity of birds following these doses. Injections were separated by an average of 7 days (range: 6-8), and birds were returned to group cages between experimental days.

To reveal the effect of amphetamine on song organization, we analyzed the first 30 songs produced after each injection. The latency to sing after injection was 32.5±3.7 (mean±SEM) min and was not significantly affected by amphetamine administration (RM-ANOVA, $p>0.05$).

Sound was recorded continuously using an omnidirectional microphone positioned above the male's cage. A computerized, song-activated recording system was used to detect and digitize song [Sound Analysis Pro v.2011.104 (http://ofer.sci.ccny.cuny.edu/html/sound_analysis.html); digitized at 44.1 kHz]. Recorded songs were digitally filtered at 0.3-10 kHz for analysis using software written in the Matlab programming language (The MathWorks, Natick, MA, USA). Following amplitude-based syllable segmentation, we labeled syllables manually based on visual inspection of spectrograms (e.g., Okanoya & Yamaguchi, 1997; Sakata & Brainard, 2006; Sakata et al., 2008). Song labeling was done blind to experimental condition.

Bengalese finch song consists of acoustic elements ('syllables') arranged into sequences ('motifs') that are embedded within bouts of song (Figure 4). Syllables are individual acoustic elements separated by ≥10 ms of silence (Okanoya & Yamaguchi, 1997; Sakata et al., 2008), and distinct syllables have distinct spectral structure and durations. Motifs are stereotyped sequences of syllables that can be readily detected because of the consistency of sequencing within the motif and because silent gaps between syllables tend to be shorter within motifs than between motifs (e.g., Glaze & Troyer, 2006; Okanoya, 2004; Takahasi et al., 2010; Vu et al., 1994; Williams & Staples, 1992). Song bouts consist of a series of consecutively produced motifs and are defined as periods of singing separated by silent intervals >500 ms (e.g., Hampton et al., 2009; Sakata & Brainard, 2006; Sakata et al., 2008). Because social context affects the structure of song at all these levels (e.g., Kao & Brainard, 2006; Sakata et al., 2008; Stepanek & Doupe, 2010; Teramitsu & White, 2006), we analyzed the effect of amphetamine administration on spectral and temporal features of Bengalese finch song.

The social context in which song is produced robustly affects the variability of syllable structure (Hampton et al., 2009; Kao et al., 2005; Kao & Brainard, 2006; Sakata et al., 2008). Therefore, we measured the effects of amphetamine on syllable structure by computing the fundamental frequency (FF) of syllables with a flat, harmonic structure (n=43 syllables in 15 males; e.g., syllables 'b', 'k', and 'l' in Figure 2). For such syllables we calculated the autocorrelation of a segment of the sound waveform. The FF was defined as the distance, in Hz, between the zero-offset peak and the highest peak in the autocorrelation function. To improve the resolution of frequency estimates, we performed a parabolic interpolation of the peak of the autocorrelation function (de Cheveigné & Kawahara, 2002). We computed the mean and variance of the FF of such syllables under each experimental condition and used

the coefficient of variation (CV: σ/μ) as our measure of variability across renditions (Kao et al., 2005; Kao & Brainard, 2006; Sakata et al., 2008; Stepanek & Doupe, 2010).

Bengalese finch song is preceded by a number of brief, low amplitude vocal elements with simple acoustic structure called 'introductory notes', and the number of introductory notes preceding song is affected by social context (e.g., Kao & Brainard, 2006; Rajan & Doupe, 2013; Sakata et al., 2008; Stepanek & Doupe, 2010). We counted introductory notes beginning with the first introductory note preceding the first song syllable, counting backwards until we reached >500 ms of silence (Hampton et al., 2009; Sakata et al., 2008).

In addition to stereotyped sequences of syllables, Bengalese finch song is also characterized by sequence variability that is modulated by social context (Okanoya 2004; Sakata & Brainard, 2006, 2009; Sakata et al., 2008). In particular, Bengalese finch song consists of sequences in which syllable transitions vary from rendition to rendition. Such sequences are called 'branch points' and often occur between motifs. We analyzed experimental effects on syllable sequencing at branch points by computing the probability of different syllable transitions. For each branch point, sequence variability was quantified as the transition entropy:

$$\text{transition entropy} = \Sigma\text{-}p_i * \log_2(p_i)$$

where the sum is over all possible transitions in the branch point, and p_i is the probability of the i^{th} transition across all songs (Gentner & Hulse, 2000; Sakata et al., 2008). Branch points with transitions that are more variable (i.e., closer to uniform probability) have higher transition entropy scores. Instances in which song was

terminated immediately following the branch point were not included in the calculation of entropy, and sequences in which the dominant transition occurred >95% of the time were not considered branch points. Recursive transitions (i.e., repeating of syllables) were not included in branch point analyses and were considered independently (see Results). Only branch points that occurred at least 15 times on each experimental day were analyzed (n=56 branch points in 15 males).

Social context acutely affects song tempo (Cooper & Goller, 2006; Hampton et al., 2009; Kao & Brainard, 2006; Sakata et al., 2008; Sakata & Brainard, 2009; Stepanek & Doupe, 2010). To analyze the effect of amphetamine on song tempo, we calculated the duration of frequently produced sequences of syllables, using the interval between the onset of the first syllable and the onset of the last syllable in the sequence (e.g., Kao & Brainard, 2006; Sakata & Brainard, 2006; Sakata et al., 2008). Onsets were used as boundaries because the change in amplitude is sharper and less variable for onsets than for offsets, allowing for a more accurate estimate of sequence duration (n=19 sequences in 15 males).

In addition to song features that are influenced by social context, we also examined another form of motor stereotypy that has been found to be regulated by catecholamines in mammals. In rodents, administration of dopaminergic agonists increase the probability that stereotyped sequences of grooming behaviors are completed before the termination of grooming (reviewed in Aldridge et al., 2004). Song motifs are stereotyped sequences of syllables, and we analyzed the extent to which amphetamines affect motif completion before song termination. Motifs were considered complete if all syllables in the motif were produced to completion.

Statistical analysis

To analyze the effects of social context on EGR-1 expression, we used an ANOVA with Condition (silent, UD, and FD) as the independent variable and proportion of TH-ir neurons expressing EGR-1 as the dependent variable. Prior to analysis, data for all midbrain areas were cube-root transformed to improve normality. Because of variation in staining across batches, we also included "Batch" as a variable in the analysis; all experimental groups were represented in equal numbers within each batch. Because we divided the VTA into three distinct subregions, we used a repeated-measures ANOVA (RM-ANOVA) to analyze social influences on activity across all subregions of the VTA (fixed effects: Condition, Subregion, and Condition*Subregion; random effects: ID(Condition), and Batch), followed by individual ANOVAs within each subregion.

We analyzed amphetamine effects on song features in different ways, depending on the nature of the data. We measured the FF of multiple syllables, the duration of multiple sequences, and the transition entropy of multiple branch points per bird. To analyze these song features, we used a mixed effects (random intercepts) model with dose (0, 1 and 2 mg/kg) as a fixed effect, bird ID as a random effect, and syllable, sequence, or branch point ID as a random effect nested within bird ID. We used a random intercepts model instead of a random slopes model because there was little variation in the slopes across individuals (e.g., for FF, sequence durations, and branch point entropy, variance in slopes accounted for <5% of the total variance component). For introductory notes, introductory-like notes, and motif completions, only one measure was taken per bird; therefore, we performed a RM-ANOVA to analyze amphetamine effects for these features (fixed effect: dose; random effect: bird ID). To improve normality, behavioral data were transformed using either a

cube-root transformation (introductory notes) or logarithmic transformation (CV of FF, sequence durations), depending which transformation best normalized the data.

While mixed effects models account for the repeated sampling of individual features within a bird (e.g., durations of multiple sequences within a bird), mixed models do not take into account differences in sample sizes across individual examples of features. Take for example a bird that produced 100 renditions of a sequence that changed in duration by +10% following amphetamine and 900 renditions of another sequence that changed by -10%. In the mixed model, both sequences are weighted equally since only one number represents each sequence, and the analysis would suggest no change in song tempo following amphetamine. Another approach that takes into account differences in sample sizes across examples is to compute the weighted average of the change for each example of a song feature within each bird. In the example above, because the more frequently produced sequence is weighted more than the rarely produced sequence, the weighted change in sequence duration would be -8%. To further analyze the effect of amphetamine on song organization, we computed the weighted change from the control dose to each dose of amphetamine then tested whether the average weighted change was significantly different than zero using a t-test (two-tailed; H_0: mean=0).

For all RM-ANOVAs, we tested sphericity using the Mauchly criterion, and when sphericity was violated, we performed the Greenhouse-Geisser correction (Quinn & Keough, 2002). We used a Tukey's HSD for post-hoc contrasts for both experiments (Quinn & Keough, 2002). Means and standard errors were computed for figures following back-transformation (Bland & Altman, 1996).

All statistical analyses were performed using JMP 10.0 (SAS Institute, Cary, NC, USA) for Macintosh with $\alpha = 0.05$ for all tests.

RESULTS

Experiment 1: Effect of social context during song production on EGR-1 expression in midbrain catecholaminergic neurons

We analyzed the effect of singing and social context on EGR-1 expression in three subregions of the ventral tegmental area (VTA; Figures 1 and 2) - the rostral VTA (rVTA), caudal magnocellular VTA (cmVTA) and caudal parvocellular VTA (cpVTA; Goodson et al., 2009; Lammel et al., 2008). Broadly speaking, EGR-1 expression in catecholamine-producing neurons was higher in caudal than rostral subregions of the VTA, and for all subregions, EGR-1 expression in catecholaminergic neurons was highest in Bengalese finches exposed to females and allowed to produce female-directed song (FD birds; Figure 5a). Although the degree to which social context during singing affected EGR-1 expression significantly varied across the rVTA, cmVTA and cpVTA (Condition x Subregion interaction: $F_{4,40}=3.57$, $P=0.01$), the pattern of group differences was similar across subregions. In both the cpVTA ($F_{2,14}=17.71$, $P<0.0001$) and cmVTA ($F_{2,14}=5.73$, $P=0.02$), the proportion of TH-ir neurons expressing EGR-1 was significantly greater in FD birds than in UD and silent birds (Tukey's HSD; $P<0.05$) and not significantly different between UD and silent birds. The proportion of TH-ir neurons expressing EGR-1 in the rVTA was not significantly different among silent, FD, and UD birds ($F_{2,14}=3.66$, $P=0.0528$). Group differences in the percent of VTA TH-ir neurons that expressed EGR-1 were not due to differences in the abundance of TH-ir neurons ($P>0.15$ for all subregions).

Similar to the VTA, social context affected EGR-1 expression in catecholaminergic neurons in the substantia nigra (SN; Figure 5b; $F_{2,14}$=4.56, P=0.03). Post-hoc contrasts revealed that a significantly greater proportion of TH-ir neurons expressed EGR-1 in FD birds than in UD birds (Tukey's HSD; P<0.05). There was no significant difference in the proportion of TH-ir neurons expressing EGR-1 between silent and FD birds. As in the VTA, there were no significant differences in the abundance of TH-ir neurons in the SN across experimental groups (P>0.50).

Unlike the VTA and SN, social context did not modulate EGR-1 expression in catecholamine-synthesizing neurons in the central grey (CG). However, singing, regardless of social context, significantly increased the proportion of TH-ir neurons expressing EGR-1 (Figure 5c; $F_{2,13}$=8.31, P=0.0047). Post-hoc comparisons revealed that a greater proportion of TH-ir neurons expressed EGR-1 in FD and UD birds than in silent birds (Tukey's HSD; P<0.05), but there was no difference between FD and UD birds. Group differences in the proportion of TH-ir neurons expressing EGR-1 were not due to differences in the number of TH-ir neurons in the CG (P>0.85).

The pattern of EGR-1 expression in the locus coeruleus (LC) and subcoeruleus (SC) was different than the other catecholamine-producing midbrain areas examined. Overall, few TH-ir neurons in the LC and SC expressed EGR-1, and neither singing nor social context significantly affected the proportion of TH-ir neurons expressing EGR-1 (Figure 5d,e; LC: $F_{2,11}$=0.41, P=0.67; SC: $F_{2,11}$=0.29, P=0.76).

Experiment 2: Effect of amphetamine administration on song organization

The previous analysis of EGR-1 expression in midbrain catecholaminergic populations suggests that more catecholamines could be released from VTA and SN neurons when Bengalese finches produce FD song than when they produce UD song.

Therefore, it is plausible that experimental increases in catecholamine concentrations in the brain could cause UD song to more closely resemble FD song. Because amphetamine increases the concentration of catecholamines in the brain (Fleckenstein et al., 2007; Seiden & Sabol, 1993), we assessed the degree to which systemic administration of amphetamine (1 and 2 mg/kg) affected the structure of UD song using a balanced, repeated-measures design.

The variability of the fundamental frequency (FF) of syllables with flat, harmonic structure is lower when male songbirds produce FD song than UD song (e.g., Kao & Brainard, 2006; Hampton et al., 2009; Sakata et al., 2008). Similar to the effect of social context, amphetamine administration reduced the variability of FF (as measured using the coefficient of variation (CV); mixed effect model: $F_{2,84}$=8.01, P=0.0007). In the example provided in Figure 6a, the bird produced the syllable with less variability following administration of 1 and 2 mg/kg of amphetamine than following control injections. Such decreases following amphetamine administration were consistent across birds, and post-hoc contrasts revealed that both the 1 and 2 mg/kg dose significantly decreased the CV of FF (Figure 6b; Tukey's HSD; P<0.05). Analysis of weighted percent changes indicated that the 2 mg/kg dose significantly decreased the CV of FF (-9.7±3.7% (mean±SEM); t_{14}=2.60; P=0.02). The weighted percent change in the CV of FF following the 1 mg/kg dose was negative but not significantly different than zero (-7.1±3.4%; t_{14}=2.08; P=0.06).

Female-directed courtship song is preceded by more introductory notes than UD song (e.g., Cooper & Goller, 2006; Kao & Brainard, 2006; Sakata et al., 2008), and amphetamine administration replicated the effect of social context (Figure 7; RM-ANOVA: $F_{2,28}$=11.53, P=0.0002). In the example provided in Figure 7b, the bird produced, on average, 4.7±0.2, 6.1±0.3, and 5.6±0.3 (mean±SEM) introductory notes

following administration of 0, 1, and 2 mg/kg doses of amphetamine, respectively. Such increases in introductory notes were observed for 13 of the 15 birds following the 1 mg/kg dose and for 12 out of 15 birds for the 2 mg/kg dose. Post-hoc contrasts revealed that both doses significantly increased the number of introductory notes by 21.2±6.4% and 20.7±7.1%, respectively (Figure 7c; Tukey's HSD; $P<0.05$).

Some birds (n=9) repeat acoustic elements that resemble introductory notes (i.e., soft, short notes with simple acoustic structure) within song, for example between song motifs (see example in Figure 7a). However, amphetamine administration did not significantly affect the number of times these notes were repeated (RM-ANOVA: $F_{2,16}=3.30$, $P=0.06$; Figure 7d).

Syllable transitions at branch points are another form of syllable sequencing that is affected by social context and that could be influenced by amphetamines (Sakata et al., 2008; Hampton et al., 2009; Sakata & Brainard, 2009). An example of the effect of amphetamine on syllable sequencing at branch points is depicted in Figure 8a. In this example, the sequence 'abcdef' could be followed by the syllables 'g', 'h', and 'i'. Both the 1 and 2 mg/kg doses of amphetamine caused the transition entropy, a measure of sequence variability, to decrease for this branch point. Across all branch points, amphetamine administration significantly affected transition entropy (mixed effects model: $F_{2,110}=3.27$, $P=0.04$). While none of the post-hoc contrasts were significant, this effect was driven by a decrease in transition entropy following the 1 mg/kg dose of amphetamine. This decrease was corroborated in our analysis of weighted changes in transition entropy: the weighted change following the 1 mg/kg dose was significantly less than zero (-0.11±0.06; $t_{14}=2.40$, $P=0.03$). The weighted

change following the 2 mg/kg dose was negative but not significantly different than zero (-0.08±0.04; t_{14}=1.91, P=0.08; Figure 8b).

The FD songs of adult Bengalese finches are faster than UD songs (Hampton et al., 2009; Sakata et al., 2008; Sakata & Brainard, 2009). In contrast to the effect of social context, amphetamine administration did not significantly affect sequence durations (mixed effect model: $F_{2,36}$=1.13, P=0.33). This lack of an effect of amphetamine on song tempo was also supported by our analysis of the weighted percent change in sequence durations (Figure 9; 1 mg/kg: 0.07±0.43%; t_{14}=0.15, P=0.89; 2 mg/kg: -0.36±0.29%; t_{14}=1.24, P=0.24).

Finally, in addition to song features that have been found to be modulated by social context, we also examined the degree to which amphetamine administration affected another form of motor stereotypy. Dopamine has been found to increase the probability that rodents complete a full grooming sequence before terminating grooming (e.g., Berridge et al., 2005; Cromwell et al., 1998). We analyzed whether amphetamine administration influenced the degree to which stereotyped sequences of syllables ('motifs') were produced to completion before birds terminated song. Similar to previous findings in other songbird species, on average, most songs (76.4±5.6%) ended following the completion of a motif (Figure 10a; e.g., Ashmore et al., 2005; Cynx, 1990: Naie & Hahnloser, 2011). Moreover, amphetamine administration significantly increased the likelihood that birds completed a motif before terminating song (Figure 10b; RM-ANOVA: $F_{2,28}$=4.60, P=0.02). Post-hoc contrasts revealed that Bengalese finches were significantly more likely to complete motifs following both the 1 and 2 mg/kg doses (Tukey's HSD, P<0.05).

DISCUSSION

Social context can influence the structure and organization of communication signals in a wide range of species, including humans, rodents, and songbirds (Giles et al., 1987; Gudykunst & Kim, 2003; Kao & Brainard, 2006; Kreutzer et al., 1999; Sakata et al., 2008; Wright et al., 2010). However, little is known about the neural mechanisms underlying this social modulation. Catecholamines such as dopamine and norepinephrine can modulate the expression and form of social and motor behaviors and could mediate the effects of social context on vocal motor signals (Ball et al., 2003; Dominguez & Hull, 2005; Vahaba et al., 2013; Woolley et al., 2004). To this end, we investigated the degree to which the social context in which song is produced affected EGR-1 expression in midbrain catecholaminergic neurons in the Bengalese finch, a songbird. Furthermore, we assessed the extent to which administration of amphetamine, a drug that increases catecholamine concentrations in the brain, mimicked the effect of social context on Bengalese finch song.

We found that social context and singing had diverse effects on EGR-1 expression across midbrain catecholaminergic areas (Figure 5). Specifically, we found that significantly more catecholaminergic neurons in the caudal ventral tegmental area (VTA) and substantia nigra (SN) expressed EGR-1 when male Bengalese finches were presented with females and produced female-directed (FD) song than when they produced undirected (UD) song in isolation. Additionally, FD birds but not UD birds had significantly more EGR-1 expression in catecholamine-producing neurons than silent controls in the caudal VTA. On the other hand, UD and FD birds had comparable increases in EGR-1 expression relative to silent birds in catecholaminergic neurons in the central grey (CG), suggesting a role of the CG in vocal production regardless of social context. In the locus coeruleus (LC) and

subcoeruleus (SC), neither singing nor social context affected EGR-1 expression in catecholaminergic neurons. These data suggest that catecholaminergic neurons in the VTA, SN and CG could generally be involved in vocal production and, moreover, that neurons in the VTA and SN are sensitive to the social context in which song is produced and could contribute to vocal motor changes across social contexts.

The effect of social context on catecholaminergic neurons in the VTA and SN is consistent with previous studies and suggests that catecholamines are elevated in target brain areas when birds direct songs to females relative to when they produce song in isolation. Across mammals, birds, and lizards, EGR-1 expression is increased in catecholaminergic neurons in the VTA and SN during copulatory and courtship interactions (e.g., Balfour et al., 2004; Charlier et al., 2005; Dominguez & Hull, 2005; Kabelik et al., 2014). In other songbird species like the zebra finch and song sparrow, immediate early gene expression is elevated in catecholaminergic neurons in the VTA during vocal and social interactions (e.g., Bharati & Goodson, 2006; Maney & Ball, 2003; Goodson et al., 2009), and putative dopaminergic neurons in the VTA show more spiking activity during the production of FD song than UD song (Yanagihara & Hessler, 2006). Furthermore, dopamine concentrations have been found to be higher when birds produce FD song than UD song in Area X, an avian basal ganglia nucleus that receives dense projections from the VTA and SN (Sasaki et al., 2006). Our data are consistent with the possibility that more dopamine is released in Area X as well as other vocal control nuclei when male Bengalese finches perform FD song than when they produce UD song.

The specific aspects of the social context that increased EGR-1 expression in the VTA and SN are unknown. For example, UD and FD birds differ not only in the type of song they produce but also in the presence of a conspecific. We hypothesize that

performing courtship song is important for the increase in EGR-1 expression because a previous study in the zebra finch found that the production of courtship song after exposure to a female was important for increases in immediate early gene expression in VTA catecholaminergic neurons (Goodson et al., 2009). However, it remains possible that simple exposure to females or other conspecifics could affect EGR-1 expression in catecholaminergic neurons of Bengalese finches. It is also possible that aspects of our experiment that were not related to social or vocal behavior could have affected EGR-1 expression in catecholaminergic neurons. For example, tapping on the soundbox to prevent UD song could cause acute stress and increase EGR-1 expression in midbrain catecholaminergic neurons (e.g., Campeau et al., 1997; Cullinan et al., 1995; Martinez et al., 1999). While it is possible that our interventions to prevent UD song affected EGR-1 expression in the midbrain, we do not believe that such interventions were the primary cause of group differences because males in all groups experienced tapping to some degree.

Increased EGR-1 expression in catecholaminergic neurons in the VTA and SN of FD birds suggest that elevated catecholamine levels during FD song could contribute to the effects of social context on song. Consequently, we administered amphetamine to test the hypothesis that increases in catecholamine levels in the brain would mimic the effects of social context on song. We specifically hypothesized that amphetamine administration would increase the stereotypy of syllable structure and sequencing, the repetition of vocal elements, and song tempo (Hampton et al., 2009; Sakata et al., 2008; Sakata & Brainard, 2009). We found support for these predictions in that amphetamine administration increased the stereotypy of syllable structure and sequencing as well as vocal motor repetition (Figures 6-8).

The precise mechanism by which amphetamine acts to affect vocal control is unknown, but cortical-basal ganglia circuits are likely to be involved to some degree. Cortical-basal ganglia circuits are densely innervated by catecholaminergic inputs and have been implicated in vocal control and motor repetition (e.g., Alm, 2004; Canales & Graybiel, 2000; Graybiel, 2008; Presti et al., 2003). For example, lesions of the nigrostriatal dopaminergic system affect the bandwidth and amplitude of rodent ultrasonic vocalizations (Ciucci et al., 2009; Gombash et al., 2013), and individuals with Parkinson's disease have articulatory deficits that affect the structure of speech (Ho et al., 1998; Logemann et al., 1978; Logemann & Fisher, 1981; Vercruysse et al., 2014). Stuttering is a form of vocal motor perseveration that is influenced by dopamine: dopaminergic activity in cortical and basal ganglia structures is elevated in stutterers (Wu et al., 1997), and antagonism of dopamine receptors has been found to decrease the prevalence and severity of stuttering (Maguire et al., 2004). These findings suggest that amphetamines could affect activity in forebrain-basal ganglia circuits to drive vocal motor changes in songbirds like the Bengalese finch.

Songbirds possess a forebrain-basal ganglia circuit called the anterior forebrain pathway (AFP) that is specialized for song control and plasticity (Brainard & Doupe, 2013; Castelino & Schmidt, 2010; Woolley & Kao, 2014). Like mammalian cortical-basal ganglia circuits, the AFP is heavily innervated by catecholamine-synthesizing areas like the VTA and SN and is important for the online control of motor behavior. For example, neural activity in the AFP regulates the spectral structure of song syllables (e.g., fundamental frequency (FF)) on a moment-by-moment basis (Kao et al., 2005; Kao et al., 2008; Ölveckzy et al., 2005, 2011; Woolley et al., 2014). Inactivations and lesions of the lateral magnocellular nucleus of the anterior nidopallium (LMAN), the output nucleus of the AFP, dramatically decrease the

variability of syllable structure (Ali et al., 2013; Hampton et al., 2009; Kao & Brainard, 2006; Ölveckzy et al., 2005; Stepanek & Doupe, 2010; Warren et al., 2011), and microstimulation of AFP neurons during ongoing song affects the spectral structure of syllables (Kao et al., 2005). Furthermore, pharmacological and genetic manipulations of dopaminergic inputs into Area X, the basal ganglia nucleus of the AFP, affect the variability of syllable structure (Leblois et al., 2010; Murugan et al., 2013). Of particular relevance is the finding that administration of D1 antagonists into Area X eliminates context-dependent differences in syllable structure (Leblois et al., 2010). It is hypothesized that dopamine from areas like the VTA and SN influences the variability of syllable structure by altering the variability of singing-related activity across renditions (Hessler & Doupe, 1999; Kao et al., 2008; Woolley et al., 2014), and we propose that amphetamine similarly affects the variability of syllable structure by modulating the variability of neural activity in the AFP.

Despite the strong evidence that the AFP contributes to the control of syllable structure, there is mixed evidence regarding the contribution of the AFP to syllable sequencing. Lesions and inactivations within the AFP do not cause significant changes to syllable sequencing or the repetition of vocal elements in adult zebra finches or Bengalese finches (e.g., Brainard & Doupe, 2001; Hampton et al., 2009; Horita et al., 2008; Kao & Brainard, 2006; Williams & Mehta, 1999). Furthermore, antagonism of D1 receptors in Area X does not affect the number of introductory notes preceding song or the social modulation of introductory notes (Leblois et al., 2012). However, some studies have found that electrolytic or neurochemical lesions of Area X transiently or persistently affect vocal repetition in Bengalese and zebra finches (Kobayashi et al., 2001; Kubikova et al., 2014). As a consequence, it remains possible that amphetamine affects the number of introductory notes and the variability of syllable sequencing at branch points by modulating activity in the AFP,

but direct manipulations of catecholamines in the AFP of Bengalese finches are required.

In contrast to the diversity of findings regarding AFP influences on vocal motor sequencing, there is consistent evidence that activity in the vocal motor pathway (VMP) regulates syllable sequencing. For example, the activity of HVC (used as proper name) neurons encodes information about syllable sequencing and introductory notes (e.g., Fujimoto et al., 2011; Kozhevnikov & Fee, 2007; Rajan & Doupe, 2013; Sakata & Brainard, 2008; Yu & Margoliash, 1996), and manipulations of HVC activity affect syllable sequencing and the production of introductory notes (Thompson et al., 2007; Thompson & Johnson, 2007; Vu et al., 1994). Computational models of vocal sequence generation focus on HVC as a critical node for sequence control (reviewed in Jin, 2009; Jin & Kozhevnikov, 2011). As such, we hypothesize that our amphetamine administrations affected the repetition of introductory notes and syllable sequencing by altering activity in HVC.

In addition to affecting the variability of syllable structure and sequencing, amphetamine also increased the degree to which stereotyped sequences of syllables ('motifs') were completed before song termination (Figure 10). This analysis was motivated by the findings that dopamine and activity in basal ganglia circuitry regulate the likelihood that rodents will complete a stereotyped "syntactic chain" of grooming sequences (Aldridge & Berridge, 1998; Berridge et al., 2005; Cromwell et al., 1998). Like stereotyped sequences of grooming behaviors, song motifs are stereotyped sequences of vocal motor elements (syllables), and our data support the notion that catecholamines like dopamine contribute to sequence completions in songbirds. The contribution of the avian basal ganglia to sequence completions, however, remains unknown. A few studies in the zebra finch have found that

manipulations of activity outside the AFP cause song terminations before motif completions. For example, inactivation of the nucleus interface of the nidopallium (NIf) leads to more incomplete song motifs (Naie & Hahnloser, 2011), and microstimulation of neurons in HVC and the robust nucleus of the arcopallium (RA) cause song motifs to abruptly stop and restart (Ashmore et al., 2005; Vu et al., 1996). Therefore, we propose that amphetamine influences motif completions by altering neurophysiological activity in vocal motor areas such as NIf, HVC, and RA.

In contrast to the effects of amphetamine on syllable structure and sequencing, amphetamine administration did not significantly affect song tempo. A number of other studies have found that amphetamines and other dopamine agonists increase the speed of vocalizations (e.g., Marrone et al., 2010; Wright et al., 2012). As such, we anticipated that amphetamines would mimic the effect of social context and increase song tempo. However, there was little evidence that our amphetamine administration affected song tempo. It is possible that different doses of amphetamine would elicit such an effect, but, nevertheless, our experiments indicate that the structure, sequencing, and timing of song syllables have different sensitivities to amphetamine administration.

The precise neurochemical mechanism by which amphetamine affected song organization is unknown. Amphetamine is known to increase the concentration of dopamine in the brain but can also increase levels of norepinephrine, serotonin, and glutamate, for example, which can affect the expression of social behaviors (Barclay et al., 1996; Dominguez & Hull, 2005; Underhill et al., 2014; Wright et al., 2012). Despite the diversity of neurochemical systems affected, we propose that amphetamine acts on the dopaminergic system to alter song organization in Bengalese finches. This is because context-dependent differences in EGR-1

expression were found in midbrain dopaminergic populations (VTA and SN) but not in midbrain noradrenergic populations (LC and SC) and because amphetamine effects on a variety of social behaviors and brain activity are blocked by dopamine receptor antagonists (e.g., Dixon et al., 2005; Hiroi & White, 1991; Jones et al., 1998; Liu et al., 2011; Vezina, 1996). Nevertheless, subsequent experiments are required to isolate the precise neurochemical mechanisms underlying amphetamine effects on vocal behavior (e.g., Wright et al., 2012, 2013).

Taken together, these data suggest that catecholamines contribute to the control of vocal communication signals in songbirds and that social context affects multiple distinct circuits to produce a diversity of vocal motor changes. Further, these data underscore the possibility that deficits in social communication in humans could be mediated by dysfunctions in catecholaminergic circuits. Neurobiological disorders such as Parkinson's disease, autism, and Tourette's syndrome are characterized by deficits in social and vocal communication (Berridge et al, 2005; Ho et al., 1998; Jankovic, 2001; Logemann et al., 1978; Logemann & Fisher, 1981; Mink, 2006; Plumb & Wetherby, 2013; Shriberg et al., 2001; Taylor et al., 2010; Wetherby et al., 1998), and manipulations of dopaminergic and noradrenergic function could help treat such communicative dysfunctions in humans (e.g., Anderson et al., 1999; Barch & Carter, 2005; Maguire et al., 2004; Stager et al., 2005; Wu et al., 1997).

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FIGURE LEGENDS

Figure 1. Catecholamine-synthesizing neurons in the midbrain of the Bengalese finch. Photomicrographs of tyrosine hydroxylase-immunoreactive (TH-ir; green) neurons in the (a) rostral ventral tegmental area (rVTA), (b) the magnocellular and parvocellular portions of the caudal VTA (cmVTA and cpVTA, respectively) and substantia nigra (SN), (c) central grey (CG), (d) subcoeruleus (SC), and (e) locus coeruleus (LC). Pictures are organized from rostral to caudal. Scale bar = 200 μm.

Figure 2. EGR-1 (red) and TH (green) expression in the ventral tegmental area (VTA) and substantia nigra (SN). Images were taken from an FD bird. (A) 10X magnification image of the rostral VTA. IP=interpeduncular nucleus. (B) 20X magnification of the rostral VTA to demonstrate the extent of colocalization. Yellow arrows point to double-labeled neurons. (C) 10X magnification image of the caudal VTA. (D) 10X magnification image of the SN. For 10X images, scale bar = 100 μm; for 20X image, scale bar = 50 μm.

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there is little EGR-1 expression in the SC. For 10X images, scale bar = 100 μm; for 20X images scale bar = 50 μm.

Figure 4. Organization of Bengalese finch song. Plotted is a spectrogram (frequency on the y-axis, time on the x-axis, darkness as intensity) of an individual Bengalese finch song. A 'bout' of Bengalese finch song is preceded by the repetition of soft, brief, and acoustically simple vocal elements called 'introductory notes'. These introductory elements are followed by vocal elements called 'syllables' arranged in stereotyped sequences called 'motifs' as well as variable sequences called 'branch points'. We label individual syllables with unique letters for song analysis. In this example, 'abcde', 'fghijkl', and 'okl' are examples of motifs, and the sequence 'abcde' is a branch point that can be followed by the motifs 'fghijkl' or 'okl'. In the Bengalese finch, social context affects the repetition of vocal elements like introductory notes, syllable sequencing at branch points, syllable structure, and song tempo (Hampton et al., 2009; Sakata et al., 2008; Sakata & Brainard, 2009). Scale bar= 500 ms.

Figure 5. Singing and social context differentially affect EGR-1 expression in catecholamine-producing neurons across midbrain areas. We measured EGR-1 expression in tyrosine hydroxylase-immunoreactive (TH-ir) neurons in Bengalese finches that were exposed to females and produced female-directed (FD) courtship song (grey bars), birds that produced undirected (UD) song in isolation (black bars), and silent birds in isolation (white bars). (a) The social context in which song is produced differentially affected EGR-1 expression in catecholaminergic neurons across subregions of the ventral tegmental area (VTA). In the caudal magnocellular and parvocellular subregions of the VTA (cmVTA and cpVTA, respectively), the proportion of TH-ir neurons expressing EGR-1 was significantly higher in FD birds

than in UD and silent birds. The proportion of TH-ir neurons expressing EGR-1 in the rostral VTA was not significantly different among silent, FD, and UD birds (rVTA; $F_{2,14}$=3.66, P=0.0528). (b) In the substantia nigra (SN), the proportion of TH-ir neurons expressing EGR-1 was significantly higher in FD birds than in UD birds. (c) In the central grey (CG), the proportion of TH-ir neurons expressing EGR-1 was significantly higher in both FD and UD birds than silent birds, but there was no difference between FD and UD birds. (d),(e) In the locus coeruleus (LC) and subcoeruleus (SC) EGR-1 expression in TH-ir neurons was not significantly different among FD, UD and silent birds. For all panels, plotted are the mean (±SEM) percent of TH-ir neurons expressing EGR-1, and groups that do not share letters are significantly different (Tukey's HSD, P<0.05). Sample sizes for each brain area are indicated below group labels on the x-axis.

Figure 6. Effect of amphetamine on the stereotypy of syllable structure. (a). An example of the effect of amphetamine on syllable structure. We measured the fundamental frequency (FF) of syllables with flat, harmonic structure and computed the variability of FF across renditions of syllables. We summarized the variability using the coefficient of variation (CV: μ/σ). On the left is a spectrogram of a syllable with flat, harmonic structure, and to the right of the spectrogram are three histograms summarizing the FF of each rendition of this syllable following administration of 0, 1 and 2 mg/kg doses of amphetamine. Numbers in the top-right corners indicate the CV of the FF under each condition, and in this example, the CVs were lower following the 1 and 2 mg/kg doses of amphetamine than following the control injection. (b). Both the 1 and 2 mg/kg doses of amphetamine significantly decreased the CV of FF (n=43 syllables in 15 birds; mixed effects model; Tukey's HSD, P<0.05). Plotted are the mean (±SEM) CVs following 0, 1 and 2 mg/kg doses of amphetamine. '*' denotes significantly different from 0 mg/kg.

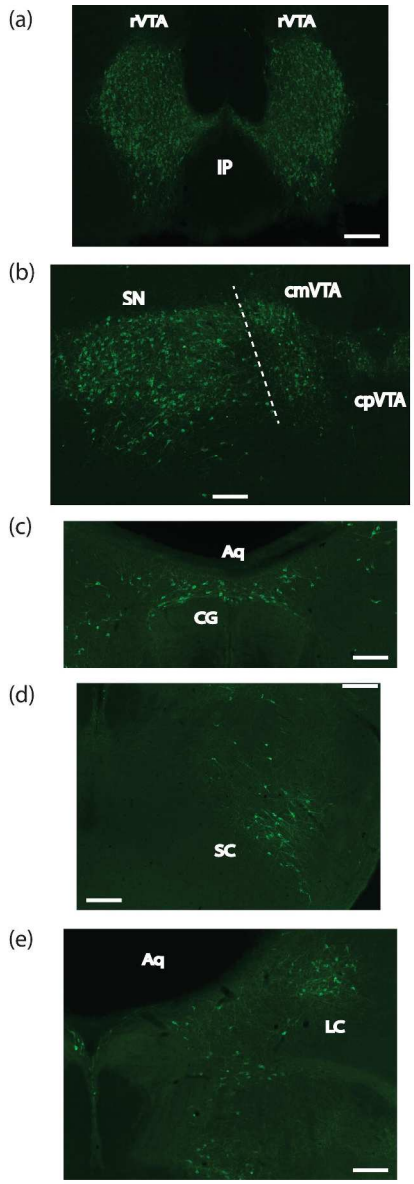
Figure 7. Effect of amphetamine administration on the repetition of vocal elements before and within song. (a). Spectrogram of a Bengalese finch song in which short and simple vocal elements are repeated before ('introductory notes') and within song ('introductory-like notes'). Scale bar= 500 ms. (b). An example of the effect of amphetamine administration on introductory notes. Plotted are three histograms summarizing the number of introductory notes preceding the bird's song following administration of 0, 1 and 2 mg/kg doses of amphetamine. Numbers above histograms represent the mean (±SEM) number of introductory notes under each condition, and in this example, the 1 and 2 mg/kg doses increased the number of introductory notes. (c). Both the 1 and 2 mg/kg doses significantly increased the number of introductory notes preceding song (n=15 birds; Tukey's HSD, $P<0.05$). Plotted are the mean (±SEM) numbers of introductory notes following 0, 1 and 2 mg/kg doses of amphetamine. '*' denotes significantly different from 0 mg/kg (Tukey's HSD). (d). Amphetamine administration did not significantly affect the repetition of introductory-like notes within song (n=9 birds; $P=0.06$). Plotted are the mean (±SEM) numbers of times such elements are repeated in succession following 0, 1 and 2 mg/kg doses of amphetamine.

Figure 8. Effect of amphetamine administration on syllable sequencing at branch point sequences. (a). An example of amphetamine effects on the transition entropy of a branch point. In this example, the sequence 'abcdef' could be followed by the syllables 'g', 'h', or 'i', and listed are the transition probabilities to each syllable following administration of 0, 1 and 2 mg/kg doses of amphetamine. For each branch point we computed the transition entropy based on the probabilities of the different transitions, and in this example, the transition entropy decreased following amphetamine administration. (b). The weighted change in transition entropy was

significantly less than zero following the 1 mg/kg dose (n=56 branch points from 15 birds; P=0.03), indicating that this dose of amphetamine decreased sequence variability at branch points. The weighted change in entropy following the 2 mg/kg dose was negative but not statistically significantly different than zero (P=0.08).

Figure 9. Amphetamine administration did not significantly affect song tempo. (a). An example of the lack of changes in sequence durations following amphetamine administration. Plotted is a spectrogram of the sequence 'abcdef' (top) and histograms of sequence durations for 'abcdef' (onset of the 'a' to the onset of the 'f') following the 0, 1, and 2 mg/kg doses of amphetamine (mean±SEM). Scale bar= 100 ms. (b). The weighted change in sequence durations was not significantly different than zero for both doses of amphetamine (n=19 sequences in 15 birds; P>0.20 for both).

Figure 10. Effect of amphetamine administration on motif completions before song termination. (a). Motifs are stereotyped sequences of syllables that are usually completed before song termination. Plotted are five randomly selected renditions of the end of song from one bird. In four of the five renditions, the bird completes the motif before ending song, but on one rendition (fourth spectrogram) the bird abruptly stops singing in the middle of the motif. Scale bar= 1 sec. (b). Bengalese finches were significantly more likely to complete a motif before terminating song following both doses of amphetamine than following control injections ($F_{2,28}$=4.60, P=0.02). Both the 1 and 2 mg/kg doses significantly increased motif completion before song termination (n=15 birds; Tukey's HSD, P<0.05). Plotted are the mean (±SEM) percent of times motifs were completed before song termination. '*' denotes significantly different from 0 mg/kg (Tukey's HSD, P<0.05).



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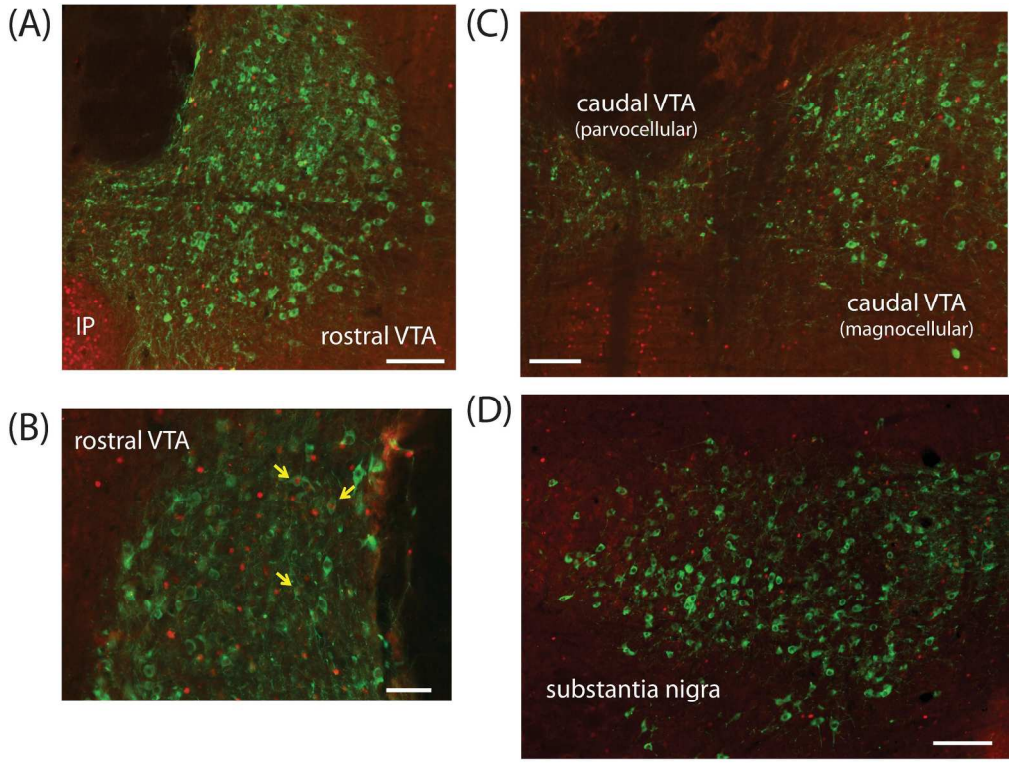

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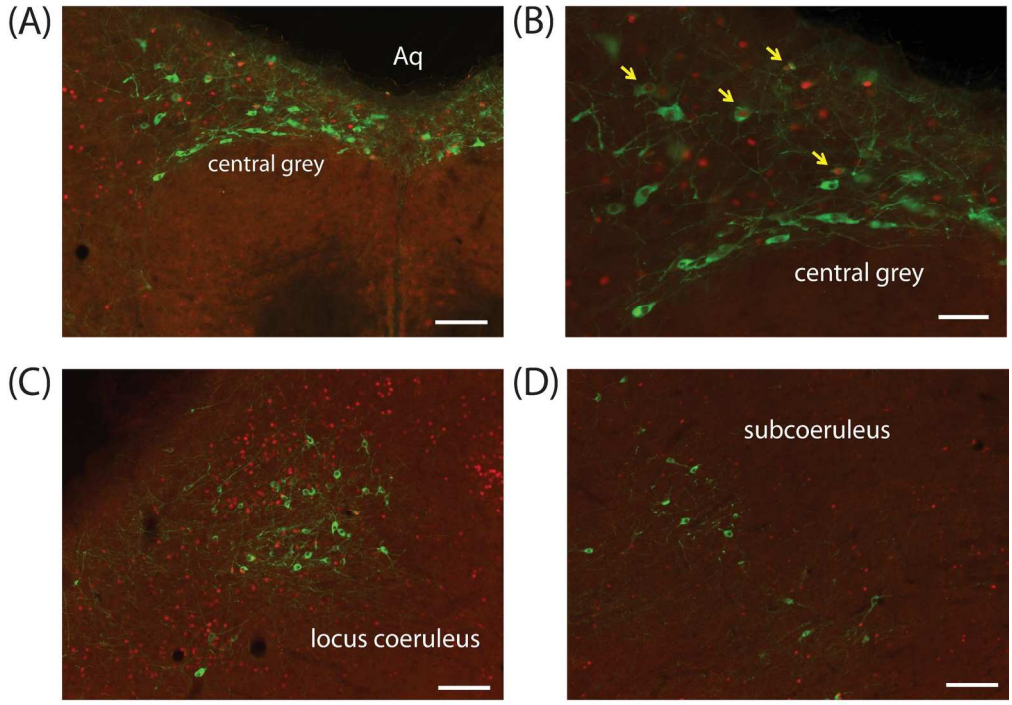

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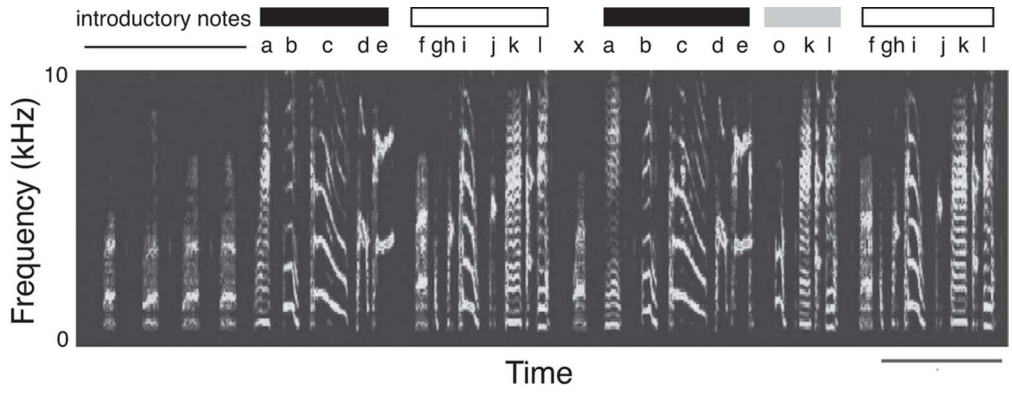

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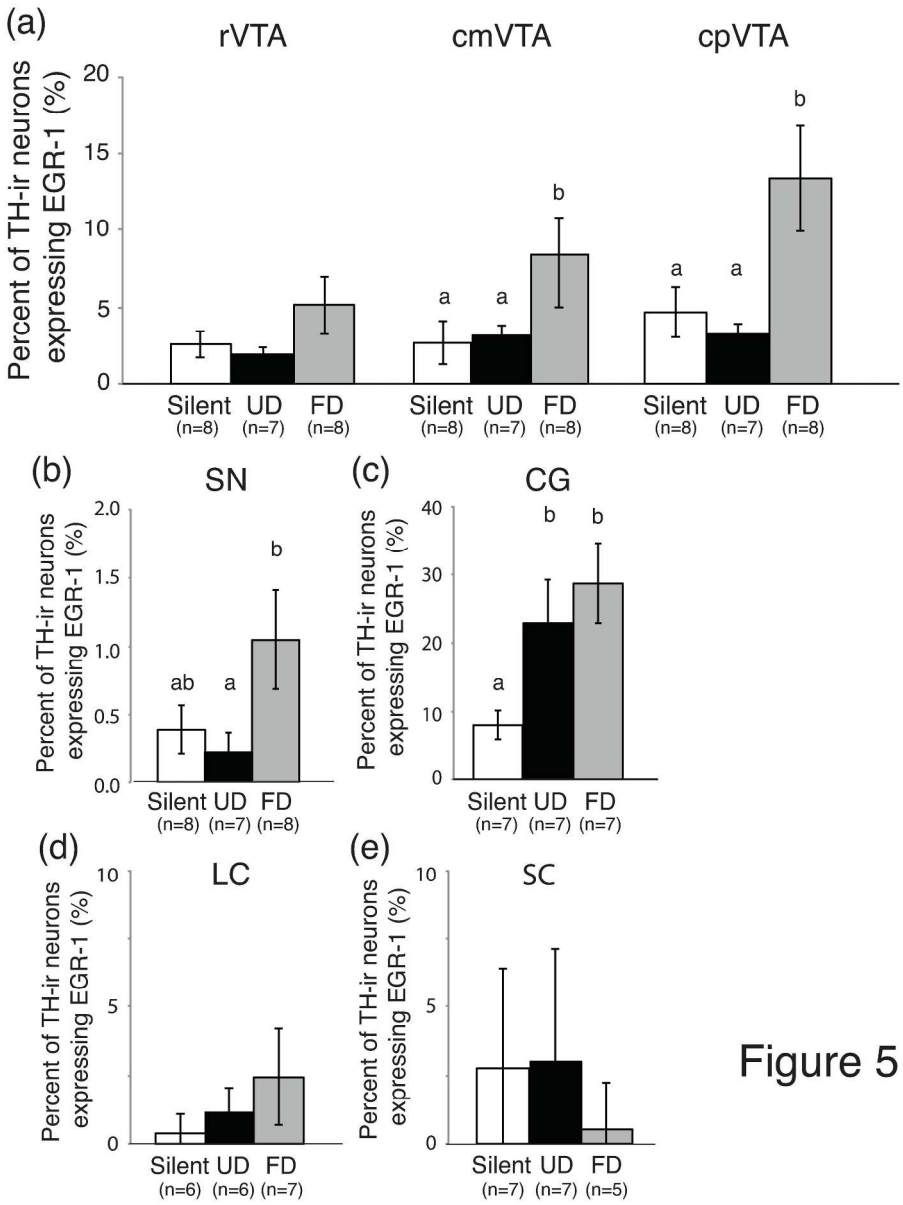

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Figure 5. Singing and social context differentially affect EGR-1 expression in catecholamine-producing neurons across midbrain areas. We measured EGR-1 expression in tyrosine hydroxylase-immunoreactive (TH-ir) neurons in Bengalese finches that were exposed to females and produced female-directed (FD) courtship song (grey bars), birds that produced undirected (UD) song in isolation (black bars), and silent birds in isolation (white bars). (a) The social context in which song is produced differentially affected EGR-1 expression in catecholaminergic neurons across subregions of the ventral tegmental area (VTA). In the caudal magnocellular and parvocellular subregions of the VTA (cmVTA and cpVTA, respectively), the proportion of TH-ir neurons expressing EGR-1 was significantly higher in FD birds than in UD and silent birds. The proportion of TH-ir neurons expressing EGR-1 in the rostral VTA was not significantly different among silent, FD, and UD birds (rVTA; $F_{2,14}=3.66$, $P=0.0528$). (b) In the substantia nigra (SN), the proportion of TH-ir neurons expressing EGR-1 was significantly higher in FD birds than in UD birds. (c) In the central grey (CG), the proportion of TH-ir neurons expressing EGR-1 was significantly higher in both FD and UD birds than silent birds, but there was no difference between FD and UD birds. (d),(e) In the locus

coeruleus (LC) and subcoeruleus (SC) EGR-1 expression in TH-ir neurons was not significantly different among FD, UD and silent birds. For all panels, plotted are the mean (+SEM) percent of TH-ir neurons expressing EGR-1, and groups that do not share letters are significantly different (Tukey's HSD, $P<0.05$). Sample sizes for each brain area are indicated below group labels on the x-axis. ±±±±±± 230x309mm (300 x 300 DPI)

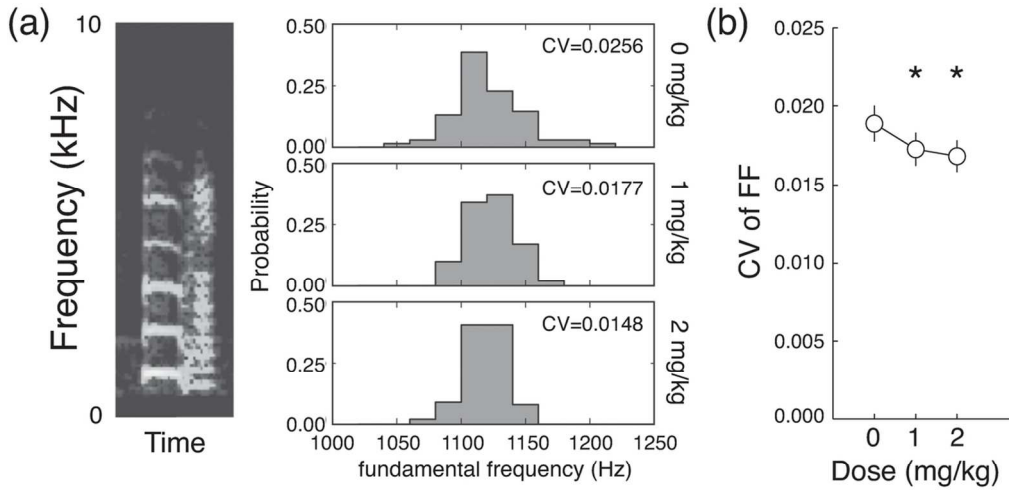

Figure 6

Figure 6. Effect of amphetamine on the stereotypy of syllable structure. (a). An example of the effect of amphetamine on syllable structure. We measured the fundamental frequency (FF) of syllables with flat, harmonic structure and computed the variability of FF across renditions of syllables. We summarized the variability using the coefficient of variation (CV: μ/σ). On the left is a spectrogram of a syllable with flat, harmonic structure, and to the right of the spectrogram are three histograms summarizing the FF of each rendition of this syllable following administration of 0, 1 and 2 mg/kg doses of amphetamine. Numbers in the top-right corners indicate the CV of the FF under each condition, and in this example, the CVs were lower following the 1 and 2 mg/kg doses of amphetamine than following the control injection. (b). Both the 1 and 2 mg/kg doses of amphetamine significantly decreased the CV of FF (n=43 syllables in 15 birds; mixed effects model; Tukey's HSD, $P<0.05$). Plotted are the mean (+SEM) CVs following 0, 1 and 2 mg/kg doses of amphetamine. '*' denotes significantly different from 0 mg/kg.
112x72mm (300 x 300 DPI)

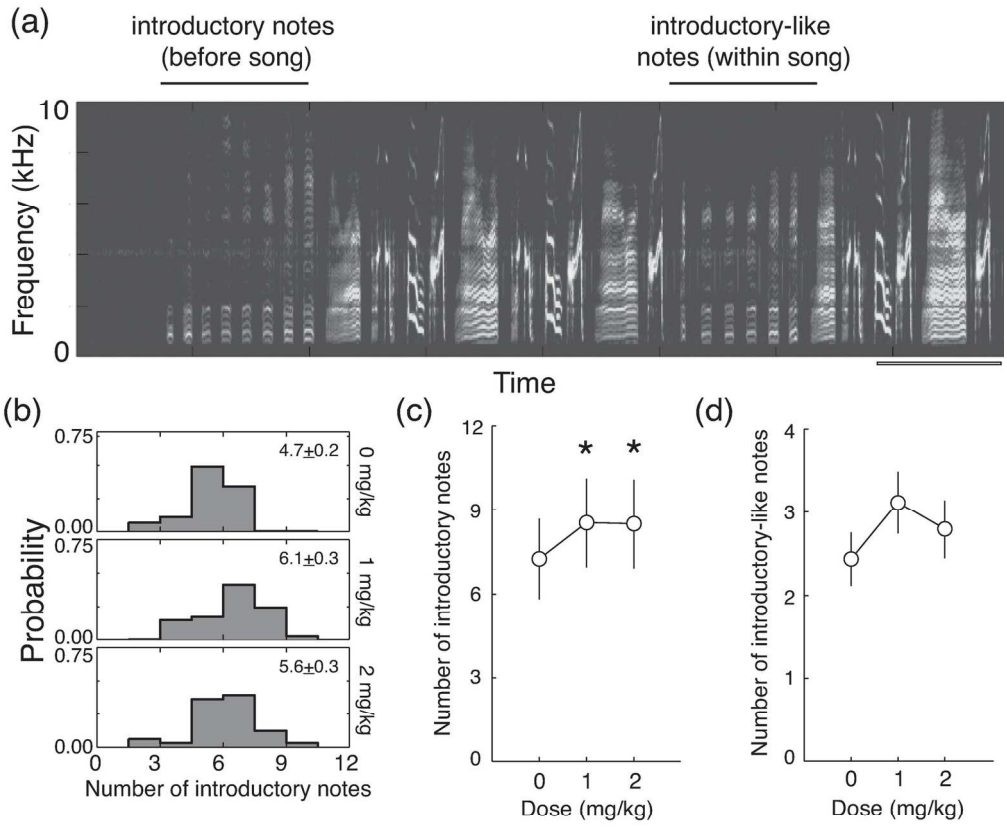

Figure 7

Figure 7. Effect of amphetamine administration on the repetition of vocal elements before and within song. (a). Spectrogram of a Bengalese finch song in which short and simple vocal elements are repeated before ('introductory notes') and within song ('introductory-like notes'). Scale bar= 500 ms. (b). An example of the effect of amphetamine administration on introductory notes. Plotted are three histograms summarizing the number of introductory notes preceding the bird's song following administration of 0, 1 and 2 mg/kg doses of amphetamine. Numbers above histograms represent the mean (+SEM) number of introductory notes under each condition, and in this example, the 1 and 2 mg/kg doses increased the number of introductory notes. (c). Both the 1 and 2 mg/kg doses significantly increased the number of introductory notes preceding song (n=15 birds; Tukey's HSD, $P<0.05$). Plotted are the mean (+SEM) numbers of introductory notes following 0, 1 and 2 mg/kg doses of amphetamine. '*' denotes significantly different from 0 mg/kg (Tukey's HSD). (d). Amphetamine administration did not significantly affect the repetition of introductory-like notes within song (n=9 birds; $P=0.06$). Plotted are the mean (+SEM) numbers of times such elements are repeated in succession following 0, 1 and 2 mg/kg doses of amphetamine.

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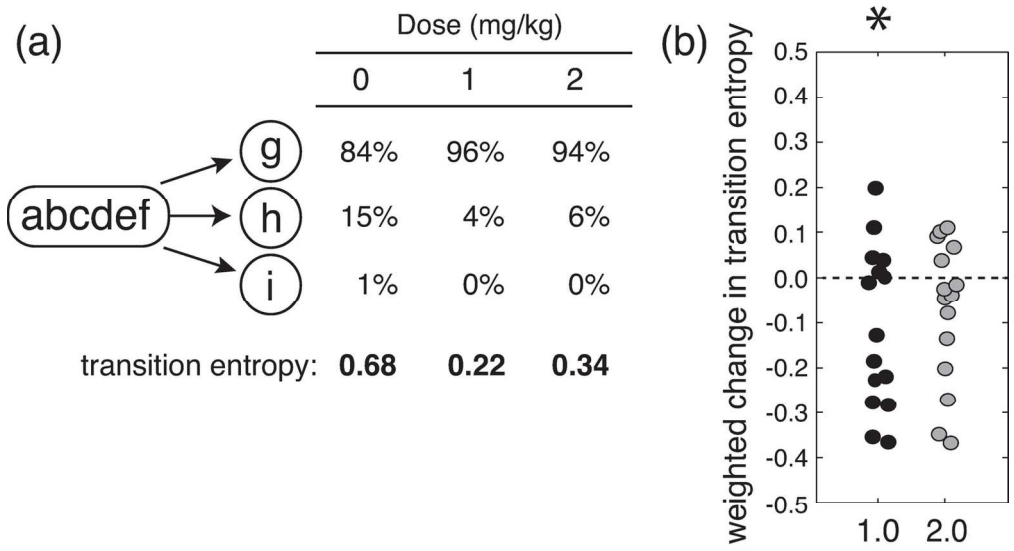

Figure 8

Figure 8. Effect of amphetamine administration on syllable sequencing at branch point sequences. (a). An example of amphetamine effects on the transition entropy of a branch point. In this example, the sequence 'abcdef' could be followed by the syllables 'g', 'h', or 'i', and listed are the transition probabilities to each syllable following administration of 0, 1 and 2 mg/kg doses of amphetamine. For each branch point we computed the transition entropy based on the probabilities of the different transitions, and in this example, the transition entropy decreased following amphetamine administration. (b). The weighted change in transition entropy was significantly less than zero following the 1 mg/kg dose (n=56 branch points from 15 birds; P=0.03), indicating that this dose of amphetamine decreased sequence variability at branch points. The weighted change in entropy following the 2 mg/kg dose was negative but not statistically significantly different than zero (P=0.08).

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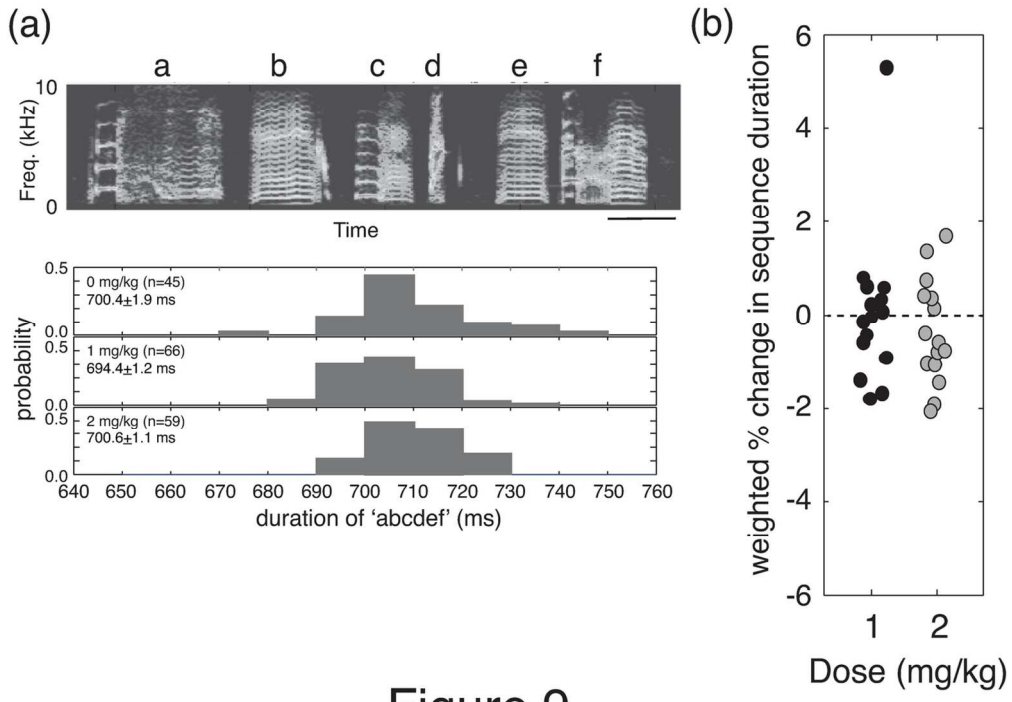

Figure 9

Figure 9. Amphetamine administration did not significantly affect song tempo. (a). An example of the lack of changes in sequence durations following amphetamine administration. Plotted is a spectrogram of the sequence 'abcdef' (top) and histograms of sequence durations for 'abcdef' (onset of the 'a' to the onset of the 'f') following the 0, 1, and 2 mg/kg doses of amphetamine (mean+SEM). Scale bar= 100 ms. (b). The weighted change in sequence durations was not significantly different than zero for both doses of amphetamine (n=19 sequences in 15 birds; P>0.20 for both).
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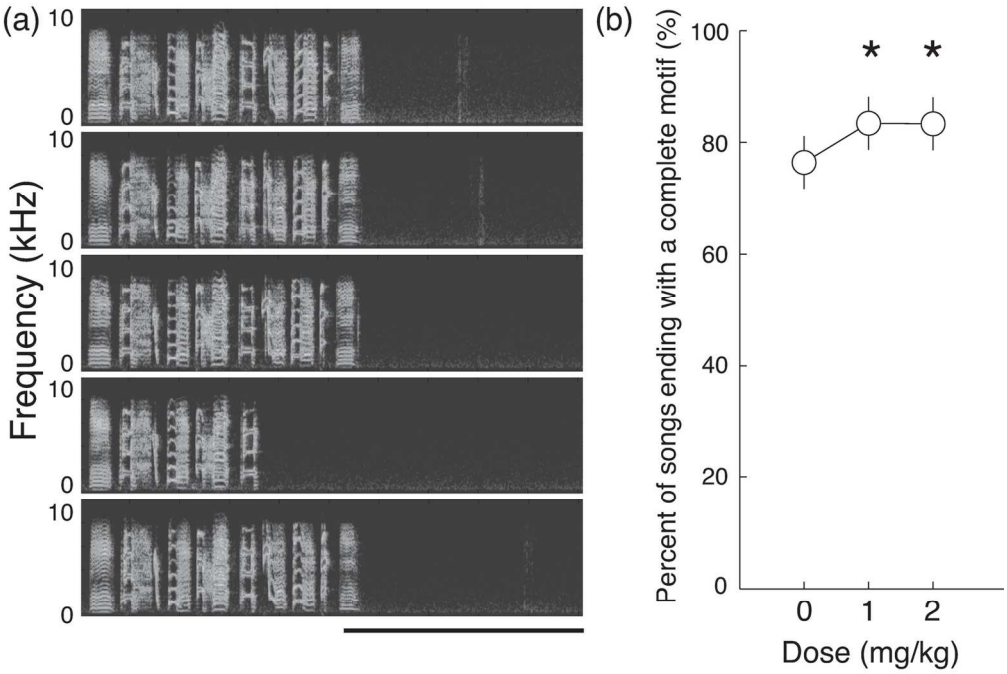

Figure 10

Figure 10. Effect of amphetamine administration on motif completions before song termination. (a). Motifs are stereotyped sequences of syllables that are usually completed before song termination. Plotted are five randomly selected renditions of the end of song from one bird. In four of the five renditions, the bird completes the motif before ending song, but on one rendition (fourth spectrogram) the bird abruptly stops singing in the middle of the motif. Scale bar= 1 sec. (b). Bengalese finches were significantly more likely to complete a motif before terminating song following both doses of amphetamine than following control injections ($F_{2,28}=4.60$, $P=0.02$). Both the 1 and 2 mg/kg doses significantly increased motif completion before song termination (n=15 birds; Tukey's HSD, $P<0.05$). Plotted are the mean (+SEM) percent of times motifs were completed before song termination. '*' denotes significantly different from 0 mg/kg (Tukey's HSD, $P<0.05$).
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