J Neurophysiol 120: 1796–1806, 2018.
First published July 11, 2018; doi:10.1152/jn.00078.2018.

RESEARCH ARTICLE | *Neural Circuits*

Vocal learning in songbirds requires cholinergic signaling in a motor cortex-like nucleus

Pavel A. Puzerey, Kamal Maher, Nikil Prasad, and Jesse H. Goldberg
Department of Neurobiology and Behavior, Cornell University, Ithaca, New York

Submitted 31 January 2018; accepted in final form 2 July 2018

Puzerey PA, Maher K, Prasad N, Goldberg JH. Vocal learning in songbirds requires cholinergic signaling in a motor cortex-like nucleus. *J Neurophysiol* 120: 1796–1806, 2018. First published July 11, 2018; doi:10.1152/jn.00078.2018.—Cholinergic inputs to cortex modulate plasticity and sensory processing, yet little is known about their role in motor control. Here, we show that cholinergic signaling in a songbird vocal motor cortical area, the robust nucleus of the arcopallium (RA), is required for song learning. Reverse microdialysis of nicotinic and muscarinic receptor antagonists into RA in juvenile birds did not significantly affect syllable timing or acoustic structure during vocal babbling. However, chronic blockade over weeks reduced singing quantity and impaired learning, resulting in an impoverished song with excess variability, abnormal acoustic features, and reduced similarity to tutor song. The demonstration that cholinergic signaling in a motor cortical area is required for song learning motivates the songbird as a tractable model system to identify roles of the basal forebrain cholinergic system in motor control.

NEW & NOTEWORTHY Cholinergic inputs to cortex are evolutionarily conserved and implicated in sensory processing and synaptic plasticity. However, functions of cholinergic signals in motor areas are understudied and poorly understood. Here, we show that cholinergic signaling in a songbird vocal motor cortical area is not required for normal vocal variability during babbling but is essential for developmental song learning. Cholinergic modulation of motor cortex is thus required for learning but not for the ability to sing.

acetylcholine; motor cortex; neuromodulation; songbird; vocal learning

INTRODUCTION

The basal forebrain cholinergic system (BFCS) is evolutionarily conserved and is implicated in sensory processing, attention, experience-dependent plasticity, and arousal (Conner et al. 2005; Coppola and Disney 2018; Hasselmo 1995; Kilgard and Merzenich 1998; Nelson and Mooney 2016; Ramanathan et al. 2009). Much less is known about BFCS projections to motor cortical areas (Ramanathan et al. 2006; Zaborszky et al. 1999). Current understanding of BFCS contribution to behavior is largely derived from studies in animals learning tasks for primary reinforcement such as food or liquid rewards (Hangya et al. 2015; Lin and Nicolelis 2008; Wilson and Rolls 1990). However, many motor skills such as speech or musical performance are driven by internal goals absent external reinforcement. Little is known about BFCS function during this type of internally guided motor learning.

Songbirds provide a tractable model system for internally guided trial-and-error learning. Like motor skills in humans, song is a complex motor sequence learned through trial and error. In the 1st month of life, juvenile zebra finches memorize a tutor song or "template." Next, they begin to practice, producing highly variable syllables akin to human babbling (Doupe and Kuhl 1999). Over several weeks, variability decreases as the bird learns to produce a highly stereotyped sequence of syllables resembling the tutor song (Tchernichovski et al. 2001). Vocal babbling in juvenile birds is characterized by highly variable syllables of different durations and sounds. Inactivation of the frontal cortical nucleus called lateral magnocellular nucleus of the anterior nidopallium (LMAN) eliminates this variability, resulting in prematurely stereotyped song (Bottjer et al. 1984; Goldberg and Fee 2011; Kao and Brainard 2006; Ölveczky et al. 2005; Scharff and Nottebohm 1991). Meanwhile, inactivation of HVC (used as a proper name), part of a separate cortical motor pathway, has little effect on juvenile song but causes adult birds with a stereotyped song to produce variable, babblelike song (Aronov et al. 2008, 2011; Chen et al. 2014).

Thus, in songbirds, there are two premotor (action-generating) circuits: LMAN actively generates trial-to-trial variability and HVC drives stereotyped vocal patterns of adults (Fig. 1*B*; Fee and Goldberg 2011; Hahnloser et al. 2002; Long et al. 2010). Both of these pathways converge via glutamatergic inputs on the robust nucleus of the arcopallium (RA), a vocal motor cortexlike region (Spiro et al. 1999). RA, which is analogous to layer 5 of primary motor cortex (Dugas-Ford et al. 2012; Pfenning et al. 2014; Wild 1993), is a major site of plasticity during song learning. Throughout the learning process, HVC axons, including those from recently born neurons, invade RA and make new synaptic connections, as LMAN axon terminals prune and become more selective (Bottjer 2004; Mooney and Rao 1994; Nottebohm 2002). The precise mechanisms that govern structural and synaptic plasticity in RA remain unclear.

Notably, a third input to RA comes from the basal forebrain cholinergic system (Fig. 1*B*; Li and Sakaguchi 1997). Cholinergic signaling in RA modulates intrinsic neuronal properties and synaptic plasticity in brain slice (Meng et al. 2017; Salgado-Commissariat et al. 2004), but its role in variability

Address for reprint requests and other correspondence: J. H. Goldberg, Dept. of Neurobiology and Behavior, Cornell Univ., W121 Mudd Hall, Ithaca, NY 14853 (e-mail: jessehgoldberg@gmail.com).

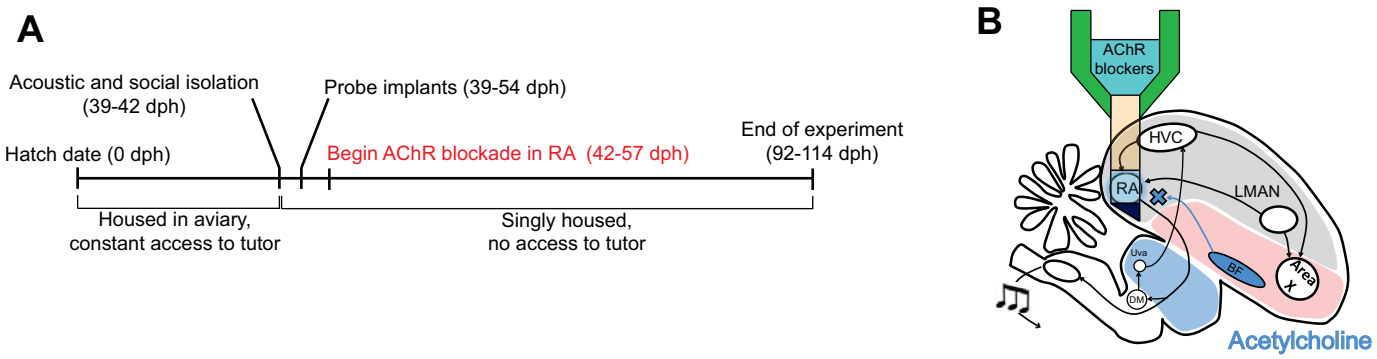

Fig. 1. Experimental timeline for acetylcholine receptor (AChR) antagonism in the robust nucleus of the arcopallium (RA) using reverse microdialysis. *A*: schematic of experimental timeline (see METHODS). *B*: schematic of the zebra finch song system. Vocal babbling is driven by lateral magnocellular nucleus of the anterior nidopallium (LMAN) inputs to RA. As song learning proceeds, premotor control is gradually transferred from LMAN to a posterior premotor nucleus HVC (used as a proper name), which drives a stereotyped vocal sequence. Basal forebrain (BF) sends cholinergic inputs to RA. We implanted miniature microdialysis probes bilaterally into RA at the onset of vocal babbling in juvenile finches and chronically infused cholinergic receptor antagonists to determine the role of cholinergic signaling in RA during vocal learning. DM, dorsomedial nucleus of the intercollicular region; dph, days posthatch; Uva, uvaeformis.

during babbling or in song learning remain unknown. Here, we use chronic pharmacological blockade of cholinergic receptors in RA across the developmental ontogeny of song to test whether cholinergic signals in RA are necessary for babbling or song learning.

METHODS

Subjects. Subjects were nine male juvenile zebra finches obtained from the Cornell University zebra finch breeding facility. All experiments were approved by the Cornell Institutional Animal Care and Use Committee. Before the start of experiments, juvenile birds were housed in a colony of conspecifics with continuous access to a tutor. Several days before surgery, juveniles were separated from the colony, placed in acoustically isolated audio recording boxes, and housed on a regular 12:12-h light-dark cycle with ad libitum access to food and water. Animals were isolated between 39 and 42 days posthatch (dph) and remained so for the entirety of the experiment (Fig. 1*A*). In the initial experimental design, ≥2 pupils were taken from each unique tutor to do side-by-side comparisons between control and treated pupils; however, given the large number of failure modes in the experiment (e.g., probes clogging and detaching from the skull), 9 of 30 implanted birds were followed to completion. Thus members of the control and drug-treatment cohorts did not always have identical tutors. Audio recordings of vocalizations were acquired continuously throughout the day. Birds were considered ready for implant surgeries after a single full day of vocal babbling in the isolation box.

Microdialysis probe implantation and use. Birds were anesthetized with 2% vapor isoflurane, and body temperature was maintained at 41°C using a homeostatic heating pad (Harvard Apparatus). Reverse microdialysis probes (Andalman and Fee 2009; Charlesworth et al. 2012) were implanted bilaterally in RA using a 60° head angle. The anteroposterior and mediolateral coordinates were −0.8 and 2.5 mm, respectively, relative to lambda, and the dorsoventral (D-V) coordinates were determined using electrophysiological mapping on the basis of strong multiunit activity, pronounced burst/pause firing patterns, and large spike widths (mean D-V = 2.21 ± 0.32 mm). Our previous studies (Goldberg and Fee 2012; Ölveczky et al. 2011) demonstrated that this path to RA avoids HVC and does not transect the HVC-RA projection. Dialysis probes were filled with phosphate-buffered saline (PBS) until the bird recovered and produced babble for ≥1 full day. For the blocked group ($n = 5$), probes were subsequently flushed and filled with a cocktail of nonselective cholinergic receptor antagonists containing scopolamine (10 mM; Tocris) to block muscarinic ACh receptors and tubocurarine (10 mM; Tocris) to block nicotinic ACh receptors. Fresh solution of cholinergic receptor antagonists was washed in every morning 1 h before lights were turned on. Chronic blockade of cholinergic receptors in RA was maintained until birds reached adulthood (92–114 dph), after which birds were euthanized and perfused with 4% paraformaldehyde solution, and their brains were sectioned to confirm probe placement in RA histologically. Control birds ($n = 4$) did not receive cholinergic receptor antagonists and instead had probes flushed daily with fresh PBS solution. Similar to the blocked group, songs of control birds were recorded until they reached adulthood (92–133 dph) and songs were fully crystallized.

Drug concentrations in the probes were determined based on principles established from previous calibration efforts. First, we (Goldberg and Fee 2012) previously recorded pallidal neurons in Area X at ever-increasing distances from the tip of the probe filled with muscimol at varying doses and determined that 1–3 mM muscimol was effective in inactivating ~1 mm^3 of tissue. This was ~500 times the dose necessary to achieve this effect by direct injection or in bath-applied brain slice. A similar approach was taken to assess the concentration of DL-amino-5-phosphonopentanoic acid (AP-V) necessary to infuse through the probes into RA that would block LMAN stimulation-induced transients (LMAN activates *N*-methyl-D-aspartate receptors on RA neurons) and to assess concentration of TTX to block activity in LMAN, as read out by reduction in song variability (Andalman and Fee 2009). About 5 mM AP-V was necessary to block LMAN stimulation-induced bursting in RA neurons, and about 25 μM TTX was necessary to abolish variability in singing, again orders of magnitude higher concentration than necessary in bath-applied brain slice. Other groups have adhered to these principles: Charlesworth et al. (2012) also used 1–5 mM AP-V in RA; Dölen et al. (2013) used these probes to block oxytocin receptor signaling in nucleus accumbens and used a concentration of the oxytocin receptor antagonist L-368,899 of 10 mM in the probes, again ~500-fold what is necessary to block those receptors by direct injection. In the present study, we adhered to these principles. We used concentrations in the probes (10 mM) that were ~500-fold higher than what is necessary to block cholinergic signaling in brain slice (Higley et al. 2011; Konopacki et al. 1992). Of course, we cannot rule out the possibility that we affected cholinergic signaling outside of RA. We note that choline acetyltransferase fiber density is profoundly higher in RA than in surrounding neuropil, suggesting that cholinergic innervation within the arcopallium is especially focused on the RA (Sakaguchi and Saito 1991).

Song analysis. All vocalizations were recorded in a sound isolation box using Sound Analysis Pro software (Tchernichovski et al. 2000). Audio recordings were manually inspected, and files containing calls or cage noise were excluded from analysis. Calls were discerned from

song bouts on the basis of being highly stereotyped and occurring in sparse succession, typically separated from other vocalizations by hundreds of milliseconds. Song bouts normally exhibit short gaps of silence between vocal elements (20–40 ms) and consist of clusters of 3–6 syllables. Song-related vocalizations (babbling, plastic song, or crystallized song) were segmented to identify the onsets, offsets, and durations of individual syllables and the gaps of silence between them using custom-written MATLAB software, as described earlier (Goldberg and Fee 2011). To assess changes in song stereotypy following acute cholinergic blockade and across the course of development, we employed three separate measures of song variability (Goldberg and Fee 2011). First, we used an entropy-based metric to analyze the variability (V^e) of syllable duration distributions. Because syllable duration distributions in juvenile birds are exponentially distributed (Aronov et al. 2011), we computed the log (base 10) of syllable durations, and binned the distribution evenly in log bins of 0.05 (n = 50 bins) between −2.5 (0.00316 s) and 0 (1 s) in log units. We then generated a probability density function, P_i, of syllable durations by normalizing the histograms by the sum of all bins. The sum of all values across all bins equals to 1. The entropy-based measure of variability was then computed as:

$$V^e = \frac{\sum_1^n P_i \log(P_i)}{\log(n)}.$$

V^e values close to 1 correspond to syllable durations being distributed across all bins (maximal variability), whereas values close to 0 correspond to high stereotypy where syllable durations are concentrated in a single bin. The same entropy-based analysis was performed on the distribution of silent gap durations. We also measured the variability index of syllable durations in adults using linear binning since adult songs do not have exponentially distributed syllable durations (Aronov et al. 2011). We also quantified song rhythmicity by taking the peak of the normalized power spectrum of sound amplitude beyond a 3-Hz cutoff for juvenile birds and an 8-Hz cutoff for adult birds, as reported previously (Goldberg and Fee 2011; Saar and Mitra 2008). Because rhythm analysis does not require syllable segmentation, it provides an unbiased measure for the periodicity of song-related vocalizations. Additionally, we used Sound Analysis Pro (Tchernichovski et al. 2000) to analyze frequency modulation, a measure of syllable complexity that has previously been used to quantify impairments associated with lesions and acoustic isolation (Fehér et al. 2009; Simpson and Vicario 1990).

Tutor similarity analysis. To compare subjects' adult songs to the songs of their tutors, we used a previously described method for measuring tutor imitation accuracy (Mandelblat-Cerf and Fee 2014). This algorithm generates a tutor imitation score, which is computed from a product of acoustic and sequence similarity scores. Acoustic similarity scores are determined by first representing songs with a set of acoustic features obtained from the power spectrum of the audio signal. The acoustic features used for this analysis are: pitch, pitch goodness, Wiener entropy, frequency modulation, gravity center, and spectral width. Similarity in acoustic space is then computed by measuring the Euclidean distance between the song feature vectors of tutor and pupil songs. The tutor song is treated as a sequence of discrete syllable segments, whereas the pupil song is treated as a single continuous stream of sound, which is at least twice the length of the tutor song sequence. The algorithm works by finding the best match within the pupil song (i.e., one that minimizes acoustic distance in feature space) to an individual tutor syllable. Sequence similarity is computed by comparing the acoustic similarity of syllables in the pupil song that follow segments having high acoustic similarity. For instance, if a segment of a pupil song has high acoustic similarity to *tutor syllable a*, then the sequence similarity score is high if the next segment of the pupil song also has high acoustic similarity to *tutor syllable b*.

Ontogeny analysis. We compared day-to-day syllable duration distributions using custom MATLAB code for song detection in which song was defined as a cluster of no less than 3 consecutive syllables separated by no more than 250–300 ms of silence. Peaks within the syllable duration distributions were detected using the MATLAB findpeaks function. Syllable duration distributions were constructed with 2-ms bin size smoothed with a 7-bin sliding window. Singing quantity was estimated by taking the daily sum of all syllable durations. For peak analysis, syllable duration distributions were taken as moving average of a 10-day window centered on a given day of interest. Because individual birds sang for a variable number of days in our experiments, we limited the peak analysis to only the 1st 47 days of singing after probe implant (Fig. 5*C*).

RESULTS

To determine the contributions of cholinergic signaling in RA to vocal learning, we used reverse microdialysis to block cholinergic receptor signaling in RA across song development (implant age = 43 ± 5 dph, n = 9 birds). Birds received regular exposure to a conspecific tutor until probe implantation, which was performed shortly after the onset of vocal babbling. We established two experimental groups: the blocked group (n = 5) had probes regularly flushed with a cocktail of nonselective nicotinic and muscarinic cholinergic receptor antagonists dissolved in saline solution (see METHODS), whereas the control group (n = 4) received a saline solution alone. We quantified the impact of acute cholinergic blockade on singing in the days immediately following pharmacological blockade as well as the long-term effects on vocal learning (block duration = 55 ± 15 days, n = 5 birds).

Chronic cholinergic blockade in RA did not impair vocal babbling in juvenile songbirds. To test the acute effects cholinergic blockade in RA on babbling, we recorded the first postoperative songs following bilateral probe implants in RA. Birds started to sing 2–24 days following probe implantation (latency to sing following probe implant = 6 ± 7 days, n = 9 birds). On the morning following the 1st full day of postoperative singing, juveniles in the blocked group received a cocktail of nonselective muscarinic and nicotinic ACh receptor antagonists (see METHODS). Most blocked birds began to produce subsong vocalizations on the same day of treatment (latency range: 0–1 days, mean latency = 0.2 ± 0.5 days, n = 5 blocked birds). We quantified the acute impact of cholinergic blockade on singing in four ways. First, we segmented individual song syllables and compared the distributions of syllable durations and the durations of the silent gaps between them in the day(s) immediately before and after drug wash-in (Aronov et al. 2011; Goldberg and Fee 2011). Second, we analyzed song rhythmicity using an unbiased metric of song temporal structure (Saar and Mitra 2008). Third, we used an entropy-based measure of song variability, V^e, to compare the stereotypy in syllable and gap durations before and after cholinergic block. V^e values close to 1 reflect a broad spread of syllable and gap durations across a range of values (high variability), whereas V^e values close to 0 reflect clustering around a single value (high stereotypy; Goldberg and Fee 2011). Finally, we examined song acoustic structure by examining the frequency modulation of song syllables (Fehér et al. 2009; Tchernichovski and Mitra 2002).

We analyzed the syllable duration distributions in the days immediately preceding cholinergic block and in the days im-

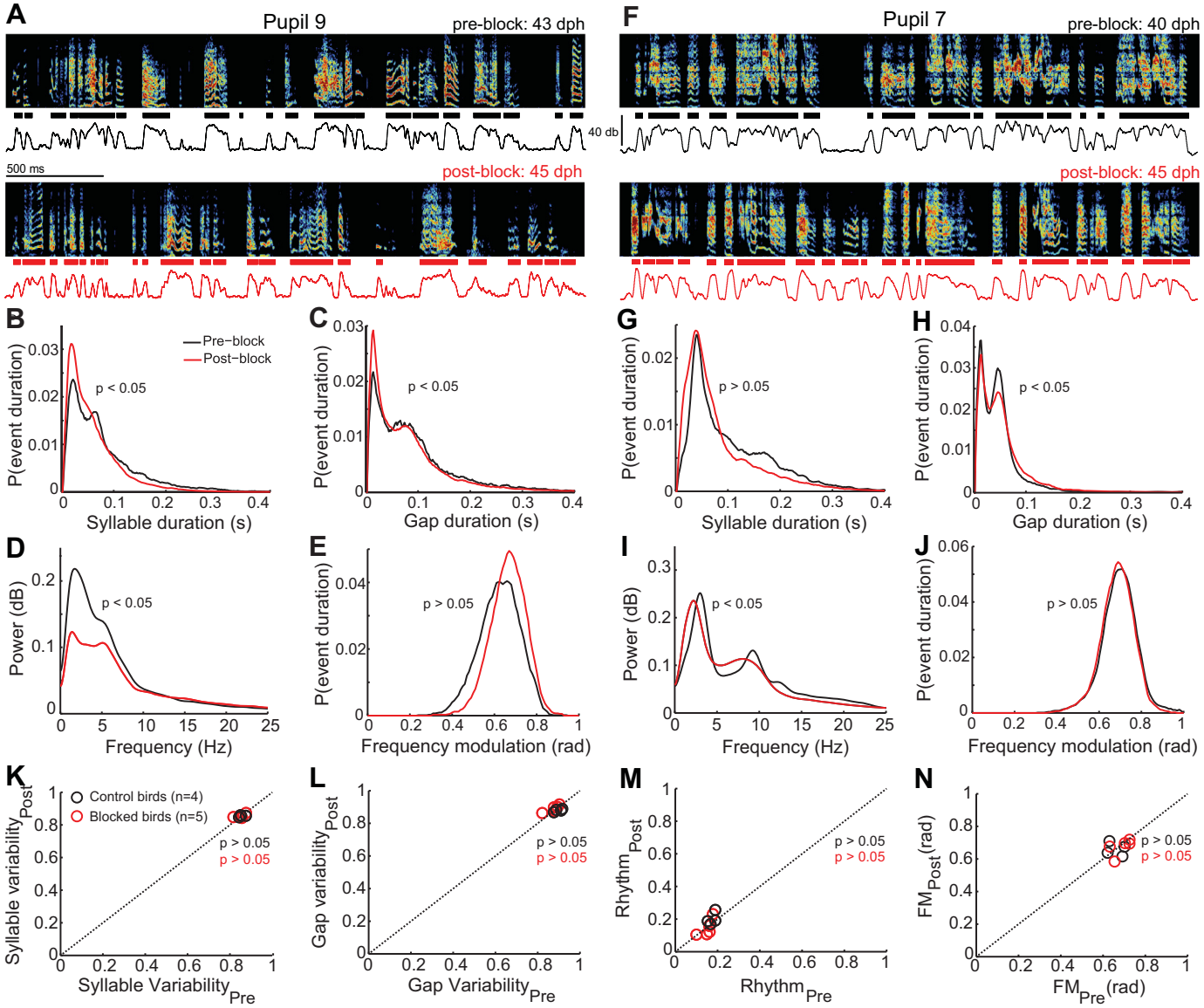

Fig. 2. Acute effects of cholinergic receptor blockade in robust nucleus of the arcopallium (RA) on vocal babbling. *A*, top to bottom: example of subsong spectrogram, corresponding sound amplitude trace, and segmented subsong syllables from a juvenile bird before (black) and after (red) acute cholinergic receptor blockade in RA. *B* and *C*: normalized syllable (*B*) and gap (*C*) duration distributions before and after block. Note the preservation of the distributions of syllable and gap durations before and after cholinergic blockade. *D*: normalized power spectra of sound amplitude from subsong vocalizations before and after block cholinergic block. *E*: normalized distribution of frequency modulation values quantified from 500 randomly selected syllables taken before and after acute cholinergic blockade. Data in *B–E* are from the pupil whose spectrograms are plotted in *A*. *F–J*: data are plotted as in *A–E* for an additional example cholinergic-blocked bird. All *P* values in *B–E* and *G–J* were obtained using Kolmogorov-Smirnov test for statistical differences between distributions. *K–N*: population analysis from all pupils during the acute phase of cholinergic block in RA. *K* and *L*: entropy-based measure of syllable (*K*) and gap (*L*) duration variability (see METHODS) before and after acute block shown here as scatter plots. *M*: scatter plots of song rhythm (see METHODS) taken before and after cholinergic blockade in blocked pupils and roughly age-matched control. *N*: median values of frequency modulation (FM) distributions of individual pupils like those shown in *E* and *J* shown here as scatter plots for comparison of pre- and postblock conditions. Color-matched *P* values in *K–N* correspond to the within-cohort differences before and shortly after acute cholinergic blockade in RA and were obtained using Wilcoxon rank sum test. dph, Days posthatch.

mediately following block. The majority of blocked birds showed no significant changes in syllable duration distributions of subsong vocalizations following acute cholinergic block ($n = 3/5$; Kolmogorov-Smirnov test, $P > 0.05$; Fig. 2, *B* and *G*). Furthermore, we observed no significant differences in the syllable duration variability metric V^e immediately before and after blockade ($V^e_{pre} = 0.85 \pm 0.02$, $V^e_{post} = 0.85 \pm 0.01$; $P > 0.05$, Wilcoxon rank sum test; $n = 5$ blocked birds; Fig. 2*K*). We also compared syllable duration distributions in control birds during developmental stages roughly age-matched to the acute effect period of blocked birds ($blocked_{pre} = 46.3 \pm 4.9$ dph, $control_{pre} = 47.6 \pm 5.8$ dph; $blocked_{post} = 49.2 \pm 5.5$ dph, $control_{post} = 54.8 \pm 9.6$ dph). Three out of four control birds showed no significant differences in syllable duration distributions (Kolmogorov-Smirnov test, $P > 0.05$). Furthermore, we analyzed the variability of syllable durations across the same acute period in control birds and found no significant differences ($V^e_{pre} = 0.86 \pm 0.01$, $V^e_{post} = 0.85 \pm 0.01$; $P > 0.05$, Wilcoxon rank sum test; $n = 4$ blocked birds; Fig. 2*K*).

To determine whether cholinergic blockade had an impact on the timing of the silent gaps between syllables, we analyzed gap duration distributions and found small but significant differences in their distributions before and immediately after cholinergic blockade ($n = 5/5$ blocked birds; Kolmogorov-Smirnov test, $P < 0.05$; Fig. 2, *C* and *H*). Similar effects were observed in the control birds ($n = 4/4$ control birds; Kolmogorov-Smirnov test, $P < 0.05$; Fig. 2*L*), reflecting the significant day-to-day changes in gap duration distributions in normal birds at this age. We then used a variability metric, V^e, to determine whether the stereotypy of gap durations changed before and after acute blockade. This metric was previously shown to be sufficiently sensitive to resolve variability reductions following lesion of LMAN or its thalamic inputs (Goldberg and Fee 2011). We show no significant differences between the pre- and postblock V^e values for gap durations ($V^e_{pre} = 0.\ 0.88 \pm 0.03$, $V^e_{post} = 0.89 \pm 0.02$; $P > 0.05$, Wilcoxon rank sum test; $n = 5$ blocked birds; Fig. 2*L*).

A key feature of vocal learning in zebra finches is the increase in rhythmicity of song temporal structure that parallels the emergence of stereotyped syllable sequences. To measure the effects of acute cholinergic block on song rhythm, we used a previously developed metric that measures song rhythm as the maximal value of the normalized power spectrum of sound amplitude (see METHODS; Saar and Mitra 2008). Acute cholinergic blockade in RA did not result in systematic differences in song rhythm in the pre- and postblock conditions ($rhythm_{pre} = 0.15 \pm 0.03$, $rhythm_{post} = 0.14 \pm 0.05$; $P > 0.05$, Wilcoxon rank sum test; $n = 5$ blocked birds; Fig. 2, *D*, *I*, and *M*).

We next examined the effect of cholinergic blockade on the acoustic structure of song syllables. Normal zebra finch song syllables exhibit substantial time-dependent modulation of syllable pitch (i.e., frequency modulation, FM; Fehér et al. 2009). Cholinergic blockade did not have a significant acute effect on the distributions of frequency modulation in juvenile birds ($n = 5/5$ blocked birds; Kolmogorov-Smirnov test, $P > 0.05$; Fig. 2, *E* and *J*) or their centers of mass (median $FM_{pre} = 0.69 \pm 0.04$, median $FM_{post} = 0.67 \pm 0.05$; $P > 0.05$, Wilcoxon rank sum test; $n = 5$ blocked birds; Fig. 2*N*). Together, these findings demonstrate that acute cholinergic blockade in RA did not abolish vocal production and had minimal impact on the timing, variability, rhythm, and spectral features of vocalizations during vocal babbling that was indistinguishable from the effects observed in control birds over comparable developmental timeframes.

Chronic cholinergic blockade in RA disrupts tutor song imitation. To test whether ACh signaling in RA is necessary for song learning, we chronically blocked cholinergic receptors in RA throughout the entirety of sensorimotor period for song learning in the same cohorts of birds described in the previous sections. A standard measure of song learning in zebra finches is the accuracy with which they imitate their tutor's song. We performed tutor similarity analysis using a previously developed algorithm that measures song similarity on the basis of acoustic features and syllable sequencing (see METHODS; Mandelblat-Cerf and Fee 2014). Unlike the control birds that sang species-typical adult songs, blocked birds produced profoundly abnormal songs (Fig. 3, *A* and *B*). Adult songs exhibited significantly lower imitation of the tutor than their control counterparts ($P < 0.05$, Wilcoxon rank sum test; $n = 5$ blocked birds, $n = 4$ control birds; Fig. 3*C*).

We next tested whether learning deficits observed in blocked birds were associated with reduced quantity of singing across song ontogeny. Although both groups increased song production in the 1st 2 wk of singing after probe implant, blocked birds sang substantially less in the following weeks compared with control birds (Fig. 3*D*), and the songs of two blocked birds became so abnormal at 70 and 73 dph, 25 and 29 days following drug infusion, respectively, that we could no longer classify their vocalizations as singing. We plotted the change in acoustic similarity score as a function of the actual time spent singing and found that, whereas control birds expectably trended better with practice, blocked birds actually degraded with practice (Fig. 3*E*).

Tutor similarity scores, however, provide only a "snapshot" of imitation quality, and this metric does not reveal specifically what aspects of song are abnormal. In the following sections, we provide a more thorough quantitative characterization of song abnormalities that followed chronic cholinergic receptor blockade in RA.

Retained vocal variability, reduced rhythm, and abnormal syllable acoustic structure following chronic cholinergic blockade in RA. Striking differences between the songs of blocked birds and controls were apparent from the spectrograms of adult song (Fig. 4*A*). The first observable difference was a lack of stereotyped syllable durations in blocked birds. To quantify this, we compared the distributions of syllable durations from songs recorded in the last stages of learning in young adults. As expected, control birds developed stereotyped syllable sequences that corresponded to syllable duration distributions with multiple prominent peaks (Fig. 4*B*). However, the syllables of blocked birds exhibited duration distributions with few peaks and shapes that more closely resembled the distributions of juvenile birds (Fig. 4*B*; see Fig. 2, *B* and *G*, for juvenile distributions; Aronov et al. 2011; Tchernichovski et al. 2001). We compared the syllable duration variability index, V^e, in adult birds and found that blocked birds had significantly higher V^e values than controls ($V^e_{control} = 0.58 \pm 0.04$, $n = 4$ control birds; $V^e_{block} = 0.66 \pm 0.03$, $n = 5$ blocked birds; $P < 0.05$, Wilcoxon rank sum test; Fig. 4*E*), consistent with higher variability (i.e., lower stereotypy) of syllable durations in blocked birds.

Another useful benchmark to assess the progress of song development is song rhythmicity. Adult male zebra finches learn to sing rhythmic motifs in which syllable onsets are spaced by ~100 ms, producing an ~10-Hz song rhythm (Saar and Mitra 2008). Control adult songs exhibited prominent peaks at frequencies corresponding to the rhythmicity of typical adult song, whereas blocked birds showed diminished power in this band (Fig. 4*C*). Thus we compared the rhythm of adult songs, taken as the peak of the power spectrum of sound amplitude above an 8-Hz cutoff. We found that blocked birds had significantly reduced rhythm compared with controls (rhythm in controls $= 0.21 \pm 0.07$, $n = 4$; rhythm in blocked birds $= 0.09 \pm 0.04$, $n = 5$; $P < 0.05$, Wilcoxon rank sum test; Fig. 4*F*).

Finally, on inspecting the spectrograms, we noticed that the syllables of blocked adults exhibited an unusual preponderance of poorly modulated harmonic syllables, suggestive of de-

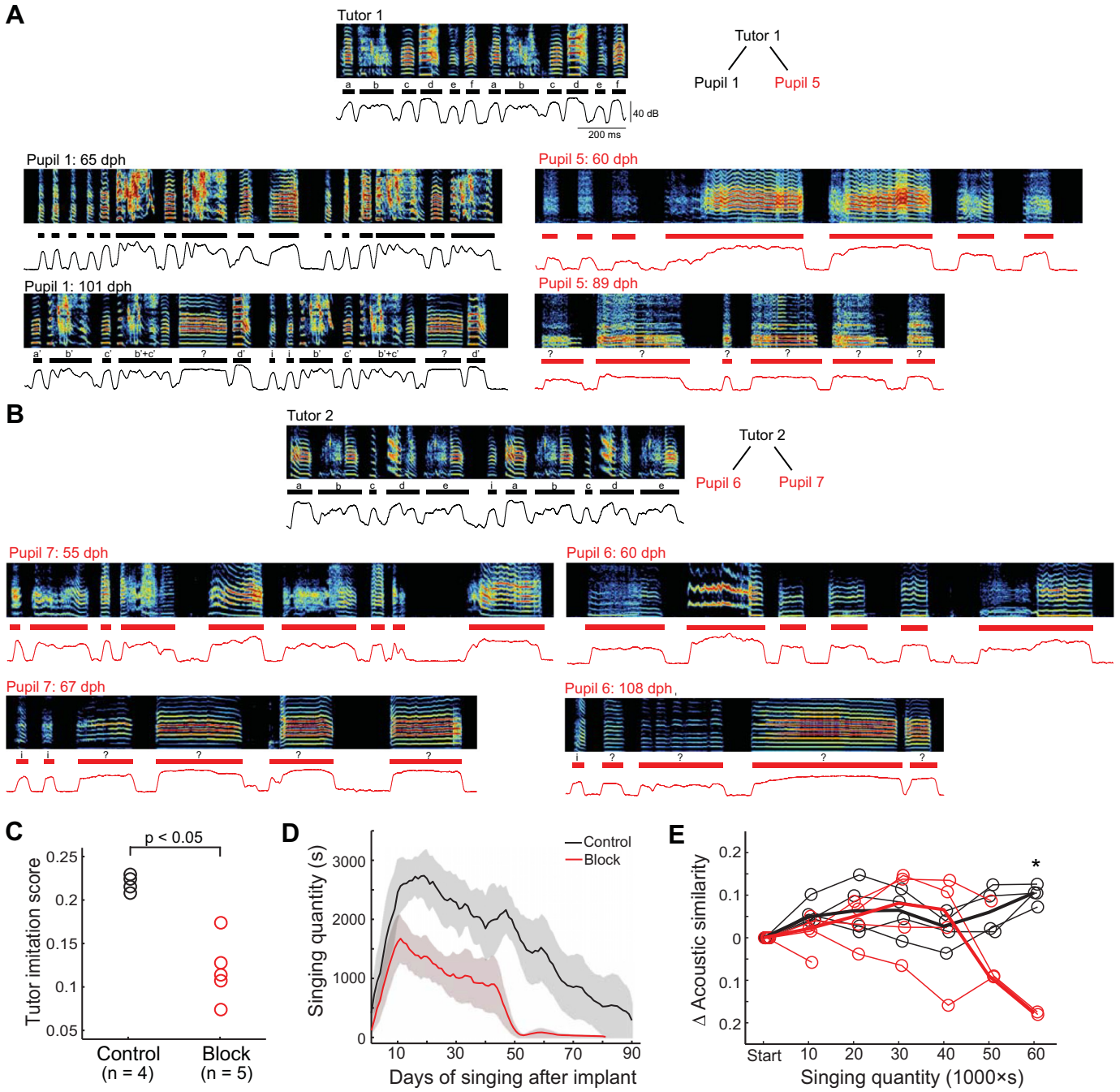

Fig. 3. Cholinergic blockade in robust nucleus of the arcopallium impairs tutor song imitation. *A*: song spectrograms, sound amplitude, and segmented syllables of a tutor song and 2 of its pupils, 1 control and 1 blocked. Spectrograms are shown for both intermediate (top pupil spectrogram) and end stages (bottom pupil spectrogram) of the experiment. The example control bird sang a stereotyped syllable sequence with some syllables that resembled the tutor song (marked with '). The song of the blocked pupil exhibited little semblance to the tutor song and had no recognizable tutor song syllables or reliable sequencing of specific syllables. *B*: data plotted as in *A* for an additional tutor and its 2 blocked pupils. *C*: scatter plots of tutor imitation scores in control and blocked (black and red, respectively) birds taken at the end of the experiment for all birds. Significance testing was performed using Wilcoxon rank sum test. *D*: developmental timeline of population averages of singing quantity, taken as the total sum of syllable durations per day, smoothed across a 7-day window. Solid lines correspond to the cohort means, and transparent patches correspond to means ± SE. *E*: scatter plots of change (Δ) in acoustic similarity score as a function of the quantity of singing for each bird. Δ Acoustic similarity is taken as the difference between scores at the start of the experiment (before drug infusion) and at elapsed amounts of singing indicated. Thin lines connect scores of the same bird taken at different times, and thick lines connect distribution medians for each cohort. Asterisks designate similarity scores at singing amount quantity with statistical significance of $P < 0.05$ using a Student's *t*-test. dph, Days posthatch.

creased FM previously reported in isolate birds and in birds with partial RA lesions (Fehér et al. 2009; Simpson and Vicario 1990). We analyzed 500 randomly selected adult syllables from each bird and observed that blocked birds exhibited song syllables with significantly reduced frequency modulation (median $FM_{controls} = 0.63 \pm 0.02$ rad, median $FM_{blocked} = 0.52 \pm 0.1$ rad; $P < 0.05$, Wilcoxon rank sum test; Fig. 4, *D* and *G*).

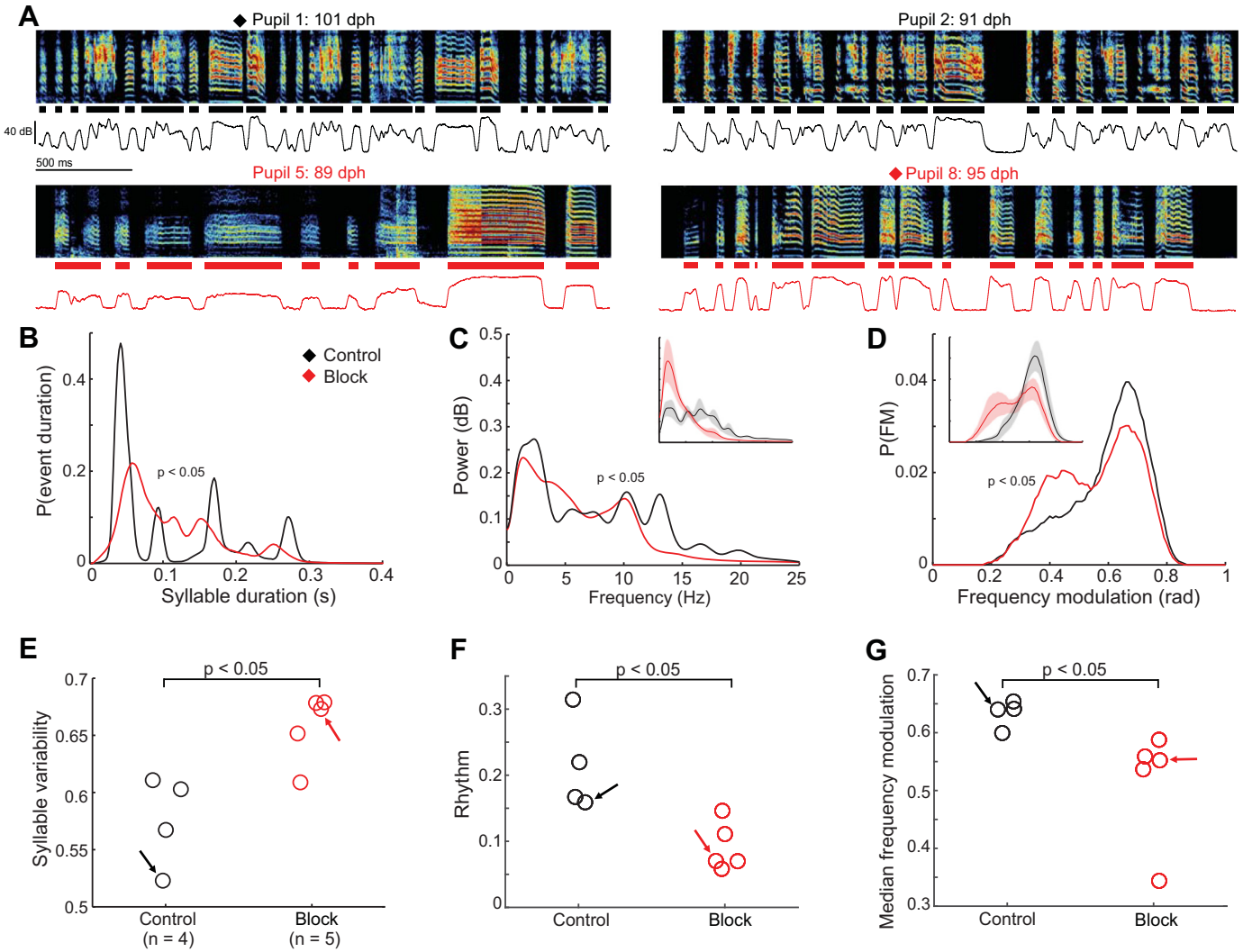

Fig. 4. Retained vocal variability and abnormal syllable structure following cholinergic blockade in robust nucleus of the arcopallium (RA). *A*, top to bottom: examples of adult song spectrograms, corresponding smoothed sound amplitude traces, and segmented song syllables from 2 control birds (top spectrograms) and 2 birds who were subjected to chronic blockade of cholinergic receptors in RA (bottom spectrograms). *B*: normalized histograms of syllable duration distributions from example birds shown in *A* (spectrograms labeled with black and red diamonds correspond to birds shown in plots *B–D*). Note the presence of prominent peaks in the syllable duration distribution of control bird (black), corresponding to unique and repeatable syllables within the adult song, that are largely absent in the distribution of the blocked bird (red). Distribution of the blocked bird has a qualitative appearance similar to the syllable duration distributions of juvenile birds (see Fig. 2, *B* and *G*). *C*: normalized power spectra of smoothed sound amplitude from the example control and blocked birds from *B*. Note the presence of prominent peaks beyond 8 Hz in the control bird (black) that are largely absent in the blocked bird (red). *Inset*: power spectra of control (black) and blocked (red) birds (mean ± SE; $n = 4$ control birds; $n = 5$ blocked birds). *D*: normalized distributions of frequency modulation (FM) values quantified from 500 randomly selected syllables taken from the birds plotted in *B*. *Inset*: average FM distributions across all birds. Note the lower FM values in blocked birds, consistent with the high proportion of unmodulated harmonic stacks seen in the spectrograms of blocked birds in *A*. All *P* values in *B–D* were obtained using Kolmogorov-Smirnov test for statistical differences between distributions. *E–G*: population analysis of adult songs from all birds. *E*: scatter plots of entropy-based syllable duration variability (*E*), adult song rhythm values (*F*), and frequency modulation shown (*G*). Significance testing in *E–G* was performed using Wilcoxon rank sum test. dph, Days posthatch.

Song development is associated with the emergence of new syllables, detectable as distinct peaks in syllable duration distributions (Aronov et al. 2011; Tchernichovski et al. 2004). We reasoned that if the production of abnormal songs in blocked birds was due to degradation after a normal phase of song development, then we would observe the emergence of distinct peaks in syllable duration distributions followed by their disappearance. Alternatively, if cholinergic blockade in RA impairs the process of song development, normal milestones such as the acquisition of protosyllables of stereotyped durations and their subsequent splitting would be blocked (Okubo et al. 2015; Tchernichovski et al. 2001). To distinguish between these possibilities, we examined the entire song ontogeny in blocked and control birds by examining daily syllable duration distributions across song development (Sasahara et al. 2015). As previously reported, in normal birds, we observed syllable splitting and differentiation into multiple peaks within the syllable duration distribution (Fig. 5, *A* and *C*). Blocked birds did not exhibit syllable splitting as control birds did, showing developmental trajectories characterized by a single prominent peak in the syllable duration distribution (Fig. 5*B*). In some instances, emergence followed by dissipation of a second peak was observed (Fig. 5*B*, *bottom*).

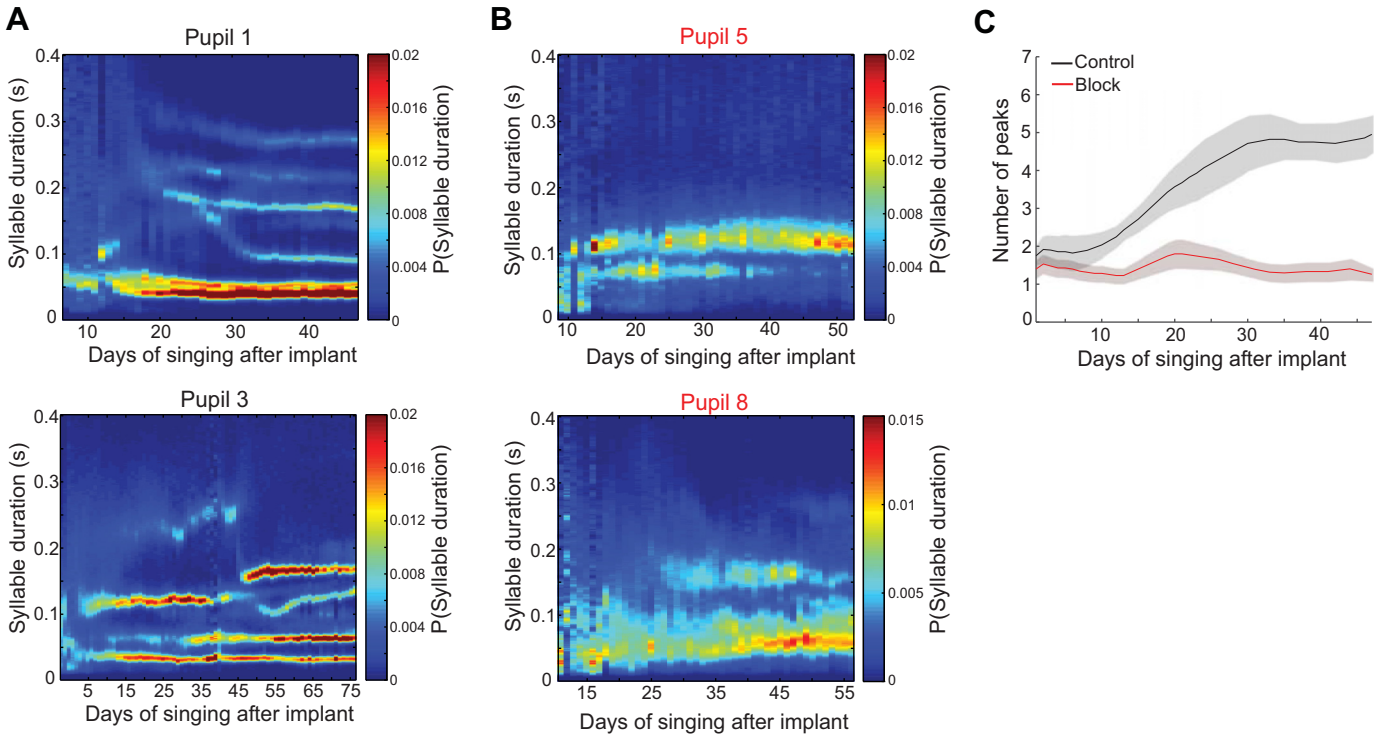

Fig. 5. Cholinergic blockade in robust nucleus of the arcopallium reduces syllable splitting and song production during song ontogeny. *A* and *B*: examples of ontogeny plots of syllable duration distributions from 2 control pupils (*A*) and 2 blocked pupils (*B*). Each column corresponds to syllable duration distributions obtained from a single day of singing, and each row represents the normalized probability of occurrence of a syllable with a given duration. Note the presence of syllable splitting in control birds and the absence or failure of splitting in blocked birds. *C*: developmental timeline of population averages of the number of peaks in the syllable duration distributions.

In summary, these results demonstrate that chronic cholinergic blockade in RA across the early development resulted in profound disruption of song production and learning. Blocked birds sang less, exhibited abnormal songs with variable syllable durations, reduced rhythmicity, altered acoustic structure, and impaired tutor song imitation.

DISCUSSION

Here, we show that cholinergic signaling in the vocal motor cortical nucleus RA is required for normal song learning but not for the production of vocal babbling. These results are consistent with a role for acetylcholine in guiding the maturation of a functional RA circuit to control song learning.

RA is an important site of plasticity during song learning. At the onset of subsong, HVC axons remain largely restricted to dorsal RA, and learning is associated with an invasion of HVC axons into RA as premotor control of RA activity, and thus song, gradually transfers from LMAN to HVC (Mooney and Rao 1994). Thus a major question is how HVC neurons precisely select which RA neurons to wire up with during development. One idea is that LMAN provides RA with a premotor signal that biases the song toward the improved (e.g., tutor memory-matching) performance and that this bias is consolidated by heterosynaptic Hebbian mechanisms and/or structural plasticity specifically in the HVC-RA pathway (Andalman and Fee 2009; Charlesworth et al. 2012; Fee and Goldberg 2011; Garst-Orozco et al. 2014; Warren et al. 2011). Both Hebbian plasticity and axonal wiring can be modulated by neuromodulators such as acetylcholine (Burbridge et al. 2014; Conner et al. 2005; Ramanathan et al. 2009; Sarter et al. 2016; Seol et al. 2007); similar mechanisms could guide plasticity in RA (Salgado-Commissariat et al. 2004). As in mammalian neocortex, synaptic plasticity and excitability mechanisms in RA appear to be complex, with established roles for neurotrophins, metabotropic glutamate receptors, as well as cholinergic, serotonergic, and dopamine receptors (Kittelberger and Mooney 2005; Liao et al. 2013; Mehaffey and Doupe 2015; Wood et al. 2011). Thus acetylcholine is likely to be just one of several modulators important for plasticity in RA and song learning more generally.

We observed the absence of stereotyped syllable sequences and abnormal song rhythmicity in adult birds following cholinergic blockade in RA. The neural mechanisms of timing in the song system have been attributed to sparsely timed spike sequences formed by feedforward synaptic chains in HVC, which drive a unique population of RA neurons at each moment in time (Fee et al. 2004; Hahnloser et al. 2002). Song timing may additionally include an interhemispheric loop including brain-stem vocal motor nuclei and thalamic nucleus uvaeformis (Fig. 1*B*; Danish et al. 2017; Hamaguchi et al. 2016; Schmidt et al. 2004). Our findings suggest that RA circuit deficits resulting from the absence of cholinergic inputs contribute to aberrant propagation of feedforward timing signals within the motor pathway as an effect of abnormal synaptic plasticity, likely in the HVC-RA synapses. HVC-projecting RA neurons could provide another mechanism by which reduced cholinergic tone in RA could influence timing signals in HVC circuit monosynaptic feedback (Roberts et al. 2008).

The behavioral consequences of ACh blockade in RA are substantially different from other lesions performed in the song system. First, lesions to LMAN or its thalamic input in juvenile birds result in an immediate loss of variability during vocal babbling and premature crystallization (Bottjer et al. 1984; Chen et al. 2014; Goldberg and Fee 2011). Superficially, ACh blockade in RA resembles Area X and HVC lesions, which also preserve subsong, result in sustained variability, and impair learning in the long term (Aronov et al. 2008; Scharff and Nottebohm 1991). However, nuances of song development differ across these conditions. HVC lesions result in a sustained reversion to subsong with exponentially distributed syllable durations, even in adults. Area X lesions in juvenile birds also preserve babbling but result, over development, in multisyllabic songs with sustained variability and poor resemblance to tutor. These results differ from our current study, in which birds acquire songs with one prominent, albeit broad, peak in their syllable duration distributions. Notably, our results qualitatively resemble partial RA lesions, which also result in significantly reduced frequency modulation (Simpson and Vicario 1990). Consistent with functional damage to RA, ACh-blocked birds sang significantly less, and two birds eventually stopped singing altogether. However, in contrast to our results, partial RA lesions preserved temporal patterning of song. Still, these parallels suggest that cholinergic inputs to RA may be important in establishing functional RA circuitry, potentially by regulating the invasion of HVC axons into RA during development (Mooney and Rao 1994). This idea is supported by our song ontogeny analysis showing that blocked birds failed to exhibit normal syllable splitting, a song maturation process thought to be controlled by activity patterns in RA-projecting HVC neurons (Okubo et al. 2015).

Gaining specific insight into cholinergic contributions to song learning will require clarification of what signals are carried from basal forebrain (BF) to cortical motor circuits during singing to guide plasticity. Interestingly, RA-projecting cholinergic neurons in the BF are comingled with ventral pallidal neurons that receive inputs from Area X, the basal ganglia nucleus of the song system (Gale et al. 2008). This part of the ventral pallidum also projects to the ventral tegmental area, which, in turn, sends auditory and song performance error signals back to Area X (Gadagkar et al. 2016; Gale and Perkel 2010; Hoffmann et al. 2016; Hisey et al. 2018; Xiao et al. 2018). Thus RA-projecting cholinergic neurons in the BF, as well as neighboring HVC-projecting cholinergic neurons (Li and Sakaguchi 1997; Shea et al. 2010; Shea and Margoliash 2003), reside in a region poised to perform error-related computations important for learning. However, at present, the nature of these computations and the full extent of the connectivity of that region remain unclear.

Many of the questions raised in this study, including the mechanisms by which the BFCS modulates motor cortical plasticity as well as the precise signals carried by this projection, are relevant not just to song learning in birds, but also to motor learning in mammals more generally. Our main finding that cholinergic blockade in RA impairs vocal learning in songbirds is conceptually similar to studies in mammals showing that cholinergic blockade in motor cortex impairs forelimb motor learning (Conner et al. 2003). Because of the tractable nature of the song system, the present findings further motivate the songbird as a powerful model system to identify roles of the BFCS in motor control and learning.

GRANTS

J. H. Goldberg was supported by National Institute of Neurological Disorders and Stroke (NINDS) Grant R01-NS-094667 and the Pew and Klingenstein foundations. P. A. Puzerey was supported by NINDS Grant F32-NS-098634.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

P.A.P. and J.H.G. conceived and designed research; P.A.P., K.M., and N.P. performed experiments; P.A.P. analyzed data; P.A.P. and J.H.G. interpreted results of experiments; P.A.P. prepared figures; P.A.P. and J.H.G. drafted manuscript; P.A.P. and J.H.G. edited and revised manuscript; P.A.P. and J.H.G. approved final version of manuscript.

REFERENCES

Andalman AS, Fee MS. A basal ganglia-forebrain circuit in the songbird biases motor output to avoid vocal errors. *Proc Natl Acad Sci USA* 106: 12518–12523, 2009. doi:10.1073/pnas.0903214106.

Aronov D, Andalman AS, Fee MS. A specialized forebrain circuit for vocal babbling in the juvenile songbird. *Science* 320: 630–634, 2008. doi:10.1126/science.1155140.

Aronov D, Veit L, Goldberg JH, Fee MS. Two distinct modes of forebrain circuit dynamics underlie temporal patterning in the vocalizations of young songbirds. *J Neurosci* 31: 16353–16368, 2011. doi:10.1523/JNEUROSCI.3009-11.2011.

Bottjer SW. Developmental regulation of basal ganglia circuitry during the sensitive period for vocal learning in songbirds. *Ann N Y Acad Sci* 1016: 395–415, 2004. doi:10.1196/annals.1298.037.

Bottjer SW, Miesner EA, Arnold AP. Forebrain lesions disrupt development but not maintenance of song in passerine birds. *Science* 224: 901–903, 1984. doi:10.1126/science.6719123.

Burbridge TJ, Xu HP, Ackman JB, Ge X, Zhang Y, Ye MJ, Zhou ZJ, Xu J, Contractor A, Crair MC. Visual circuit development requires patterned activity mediated by retinal acetylcholine receptors. *Neuron* 84: 1049–1064, 2014. doi:10.1016/j.neuron.2014.10.051.

Charlesworth JD, Warren TL, Brainard MS. Covert skill learning in a cortical-basal ganglia circuit. *Nature* 486: 251–255, 2012. doi:10.1038/nature11078.

Chen JR, Stepanek L, Doupe AJ. Differential contributions of basal ganglia and thalamus to song initiation, tempo, and structure. *J Neurophysiol* 111: 248–257, 2014. doi:10.1152/jn.00584.2012.

Conner JM, Chiba AA, Tuszynski MH. The basal forebrain cholinergic system is essential for cortical plasticity and functional recovery following brain injury. *Neuron* 46: 173–179, 2005. doi:10.1016/j.neuron.2005.03.003.

Conner JM, Culberson A, Packowski C, Chiba AA, Tuszynski MH. Lesions of the basal forebrain cholinergic system impair task acquisition and abolish cortical plasticity associated with motor skill learning. *Neuron* 38: 819–829, 2003. doi:10.1016/S0896-6273(03)00288-5.

Coppola JJ, Disney AA. Is there a canonical cortical circuit for the cholinergic system? Anatomical differences across common model systems. *Front Neural Circuits* 12: 8, 2018. doi:10.3389/fncir.2018.00008.

Danish HH, Aronov D, Fee MS. Rhythmic syllable-related activity in a songbird motor thalamic nucleus necessary for learned vocalizations. *PLoS One* 12: e0169568, 2017. doi:10.1371/journal.pone.0169568.

Dölen G, Darvishzadeh A, Huang KW, Malenka RC. Social reward requires coordinated activity of nucleus accumbens oxytocin and serotonin. *Nature* 501: 179–184, 2013. doi:10.1038/nature12518.

Doupe AJ, Kuhl PK. Birdsong and human speech: common themes and mechanisms. *Annu Rev Neurosci* 22: 567–631, 1999. doi:10.1146/annurev.neuro.22.1.567.

Dugas-Ford J, Rowell JJ, Ragsdale CW. Cell-type homologies and the origins of the neocortex. *Proc Natl Acad Sci USA* 109: 16974–16979, 2012. doi:10.1073/pnas.1204773109.

Fee MS, Goldberg JH. A hypothesis for basal ganglia-dependent reinforcement learning in the songbird. *Neuroscience* 198: 152–170, 2011. doi:10.1016/j.neuroscience.2011.09.069.

Fee MS, Kozhevnikov AA, Hahnloser RH. Neural mechanisms of vocal sequence generation in the songbird. *Ann N Y Acad Sci* 1016: 153–170, 2004. doi:10.1196/annals.1298.022.

Fehér O, Wang H, Saar S, Mitra PP, Tchernichovski O. De novo establishment of wild-type song culture in the zebra finch. *Nature* 459: 564–568, 2009. doi:10.1038/nature07994.

Gadagkar V, Puzerey PA, Chen R, Baird-Daniel E, Farhang AR, Goldberg JH. Dopamine neurons encode performance error in singing birds. *Science* 354: 1278–1282, 2016. doi:10.1126/science.aah6837.

Gale SD, Perkel DJ. A basal ganglia pathway drives selective auditory responses in songbird dopaminergic neurons via disinhibition. *J Neurosci* 30: 1027–1037, 2010. doi:10.1523/JNEUROSCI.3585-09.2010.

Gale SD, Person AL, Perkel DJ. A novel basal ganglia pathway forms a loop linking a vocal learning circuit with its dopaminergic input. *J Comp Neurol* 508: 824–839, 2008. doi:10.1002/cne.21700.

Garst-Orozco J, Babadi B, Ölveczky BP. A neural circuit mechanism for regulating vocal variability during song learning in zebra finches. *eLife* 3: e03697, 2014. doi:10.7554/eLife.03697.

Goldberg JH, Fee MS. A cortical motor nucleus drives the basal ganglia-recipient thalamus in singing birds. *Nat Neurosci* 15: 620–627, 2012. doi:10.1038/nn.3047.

Goldberg JH, Fee MS. Vocal babbling in songbirds requires the basal ganglia-recipient motor thalamus but not the basal ganglia. *J Neurophysiol* 105: 2729–2739, 2011. doi:10.1152/jn.00823.2010.

Hahnloser RH, Kozhevnikov AA, Fee MS. An ultra-sparse code underlies the generation of neural sequences in a songbird. *Nature* 419: 65–70, 2002. [Erratum in *Nature* 421: 294, 2003.] doi:10.1038/nature00974.

Hamaguchi K, Tanaka M, Mooney R. A distributed recurrent network contributes to temporally precise vocalizations. *Neuron* 91: 680–693, 2016. doi:10.1016/j.neuron.2016.06.019.

Hangya B, Ranade SP, Lorenc M, Kepecs A. Central cholinergic neurons are rapidly recruited by reinforcement feedback. *Cell* 162: 1155–1168, 2015. doi:10.1016/j.cell.2015.07.057.

Hasselmo ME. Neuromodulation and cortical function: modeling the physiological basis of behavior. *Behav Brain Res* 67: 1–27, 1995. doi:10.1016/0166-4328(94)00113-T.

Higley MJ, Gittis AH, Oldenburg IA, Balthasar N, Seal RP, Edwards RH, Lowell BB, Kreitzer AC, Sabatini BL. Cholinergic interneurons mediate fast VGluT3-dependent glutamatergic transmission in the striatum. *PLoS One* 6: e19155, 2011. doi:10.1371/journal.pone.0019155.

Hisey E, Kearney MG, Mooney R. A common neural circuit mechanism for internally guided and externally reinforced forms of motor learning. *Nat Neurosci* 21: 589–597, 2018. doi:10.1038/s41593-018-0092-6.

Hoffmann LA, Saravanan V, Wood AN, He L, Sober SJ. Dopaminergic contributions to vocal learning. *J Neurosci* 36: 2176–2189, 2016. doi:10.1523/JNEUROSCI.3883-15.2016.

Kao MH, Brainard MS. Lesions of an avian basal ganglia circuit prevent context-dependent changes to song variability. *J Neurophysiol* 96: 1441–1455, 2006. doi:10.1152/jn.01138.2005.

Kilgard MP, Merzenich MM. Cortical map reorganization enabled by nucleus basalis activity. *Science* 279: 1714–1718, 1998. doi:10.1126/science.279.5357.1714.

Kittelberger JM, Mooney R. Acute injections of brain-derived neurotrophic factor in a vocal premotor nucleus reversibly disrupt adult birdsong stability and trigger syllable deletion. *J Neurobiol* 62: 406–424, 2005. doi:10.1002/neu.20109.

Konopacki J, Gołebiewski H, Eckersdorf B. Carbachol-induced theta-like activity in entorhinal cortex slices. *Brain Res* 572: 76–80, 1992. doi:10.1016/0006-8993(92)90453-G.

Li R, Sakaguchi H. Cholinergic innervation of the song control nuclei by the ventral paleostriatum in the zebra finch: a double-labeling study with retrograde fluorescent tracers and choline acetyltransferase immunohistochemistry. *Brain Res* 763: 239–246, 1997. doi:10.1016/S0006-8993(97)00417-4.

Liao C, Wang S, Pan X, Hou G, Li D. Dopamine modulates the excitability of projection neurons in the robust nucleus of the arcopallium in adult zebra finches. *PLoS One* 8: e82497, 2013. doi:10.1371/journal.pone.0082497.

Lin SC, Nicolelis MA. Neuronal ensemble bursting in the basal forebrain encodes salience irrespective of valence. *Neuron* 59: 138–149, 2008. doi:10.1016/j.neuron.2008.04.031.

Long MA, Jin DZ, Fee MS. Support for a synaptic chain model of neuronal sequence generation. *Nature* 468: 394–399, 2010. doi:10.1038/nature09514.

Mandelblat-Cerf Y, Fee MS. An automated procedure for evaluating song imitation. *PLoS One* 9: e96484, 2014. [Correction in *PLoS One* 9: e106169, 2014.] doi:10.1371/journal.pone.0096484.

Mehaffey WH, Doupe AJ. Naturalistic stimulation drives opposing heterosynaptic plasticity at two inputs to songbird cortex. *Nat Neurosci* 18: 1272–1280, 2015. doi:10.1038/nn.4078.

Meng W, Wang S, Yao L, Zhang N, Li D. Muscarinic receptors are responsible for the cholinergic modulation of projection neurons in the song production brain nucleus RA of zebra finches. *Front Cell Neurosci* 11: 51, 2017. doi:10.3389/fncel.2017.00051.

Mooney R, Rao M. Waiting periods versus early innervation: the development of axonal connections in the zebra finch song system. *J Neurosci* 14: 6532–6543, 1994. doi:10.1523/JNEUROSCI.14-11-06532.1994.

Nelson A, Mooney R. The basal forebrain and motor cortex provide convergent yet distinct movement-related inputs to the auditory cortex. *Neuron* 90: 635–648, 2016. doi:10.1016/j.neuron.2016.03.031.

Nottebohm F. Neuronal replacement in adult brain. *Brain Res Bull* 57: 737–749, 2002. doi:10.1016/S0361-9230(02)00750-5.

Okubo TS, Mackevicius EL, Payne HL, Lynch GF, Fee MS. Growth and splitting of neural sequences in songbird vocal development. *Nature* 528: 352–357, 2015. doi:10.1038/nature15741.

Ölveczky BP, Andalman AS, Fee MS. Vocal experimentation in the juvenile songbird requires a basal ganglia circuit. *PLoS Biol* 3: e153, 2005. doi:10.1371/journal.pbio.0030153.

Ölveczky BP, Otchy TM, Goldberg JH, Aronov D, Fee MS. Changes in the neural control of a complex motor sequence during learning. *J Neurophysiol* 106: 386–397, 2011. doi:10.1152/jn.00018.2011.

Pfenning AR, Hara E, Whitney O, Rivas MV, Wang R, Roulhac PL, Howard JT, Wirthlin M, Lovell PV, Ganapathy G, Mountcastle J, Moseley MA, Thompson JW, Soderblom EJ, Iriki A, Kato M, Gilbert MT, Zhang G, Bakken T, Bongaarts A, Bernard A, Lein E, Mello CV, Hartemink AJ, Jarvis ED. Convergent transcriptional specializations in the brains of humans and song-learning birds. *Science* 346: 1256846, 2014. doi:10.1126/science.1256846.

Ramanathan D, Conner JM, Tuszynski MH. A form of motor cortical plasticity that correlates with recovery of function after brain injury. *Proc Natl Acad Sci USA* 103: 11370–11375, 2006. doi:10.1073/pnas.0601065103.

Ramanathan D, Tuszynski MH, Conner JM. The basal forebrain cholinergic system is required specifically for behaviorally mediated cortical map plasticity. *J Neurosci* 29: 5992–6000, 2009. doi:10.1523/JNEUROSCI.0230-09.2009.

Roberts TF, Klein ME, Kubke MF, Wild JM, Mooney R. Telencephalic neurons monosynaptically link brainstem and forebrain premotor networks necessary for song. *J Neurosci* 28: 3479–3489, 2008. doi:10.1523/JNEUROSCI.0177-08.2008.

Saar S, Mitra PP. A technique for characterizing the development of rhythms in bird song. *PLoS One* 3: e1461, 2008. doi:10.1371/journal.pone.0001461.

Sakaguchi H, Saito N. Developmental change of cholinergic activity in the forebrain of the zebra finch during song learning. *Brain Res Dev Brain Res* 62: 223–228, 1991. doi:10.1016/0165-3806(91)90169-J.

Salgado-Commissariat D, Rosenfield DB, Helekar SA. Nicotine-mediated plasticity in robust nucleus of the archistriatum of the adult zebra finch. *Brain Res* 1018: 97–105, 2004. doi:10.1016/j.brainres.2004.05.051.

Sarter M, Lustig C, Berry AS, Gritton H, Howe WM, Parikh V. What do phasic cholinergic signals do? *Neurobiol Learn Mem* 130: 135–141, 2016. doi:10.1016/j.nlm.2016.02.008.

Sasahara K, Tchernichovski O, Takahasi M, Suzuki K, Okanoya K. A rhythm landscape approach to the developmental dynamics of birdsong. *J R Soc Interface* 12: 20150802, 2015. doi:10.1098/rsif.2015.0802.

Scharff C, Nottebohm F. A comparative study of the behavioral deficits following lesions of various parts of the zebra finch song system: implications for vocal learning. *J Neurosci* 11: 2896–2913, 1991. doi:10.1523/JNEUROSCI.11-09-02896.1991.

Schmidt MF, Ashmore RC, Vu ET. Bilateral control and interhemispheric coordination in the avian song motor system. *Ann N Y Acad Sci* 1016: 171–186, 2004. doi:10.1196/annals.1298.014.

Seol GH, Ziburkus J, Huang S, Song L, Kim IT, Takamiya K, Huganir RL, Lee HK, Kirkwood A. Neuromodulators control the polarity of spike-timing-dependent synaptic plasticity. *Neuron* 55: 919–929, 2007. doi:10.1016/j.neuron.2007.08.013.

Shea SD, Koch H, Baleckaitis D, Ramirez JM, Margoliash D. Neuron-specific cholinergic modulation of a forebrain song control nucleus. *J Neurophysiol* 103: 733–745, 2010. doi:10.1152/jn.00803.2009.

Shea SD, Margoliash D. Basal forebrain cholinergic modulation of auditory activity in the zebra finch song system. *Neuron* 40: 1213–1226, 2003. doi:10.1016/S0896-6273(03)00723-2.

Simpson HB, Vicario DS. Brain pathways for learned and unlearned vocalizations differ in zebra finches. *J Neurosci* 10: 1541–1556, 1990. doi:10.1523/JNEUROSCI.10-05-01541.1990.

Spiro JE, Dalva MB, Mooney R. Long-range inhibition within the zebra finch song nucleus RA can coordinate the firing of multiple projection neurons. *J Neurophysiol* 81: 3007–3020, 1999. doi:10.1152/jn.1999.81.6.3007.

Tchernichovski O, Lints TJ, Deregnaucourt S, Cimenser A, Mitra PP. Studying the song development process: rationale and methods. *Ann N Y Acad Sci* 1016: 348–363, 2004. doi:10.1196/annals.1298.031.

Tchernichovski O, Mitra P. Towards quantification of vocal imitation in the zebra finch. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* 188: 867–878, 2002. doi:10.1007/s00359-002-0352-4.

Tchernichovski O, Mitra PP, Lints T, Nottebohm F. Dynamics of the vocal imitation process: how a zebra finch learns its song. *Science* 291: 2564–2569, 2001. doi:10.1126/science.1058522.

Tchernichovski O, Nottebohm F, Ho CE, Pesaran B, Mitra PP. A procedure for an automated measurement of song similarity. *Anim Behav* 59: 1167–1176, 2000. doi:10.1006/anbe.1999.1416.

Warren TL, Tumer EC, Charlesworth JD, Brainard MS. Mechanisms and time course of vocal learning and consolidation in the adult songbird. *J Neurophysiol* 106: 1806–1821, 2011. doi:10.1152/jn.00311.2011.

Wild JM. Descending projections of the songbird nucleus robustus archistriatalis. *J Comp Neurol* 338: 225–241, 1993. doi:10.1002/cne.903380207.

Wilson FA, Rolls ET. Neuronal responses related to reinforcement in the primate basal forebrain. *Brain Res* 509: 213–231, 1990. doi:10.1016/0006-8993(90)90546-N.

Wood WE, Lovell PV, Mello CV, Perkel DJ. Serotonin, via HTR2 receptors, excites neurons in a cortical-like premotor nucleus necessary for song learning and production. *J Neurosci* 31: 13808–13815, 2011. doi:10.1523/JNEUROSCI.2281-11.2011.

Xiao L, Chattree G, Oscos FG, Cao M, Wanat MJ, Roberts TF. A basal ganglia circuit sufficient to guide birdsong learning. *Neuron* 98: 208–221.e5, 2018. doi:10.1016/j.neuron.2018.02.020.

Zaborszky L, Pang K, Somogyi J, Nadasdy Z, Kallo I. The basal forebrain corticopetal system revisited. *Ann N Y Acad Sci* 877: 339–367, 1999. doi:10.1111/j.1749-6632.1999.tb09276.x.