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Neural mechanisms of hierarchically distinct forms of vocal learning in adult songbirds

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Neural Mechanisms of Hierarchically Distinct Forms of Vocal Learning in Adult Songbirds

by

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Neural mechanisms of hierarchically distinct forms of vocal learning in adult songbirds

Timothy L. Warren

Abstract

Birdsong is an ideal behavior for testing the neural mechanisms that underlie motor learning, as the dedicated neural circuits that control song production and learning are well-elucidated and amenable to experimental manipulation. Here I investigate how the anterior forebrain pathway (AFP), an avian cortical-basal ganglia circuit long implicated in vocal plasticity, contributes to two hierarchically distinct forms of adaptive song modification in adult songbirds. First, I tested how the AFP contributes to adaptive modifications of the acoustic structure of individual song syllables, both in the context of reinforcement-driven learning and a self-driven recovery process. I found that, in both contexts, transiently interfering with the AFP's output to the primary motor pathway reversed the expression of newly learned changes to syllable acoustic structure, but that over multiple days, the expression of learning consolidated to become AFP-independent. This finding supports an emerging view that cortical-basal ganglia circuits can direct the initial expression of learning via top-down influences on primary motor circuits. Second, I tested how adult Bengalese finches control the stochastic sequence transitions that occur between syllables. I found that the probabilities of transitions are ordinarily stable, yet birds have a previously unrecognized capacity to adaptively modify these probabilities in response to differential reinforcement. Despite this capacity for modification, when reinforcement was terminated, birds gradually restored transition probabilities to their baseline values, indicating that these values are set points that the nervous system has the impetus to restore. These findings suggest that the

statistics of sequence variation in a motor skill can reflect an end point of learning that is actively maintained via continual self-monitoring. Finally, I tested the extent to which the adaptive modification of syllable acoustic structure and syllable sequencing rely on the same basal ganglia circuitry. I found that lesions of LMAN, the AFP's cortical outflow nucleus, prevented the adaptive modification of syllable structure but did not affect birds' capacity to adaptively modify syllable sequencing. Therefore, although similar principles of differential reinforcement can be used to drive changes to syllable structure and syllable sequencing, an avian basal ganglia circuit contributes differently to these hierarchically distinct forms of learning.

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Chapter 1: Mechanisms and time course of vocal learning and consolidation in the adult songbird

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Abstract

In songbirds, the basal ganglia outflow nucleus LMAN is a cortical analogue that is required for several forms of song plasticity and learning. Moreover, in adults, inactivating LMAN can reverse the initial expression of learning driven via aversive reinforcement. Here, we investigate how LMAN contributes both to reinforcement-driven learning and to a self-driven recovery process in adult Bengalese finches. We first drove changes in the fundamental frequency of targeted song syllables and compared the effects of inactivating LMAN with the effects of interfering with NMDA receptor dependent transmission from LMAN to one of its principal targets, the song premotor nucleus RA. Inactivating LMAN and blocking NMDA receptors in RA caused indistinguishable reversions in the expression of learning, indicating that LMAN contributes to learning through NMDA receptor mediated glutamatergic transmission to RA. We next assessed how LMAN's role evolves over time by maintaining learned changes to song while periodically inactivating LMAN. The expression of learning consolidated to become LMAN-independent over multiple days, indicating that this form of consolidation is not completed over one night, as previously suggested, and instead may occur gradually during singing. Subsequent cessation of reinforcement was followed by a gradual self-driven recovery of original song structure, indicating that consolidation does not correspond with the lasting retention of changes to song. Finally, for self-driven recovery, as for reinforcement-driven learning, LMAN was required for the

expression of initial, but not later, changes to song. Our results indicate that NMDA receptor dependent transmission from LMAN to RA plays an essential role in the initial expression of two distinct forms of vocal learning and that this role gradually wanes over a multi-day process of consolidation. The results support an emerging view that cortical-basal ganglia circuits can direct the initial expression of learning via top-down influences on primary motor circuitry.

Introduction

Birdsong is an ideal behavior for probing how basal ganglia circuits contribute to learning, as the dedicated neural pathways that control song production and song learning are well-elucidated and highly amenable to experimental monitoring and manipulation. These neural pathways include a song motor pathway that controls much of the moment-by-moment structure of song as well as a cortical-basal ganglia circuit, the anterior forebrain pathway (AFP), that plays a crucial role in juvenile song learning and adult vocal plasticity (Figure 1; Andalman and Fee 2009; Bottjer et al. 1984; Brainard and Doupe 2000; Nordeen and Nordeen 2010; Scharff and Nottebohm 1991; Williams and Mehta 1999).

The influence of the AFP on song production and learning has been demonstrated by silencing the lateral magnocellular nucleus of the anterior nidopallium (LMAN). LMAN is a cortical analogue that receives input from the basal ganglia via the thalamus and projects to multiple targets including the song premotor nucleus RA, an analogue of primary vocal motor cortex (Fig. 1). Lesions or inactivations of LMAN in juvenile birds cause an abrupt and premature stabilization of an abnormally simple song and prevent the subsequent progression of song learning (Bottjer et al. 1984; Olveczky et al. 2005; Scharff and Nottebohm 1991). In contrast, lesions or inactivations of LMAN in adults reduce rendition-

to-rendition variation in the acoustic structure of song syllables but do not affect the gross structure of stable adult song (Hampton et al. 2009; Kao and Brainard 2006; Kao et al. 2005; Nordeen and Nordeen 1993; Stepanek and Doupe 2010).

Lesions of LMAN, however, do prevent several forms of adult song plasticity that can normally be elicited by altered sensory experience (Brainard and Doupe 2000; Morrison and Nottebohm 1993; Thompson and Johnson 2006; Williams and Mehta 1999). Moreover, lesions or inactivations of LMAN can also reverse the expression of recently induced adult song plasticity and learning. For instance, lesions of LMAN partially reverse both the song deterioration that results from deafening and the song deterioration that results from microlesions made within the song motor pathway (Nordeen and Nordeen 2010; Thompson et al. 2007). Similarly, Andalman and Fee (2009) found that pharmacological inactivation of LMAN in adult birds partially reverses changes to syllable structure induced in an aversive reinforcement learning paradigm. The extent to which silencing LMAN reverses the expression of plasticity seems to diminish over time, indicating a consolidation process occurs in which the expression of learning becomes LMAN-independent (Andalman and Fee 2009; Nordeen and Nordeen 2010).

Here we address several questions about how LMAN contributes to the expression of adult learning: 1) Via which circuitry does LMAN contribute to the expression of learning? 2) Over what time course does learning become consolidated so that its expression no longer depends on LMAN? 3) Does this consolidation correspond with the establishment of lasting changes to song structure? 4) Do LMAN's contributions generalize across distinct forms of adaptive adult learning?

First, we investigate the neural circuitry through which LMAN influences the expression of learning (Andalman and Fee 2009). Ultimately, the expression of learning is

likely mediated by changes in the pattern of activity in the song premotor nucleus RA during singing; RA projects to the brainstem circuitry controlling vocal musculature and RA activity has been strongly linked to the moment-by-moment control of song structure (Leonardo and Fee 2005; Sober et al. 2008; Vu et al. 1994; Yu and Margoliash 1996). There are several direct and indirect pathways by which LMAN could affect RA activity (Fig. 1). One attractive hypothesis is that LMAN exerts its primary influence on the initial expression of adult song learning via direct projections from its central region, LMAN-core, to RA. Projections from LMAN-core to RA could influence RA activity through the release of glutamate or the release of neurotrophins (Akutagawa and Konishi 1998; Johnson et al. 1997; Kittelberger and Mooney 2005; Mooney 1992; Mooney and Konishi 1991; Stark and Perkel 1999). However, neurons in LMAN-core also project to Area X, a basal ganglia homologue that is well-positioned to influence RA activity via projections from Area X to neuromodulatory nuclei thought to participate in acute control of song production and learning (Fig. 1; Appeltants et al. 2002; Gale and Perkel 2010; Li and Sakaguchi 1997). Additionally, neurons in LMAN-shell, surrounding LMAN-core, project to Ad, which is part of a distinct circuit involved in juvenile song learning (Bottjer and Altenau 2010; Iyengar et al. 1999). Pharmacological inactivations targeting LMAN presumably affect neural activity in both LMAN-core and LMAN-shell. Moreover, such inactivations could exert their influence either by preventing excitatory glutamatergic transmission or by preventing neurotrophin release at diverse downstream targets. Hence, silencing LMAN activity could affect the expression of learning via multiple pathways and mechanisms.

Here we directly test the possibility that glutamatergic transmission from LMAN to RA mediates LMAN's role in the expression of learning. To do so, we compare the effects of inactivating LMAN with the effects of blocking NMDA receptors in RA. Glutamatergic

transmission from LMAN-core to RA relies primarily on activation of NMDA receptors in RA, while glutamatergic transmission within the direct motor pathway is mediated by a mixture of NMDA and AMPA receptors (Mooney 1992; Mooney and Konishi 1991; Stark and Perkel 1999). Therefore, blocking NMDA receptors in RA should preferentially block glutamatergic transmission from LMAN to RA while having a lesser effect on transmission within the motor pathway itself. Indeed, a previous study showed that infusing AP5, an NMDA receptor antagonist, into RA of juvenile birds caused similar effects on the variability of song as inactivating LMAN (Olveczky et al. 2005), suggesting that blocking NMDA receptors in RA functionally de-afferents RA from its glutamatergic LMAN inputs. Here we therefore infuse AP5 into RA to ask whether LMAN-RA glutamatergic transmission mediates LMAN's contributions to the expression of song learning. We find that infusion of AP5 into RA and inactivation of LMAN similarly reverse the initial expression of learned changes to syllable structure, suggesting that LMAN's contributions to the expression of learning are mediated by direct glutamatergic projections from LMAN-core to RA, rather than through LMAN's projections to other targets.

Second, we address how the contributions of LMAN to the expression of learning change over time. Andalman and Fee (2009) inactivated LMAN while driving continuous changes to the fundamental frequency (FF) of targeted syllables via aversive reinforcement. The amount of reversion caused by inactivating LMAN on a given day tended to correspond with the amount of learning that had already occurred on that day. This correspondence indirectly suggests that learning from prior days may become completely consolidated overnight in the sense that the expression of prior learning no longer requires LMAN activity. However, this possibility was not tested directly because learned changes to FF were not held at a constant value from one day to the next. Here we test the time course of consolidation

directly by maintaining FF at a fixed, learned offset from baseline for several days while inactivating LMAN periodically. We find, as reported by Andalman and Fee (2009), that LMAN inactivation initially causes a large reversion of learned changes in FF back toward the initial (pre learning) baseline value. However, we find that the effects of LMAN inactivation on the expression of learning only gradually decline, over a period of multiple days. These results are incompatible with a model in which learned changes to song become completely consolidated in a single night and instead suggest a multi-day process of consolidation.

Third, we test whether this slow consolidation process results in the stable retention of learned changes to song. Indeed, in other forms of motor learning, analogous processes of consolidation result in the lasting retention of learned changes to behavior (e.g. Brashers-Krug et al. 1996; Joiner and Smith 2008; Shadmehr and Holcomb 1997). Here, however, we find that this is not the case; even after learning becomes consolidated (in the sense that LMAN is no longer required for the expression of learned changes to FF), cessation of external reinforcement results in a gradual recovery of song to its original structure. This indicates that the brain retains a lasting representation of the original song structure and both the capacity and impetus to restore song to that structure even following a consolidation process in which control over the expression of learning has been transferred to song motor circuitry that can operate independently of LMAN.

Finally, we take advantage of this self-driven recovery, which occurs spontaneously in the absence of external instruction, to test whether LMAN's contributions to the expression of learning generalize across different forms of learning. The mechanisms by which LMAN contributes to learning driven by aversive reinforcement (Andalman and Fee 2009; Charlesworth et al. 2011; Tumer and Brainard 2007) may be distinct from the

mechanisms by which LMAN contributes to juvenile song learning or to other forms of song learning that rely on error correction rather than external reinforcement (Mooney 2009a; Sober and Brainard 2009). We find that inactivating LMAN and blocking NMDA receptors in RA during the process of self-driven recovery cause a re-emergence of previously learned changes to song (and a shift in song structure away from the original baseline). This indicates that for self-driven song recovery, as for externally instructed learning, the initial expression of changes to song structure relies on LMAN while later expression of maintained changes relies on LMAN-independent components of song motor circuitry.

Materials and Methods

Subjects

Seventeen adult (>120 d old) Bengalese finches (*Lonchura striata domestica*) were used in this study. All birds were bred in our colony and housed with their parents until at least 60 d of age. During experiments, birds were isolated and housed individually in sound-attenuating chambers (Acoustic Systems) on a 14 hr on/10 hr off light cycle. All song recordings were of undirected song (i.e. no female was present). All procedures were performed in accordance with protocols approved by the University of California, San Francisco Institutional Animal Care and Use Committee.

Computerized song recording and control of reinforcement

Song recording and delivery of experimental stimuli were controlled using EvTAF, a computer program that drives learning through delivery of auditory feedback that is contingent on ongoing song performance (Charlesworth et al. 2011; Tumer and Brainard 2007). For each experiment, a set of spectral templates was constructed which enabled

detection of a specific 8 ms segment (the 'contingency segment') within a specific syllable of song (the ‘target syllable’). To detect the contingency segment, the program continuously monitored successive 8 ms segments of song as they were produced. Detection depended on the spectral structure of song during the contingency segment as well as preceding segments up to 200 ms earlier. A Fast Fourier Transform (FFT) was performed on each recorded segment and the resulting spectrum (power spectral density) was compared against the set of spectral templates. A match to a specific spectral template occurred when the difference between that spectral template and the recorded song spectrum was below a threshold value. Detection of the contingency segment required that a series of 8 ms segments satisfy a pre-specified sequence of matches to a set of spectral templates. The template matching sequence was specified so that the contingency time (the midpoint of the contingency segment) occurred within a portion of the syllable in which the harmonic structure was well-defined and stable. Across all target syllables, the contingency time, measured relative to syllable onset, had a median value of 38 ms. Detection was temporally precise; the standard deviation of the contingency time (calculated from the empirically measured distribution of contingency times for each syllable) had a median value of 4.5 ms. The variation in the contingency time for a given syllable was due both to random variation in the onset of 8 ms segments relative to syllable onset and also due to biological variation that affected when song spectral structure matched templates. Across all experiments, the percent of target syllable renditions that were correctly detected ranged from 98% to 100%. The percent of non-target syllable renditions that were incorrectly detected as target syllables ranged from 0% to 4%.

Following detection of the contingency segment, the fundamental frequency (FF) of the 8 ms contingency segment was computed. This value was compared to a previously set FF

threshold. To drive the FF of the target syllable higher, a 60 ms white noise (WN) stimulus was delivered (within 1 ms of the end of the contingency segment) if the calculated FF was below the threshold. To drive FF lower, WN was delivered if the calculated FF was above the threshold.

Trajectories of learning

Twenty-four distinct multi-day learning trajectories (in 15 birds) were driven using WN reinforcement in this study. We first drove a shift in the FF of the targeted syllable during a 3-4 day period (e.g. Fig. 2; 'initial shift'). The threshold for reinforcement was initially set so that the hit rate (percentage of syllables receiving WN) was ~75 percent. Birds gradually modified FF of the target syllable to lower this hit rate and the threshold was adjusted daily to propel further learning. For 12 learning trajectories (in 10 birds), the threshold was then fixed to maintain FF at a constant offset from its original baseline value (e.g. Fig. 2; 'maintained shift'). Following the period of maintained shift, in 5 experiments (n=5 birds), reinforcement with WN was terminated in order to evaluate the extent to which learned changes to FF were retained without external reinforcement. In 5 other experiments (n=3 birds), recovery of FF towards baseline was initiated by switching the contingency for reinforcement so that the syllables with values of FF closer to baseline escaped WN.

Reversible inactivation of LMAN via retrodialysis

We transiently inactivated LMAN via a retrodialysis technique (Lindefors et al. 1989; Stepanek and Doupe 2010), in which solutes diffuse into targeted brain areas across implanted dialysis membranes. Zero net volume crosses the membrane, making the technique well suited for long-term experiments. In 9 birds, we bilaterally implanted guide

cannulae (CMA 7; CMA Microdialysis) by stereotaxic coordinates. The bird was positioned so that the ventral surface of the upper beak was tipped downward by 40 degrees relative to horizontal. The tips of the cannulae were then positioned 5.3 mm rostral and 1.5 mm lateral to the caudal junction of the mid-sagittal sinus and transverse sinus and lowered to a depth of 0.7 mm from the surface of the brain. After birds recovered from surgery (3-4 days), we inserted microdialysis probes (CMA 7; 0.24 mm diameter, 1 mm diffusion membrane, diffusion pore size 6 kD) into the cannulae. Probe tips extended 1.2 mm beyond the guide cannulae so that they were centered in LMAN. Probes were connected to infusion pumps via flexible tubing, a dual channel liquid commutator (Instech Labs 375/D/22QM), and a fluid switch (BASi Uniswitch) that allowed birds to sing and move around their cage while the solution flowing through the probes was switched remotely. The two probes were connected serially (i.e. the outflow of one probe was the inflow of the second probe).

During control periods, probes were perfused continuously with artificial cerebro-spinal fluid (ACSF; c.f. Stepanek and Doupe 2010). To inactivate LMAN, we remotely switched the dialysis solution to the $GABA_A$ agonist muscimol (100-500 μM; Sigma; 7 birds) or the Na^+ channel blocker lidocaine (2%; Hospira; 2 birds) at a flow rate of 1 μl/min. Inactivations began 3-5 hours after lights were turned on and lasted for 3-4 hours, during which a 1 μl/min flow rate was maintained. At the conclusion of the inactivation, the dialyzing solution was switched back to ACSF. Under our experimental conditions, it took 6 minutes from the time at which solutions were switched at the fluid switch until the new solution reached the tips of both probes. For all analyses and plots we therefore consider drug infusion (time = 0) to begin 6 minutes after switching solutions at the fluid switch. Pharmacological inactivations were performed every other day, except for 8 (out of 59) cases in which inactivations were performed after a 1 day interval. In several cases (e.g. Fig. 3c),

inactivations were not performed until the third day of learning, in order to elicit a large change in FF prior to inactivating LMAN.

We evaluated the efficacy of LMAN inactivations during baseline conditions (prior to any learning) by measuring the effect of LMAN inactivations on the rendition-to-rendition variability of FF for individual syllables. Previous studies in the adult Bengalese finch and zebra finch (*Taeniopygia guttata)* have shown that lesions and inactivations of LMAN cause a reduction in the rendition-by-rendition variability of FF (Andalman and Fee 2009; Hampton et al. 2009; Kao et al. 2005; Stepanek and Doupe 2010). In 7 out of the 9 implanted birds, we observed a significant and reliable reduction in the variability of FF following infusion of muscimol (or lidocaine) into LMAN ($P < 0.05$ in all 7 cases, permutation test; see Results). These 7 birds were used for subsequent learning experiments; the two remaining birds were perfused and found (via histology; see below) to have incorrect targeting (n=1) or a faulty dialysis probe (n=1).

In principle, the effects of infusions could diminish over time, either due to changes in efficacy of dialysis probes or due to changes induced in LMAN over the course of repeated inactivations. To assess whether this was the case, we monitored for any changes in the influence of LMAN inactivations on the rendition-to-rendition variability of FF over the course of each experiment. We found no trend for a change in the efficacy of LMAN inactivations over the course of experiments (see Results). In six birds, in which we drove at least two distinct trajectories of learning, we additionally tested for any changes over time in the influence of LMAN inactivations on the expression of learning. In these cases, the first learning trajectory was preceded by only a small number of LMAN inactivations during a baseline period (see above). In contrast, the second trajectory occurred after several weeks during which LMAN was repeatedly inactivated (~10 prior inactivations of LMAN). There

was no significant difference between the effects of LMAN inactivations on the expression of learning for first versus second trajectories ($P > 0.4$, paired t-test, $n = 6$), providing further evidence that neither deterioration of probes nor changes to LMAN function accumulated over time.

Reversible interference with NMDA-receptors in RA

In 5 birds, we targeted the song premotor nucleus RA with the same type of cannulae and probes used for LMAN inactivations. RA was mapped electrophysiologically during implantation in order to direct probes toward the center of the nucleus (see Fig. 4b). RA implants were angled in a posterior direction by 20 degrees relative to LMAN implants to avoid nucleus HVC. During control conditions, ACSF was dialyzed through RA probes. For experimental infusions, the NMDA-receptor antagonist DL-AP5 (2-5 mM; Ascent) was dialyzed at a flow rate of 1 μl/min.

Post-experiment localization of dialysis probes

Probe positioning and the path of drug diffusion were evaluated post mortem by histological staining of sectioned tissue. Tissue damage caused by cannulae enabled confirmation that probes were accurately targeted to LMAN in all 7 experimental birds, and in RA for all 5 experimental birds. Additionally, in 5 of the 7 birds targeted for LMAN infusion, and in all 5 birds targeted for RA infusion, biotinylated muscimol (EZ-link biotin kit; Pierce; diluted to 500 μM, matching the highest concentration used across all experiments) was dialyzed across the diffusion membrane for 4 hours in order to estimate the path of diffusion from the membrane (Stepanek and Doupe 2010). In these birds, probe position was determined post mortem by histological staining for biotin and by comparing

interleaved sections stained for anti-CGRP (to stain LMAN, mMAN, and area X; Bottjer et al. 1997; Brainard and Doupe 2001) or for Nissl bodies (RA). In the two remaining birds used for LMAN inactivations, ibotenic acid, an excitotoxic agent, was dialyzed across the membranes at the conclusion of the experiment, and the spatial extent of drug-induced lesions was assessed histologically.

Diffusion paths assessed with biotinylated drug indicated that the radius of maximal spread of drug along the dorso-ventral axis of the probe was ~1.0 mm and of maximal spread perpendicular to the axis of the probe was ~0.75 mm. Effects on syllable FF developed within 20 minutes to 1 hour of drug infusion and thereafter remained stable, consistent with prior results indicating that diffusion from a point source results in the gradual development of a stable sphere of drug spread (Amberg and Lindefors 1989; Lindefors et al. 1989).

In experiments targeting LMAN, the sphere of drug spread assessed histologically encompassed the entirety of both LMAN-core and LMAN-shell. No evidence of drug spread into mMAN was observed. In 2 of 7 LMAN-targeted birds, there was modest drug spread into the most dorsal regions of Area X on at least one side of the brain. The observed effects on variability reduction and the expression of learning in these birds were similar to those observed in other LMAN-targeted birds, and exclusion of these birds did not affect any conclusions about the significance of any reported results. In experiments targeting RA, any drug spread outside RA tended to be in regions dorsal to RA, along the cannula, but not into the lateral areas where Ad is located (Fig. 4b; Bottjer and Altenau 2010).

Fundamental frequency (FF) measurement

FF was measured 'online' in order to determine whether WN would be played back following detection of a target syllable (see above). However, for all analyses presented in

Results, FF was measured separately offline, so that measurements for a given syllable could be applied to a segment of song that was consistent across all renditions of the syllable. FF was measured for 8 or 16 ms segments of song; for syllables targeted with WN feedback, the segment for which FF was measured was centered at the median point at which feedback was delivered (onset of WN). Previous experiments using the same learning paradigm indicate that any reinforcement-driven changes to FF are likely to be maximal for this portion of the syllable (Charlesworth et al. 2011). We determined FF by calculating the FFT of the sound waveform and measuring peak spectral power via interpolation within a frequency window that spanned the first harmonic or a multiple of the first harmonic (Tumer and Brainard 2007). For each bird, FF was calculated both for the syllable targeted for learning and for a control syllable that did not receive WN. In all birds we analyzed FF in a random selection of songs (10-20%). In some birds, FF was measured for a random subset of "catch" syllables for which WN was omitted during the experiment (as in Tumer and Brainard, 2007). In other birds, FF was measured for a random subset of syllables in which a low-pass filtered WN stimulus was used that allowed measurement of higher frequency harmonics even when WN was played (as in Fig. 2). All analyses were performed with custom software written in MATLAB (Mathworks).

Assessing effects of drug infusion on song

In each instance of drug infusion (e.g. muscimol, lidocaine, or AP5), we compared the values of FF for renditions of the target syllable during the experimental infusion with the values of FF during a 4 hour control (pre-ACSF) period immediately prior to the infusion. Because of the gradual onset of drug effects, we excluded from analysis the first hour of songs after the dialysis solution was changed (either from ACSF to drug, or from drug to

ACSF). In one bird, recovery from AP5 infusion took longer than one hour (e.g. Fig. 4c). In this bird, we analyzed recovery data from the first two hours of song the morning after drug infusion.

To test whether distinct manipulations (playback of WN, inactivation of LMAN, NMDA receptor blockade) affected the rate of song production, we compared the number of occurrences of the targeted song syllable in a matched two-hour period for each bird during baseline ACSF infusion and during each of the manipulations. For each manipulation, there were no systematic changes in amount of singing relative to that observed during control conditions (ACSF dialysis with no WN), and no manipulation had a significant effect on the rate of production of the target syllable ($P > 0.7$, paired t-tests).

For analysis of the effects of drug infusions on FF during the initial shift period (i.e. 'initial shift', Fig. 3d and Fig. 4d), the pre-ACSF FF was shifted at least 1.5 s.d. from the baseline FF distribution and reinforcement had been ongoing for 4 or fewer days. For analysis of the effects of drug infusions during the period when FF was maintained at a fixed offset from its baseline value (i.e. 'maintained shift', Fig. 3e), the daily median FF offset from baseline was at least 2.5 s.d. and was maintained within 0.75 s.d. of the fixed offset value for each of at least 5 consecutive days. During maintained shifts (when the FF remained constant), the effects of drug infusions were compared across each of three periods: days 1-2, days 3-4, and days 5-6. The effects of LMAN inactivation on FF were analyzed relative to the pre-ACSF FF on the day of the inactivation.

During recovery to baseline (Fig. 6), we compared the effects of blocking input from LMAN to RA across five time stages: 1. *Baseline*, prior to WN onset, 2. *Initial shift* (defined above), 3. *Maintained shift* (defined above, range of 4-7 days), 4. *Initial recovery* (days 1-3 of recovery), and 5. *Maintained recovery*, in which FF had been restored to within 1 s.d. of

the original baseline level for at least three days (3-14 days at baseline in 5 experiments and 43 days at baseline in 1 experiment). For summary analysis in these recovery experiments, we normalized shifts in FF by the magnitude of the maintained offset of FF from baseline (Fig. 6b).

In cases where multiple infusions occurred during a given stage of learning (e.g. more than one infusion occurred during the initial shift period for a single trajectory of learning), effects were averaged across infusions and contributed only one data point to summary analyses, in order to avoid pseudoreplication.

Statistics

We used three types of statistical tests: Permutation test, T-test and ANOVA, as described below. The term 'significant' refers to results that had P values of < 0.05. Unless otherwise noted, mean values are reported as mean +/- standard error of the mean (SEM).

Permutation test: We tested for significant differences in test statistics (e.g. mean difference) between unpaired data in two groups via a permutation test in which we tested the null hypothesis that the data from the two groups came from the same underlying distribution. We randomly permuted all data values across the two groups 10,000 times while maintaining the original size of each group. By determining the frequency at which differences in the test statistic in the resampled distributions were as large as the originally observed differences, we generated a P value at which we could reject the null hypothesis that the two groups came from the same underlying distribution.

T-test: We used paired and unpaired t-tests (as specified in Results) to test whether two groups had significantly different means. In cases where we were testing a specific directional hypothesis (e.g. that LMAN inactivation reduced acoustic variability, or that

LMAN inactivation caused a reversion to the original baseline), we used a one-tailed t-test. These cases are indicated in Results.

ANOVA: We used a one-way ANOVA to test for significant differences in the mean across multiple groups in which data were paired (e.g. Fig. 3e). In cases where an ANOVA indicated a significant effect, we used a post-hoc Tukey's HSD test (which accounts for multiple comparisons) to determine which specific means were significantly different.

Results

Trajectory of reinforcement-driven learning

We used a previously established reinforcement paradigm to elicit adult vocal learning (Figure 2; Charlesworth et al. 2011; Tumer and Brainard 2007). An individual 'target syllable' (Fig. 2a) was selected for online monitoring and directed modification. An online, automated system detected each rendition of the target syllable and delivered white noise (WN) feedback that was contingent on the measured fundamental frequency (FF) for each rendition of the target syllable. In the example in Figure 2b, feedback was delivered to renditions of the target syllable with FF below a threshold value (Fig. 2b, 'hit'), but not to renditions with FF above that threshold (Fig. 2b, 'escape'). As in previous studies, (Andalman and Fee 2009; Charlesworth et al. 2011; Tumer and Brainard 2007), either upward or downward shifts in FF of a target syllable could be directed depending on whether WN was applied to renditions of the target syllable with FF below or above the experimentally imposed threshold.

We used this aversive reinforcement paradigm to drive controlled trajectories of learning in order to assess contributions of LMAN, and its glutamatergic inputs to RA, at distinct stages of learning. Following an initial period of learning during which FF was

shifted upward (or downward) by the progressive adjustment of the threshold for reinforcement (Fig. 2c, 'initial shift'), we maintained the learned FF at a fixed offset from baseline by keeping the threshold for reinforcement at a fixed value (Fig. 2c, 'maintained shift').

Transient inactivation of LMAN reverses the initial expression of learning

We first assessed the contributions of LMAN activity to the expression of learning by using bilateral dialysis of either the sodium channel blocker lidocaine or the $GABA_A$ agonist muscimol to inactivate LMAN reversibly (Fig. 3a). At baseline, prior to any learning, inactivation of LMAN had little gross effect on song structure or on the mean FF at which target syllables were sung. An example of this is illustrated in Figure 3b, which shows spectrograms of song structure during ACSF and muscimol infusions, and Figure 3c ('baseline' period), which shows the mean FF for each day during ACSF (black points) and muscimol (red points) infusions (top and bottom panels show daily means and expanded timeline for single day, respectively). While there was no significant effect on the mean FF at baseline, there was a significant reduction in the rendition-to-rendition variation in FF, which can be seen as a decrease in the range of FF values at which individual syllables were produced during muscimol infusion (change in mean, $P > 0.7$; change in the coefficient of variation (CV), $P < 0.01$, permutation test). On average, across 16 experiments in 7 birds, inactivation of LMAN at baseline caused no significant shift in mean FF ($P = 0.39$, paired t-test), but did cause a significant reduction in the coefficient of variation of FF (28.4 +/- 6.0% reduction in CV; $P < 0.001$, one-tailed paired t-test; Fig. 3d, 'baseline'). The CV rapidly recovered to its baseline value upon subsequent dialysis with ACSF (Fig. 3c-d). The post-ACSF CV was not significantly different than the pre-ACSF CV ($P = 0.25$; paired t-test).

Such a reduction in variability of syllable structure without other systematic changes to song is consistent with previously reported effects of lesions and inactivations of LMAN in adult zebra and Bengalese finches (Andalman and Fee 2009; Hampton et al. 2009; Kao and Brainard 2006; Kao et al. 2005; Stepanek and Doupe 2010). Indeed, the reduction in CV for FF (28.4%) was not significantly different from that observed following lesions of LMAN in Bengalese finches (34% +/- 5%; Fig. 3d, dashed line, data from Hampton et al. 2009; $P = 0.52$, unpaired t-test). This indicates that infusions of pharmacological agents were effective in inactivating LMAN.

In contrast to the effects of LMAN inactivation at baseline, LMAN inactivation during initial learning caused a large reversion of FF back towards the baseline value of FF. In the example shown in Figure 3c, by day 3 of learning, the mean FF of the targeted syllable produced under control conditions had been driven upward from the original baseline by 126 Hz. Inactivation of LMAN on day 3 again reduced the variability of FF, but additionally caused a significant reversion of the mean FF back towards the original baseline by 72 Hz, or 57% (change in CV, $P < 0.001$; change in mean, $P < 0.001$; permutation tests). Across experiments, such a reversion of FF during initial learning (days 2-4 of learning) consistently occurred in response to LMAN inactivation. In all 9 experiments where FF had been shifted upwards, inactivation of LMAN caused FF to revert downwards; in all 7 experiments where FF had been shifted downwards, inactivation of LMAN caused FF to revert upwards. The mean reversion of FF towards baseline was 46.9 +/- 7.0% (Fig. 3d) and did not differ significantly between experiments in which LMAN was inactivated with lidocaine (43.7 +/- 32.9%, $n = 3$) or muscimol (47.6 +/- 5.9%, $n = 13$; $P = 0.84$, unpaired t-test). FF rapidly recovered to the learned level upon subsequent dialysis with ACSF (Fig. 3c-d). The post-ACSF FF was not significantly different than the pre-ACSF FF ($P = 0.86$; paired t-test). The

average reduction in the variability of FF for the target syllables in these experiments was 36.5 +/- 7.7%, not significantly different from the 28.4 +/- 6.0% reduction in variability observed at baseline (P = 0.43, unpaired t-test). The systematic shifts in FF in response to LMAN inactivation were specific to the syllables targeted for learning; the mean FF of control syllables not targeted with WN did not exhibit significant shifts in response to LMAN inactivations either during the baseline or initial shift period (P = 0.7, paired t-test)

These data qualitatively parallel results from Andalman and Fee (2009), who found that infusing the sodium channel blocker TTX to inactivate LMAN in the adult zebra finch caused a reversion of recent learning induced in a similar aversive reinforcement paradigm. Our finding that muscimol, a $GABA_A$ agonist, is sufficient to cause such a reversion additionally indicates that the reversion of learning arises from inactivation of cell bodies rather than inactivation of fibers passing through or near LMAN.

The expression of learning becomes LMAN-independent via a slow, multi-day process

Prior studies suggest that a consolidation process occurs in which the contributions of LMAN to adult plasticity gradually diminish over time (Andalman and Fee 2009; Nordeen and Nordeen 2010). However, the time course of this consolidation process has not been tested directly. Here, we tested the time course of consolidation by maintaining learned changes to FF at a fixed offset from baseline and performing successive LMAN inactivations over a period of 5-6 days. An example is illustrated in Figure 3c ('maintained shift'). Following a period in which FF was directed upwards, we maintained FF at a fixed offset of ~170 Hz from its baseline value for 5 days. We inactivated LMAN on days 2, 4, and 5 of this maintained shift (corresponding to days 5, 7, and 8 since initiation of reinforcement). In this example, the contributions of LMAN activity to the expression of learning gradually declined

over the 5 day period of maintained shift in FF. Such a gradual decrease in the contribution of LMAN to the expression of learning was typical during periods of maintained shift. On average (across 6 experiments in 4 birds), during the first two days of the maintained shift, LMAN inactivation caused a significant reversion of 45.0 +/- 8.2% toward baseline ($P < 0.001$, one-tailed paired t-test, Bonferroni corrected for 3 comparisons). After 3-4 days of maintained shift, LMAN inactivation caused a reduced reversion of 27.2 +/- 5.1 % that was still significant ($P < 0.001$, one-tailed paired t-test, Bonferroni corrected), and after 5-6 days, caused a further reduced reversion of 15.1 +/- 7.1% that did not achieve statistical significance (Fig. 3e, top panel; $P = 0.11$, one-tailed paired t-test, Bonferroni corrected).

These data support two conclusions about consolidation. First, they confirm that learned changes to FF eventually consolidate so that their expression becomes largely independent of LMAN (Andalman and Fee 2009); in particular, we found that over the period in which FF was maintained at a constant value, the magnitude of reversion caused by LMAN inactivation decreased significantly ($P < 0.01$, ANOVA for difference in mean reversion over time, post hoc Tukey-HSD test for reduced reversion from days 1-2 to days 5-6, $P < 0.05$). This decreasing reversion caused by LMAN inactivations could not be attributed to decreasing efficacy of the inactivations, because LMAN inactivations continued to cause a stable reduction in the variability of FF throughout this period (Fig. 3e, bottom panel; mean reduction of CV across three time periods, 40.3 +/- 4.6%; ANOVA for difference in CV reduction across these periods, $P = 0.6$).

Second, in contrast to results of Andalman and Fee (2009), which indirectly suggest that consolidation of learning in a similar paradigm is completed over a single night, our results indicate that consolidation is a gradual, multi-day process. Indeed, after 3-4 days over which FF was maintained at a fixed, learned value, 27 percent of the learned changes to FF

remained dependent on LMAN (Fig. 3e). To ensure that this continuing influence of LMAN inactivation on the expression of learning was not due to the recent history of disrupting LMAN activity, we examined a subset of the experiments (n=4) in which inactivation of LMAN on day 4 of a maintained shift in FF was preceded by at least two full days and nights since prior inactivation of LMAN. The reversion of learning in these cases was significant (mean reversion: 23.2 +/-5.0%; $P < 0.003$, one-tailed paired t-test). This indicates that consolidation is not fully completed even over a 48 hour period in which LMAN remains active.

Initial expression of learning relies on NMDA receptor activation in RA

We next tested the hypothesis that LMAN activity contributes to the initial expression of learning via glutamatergic projections from LMAN-core to RA rather than through action of LMAN at its other known targets (Fig. 1, Bottjer and Altenau 2010; Gale et al. 2008; Johnson et al. 1997; Kittelberger and Mooney 2005; Mooney 2009b). To test the importance of glutamatergic transmission from LMAN-core to RA in the expression of learning, we reversibly dialyzed the NMDA receptor antagonist, DL-AP5, into RA (Fig. 4a-b). Since synaptic input from LMAN-core to RA is predominantly mediated by NMDA receptors while input from HVC is mediated by a mixture of NMDA and AMPA receptors (Mooney 1992; Mooney and Konishi 1991; Stark and Perkel 1999), blocking NMDA receptors in RA should preferentially block glutamatergic transmission from LMAN to RA while having a lesser effect on transmission within the motor pathway itself (see also Introduction).

Dialysis of AP5 into RA reversed the expression of initial learning in a qualitatively and quantitatively similar manner to LMAN inactivation. An example is shown in Figure 4c. At baseline, before any learning, dialysis of AP5 significantly reduced the variability in the

FF of the target syllable (CV reduction of 47%; $P < 0.01$, permutation test) while causing little change to the mean FF of the syllable or other aspects of song (Fig. 4c, 'baseline'). However, when the mean FF had been shifted over several days in response to aversive reinforcement with WN, the dialysis of AP5 into RA caused a 66% reversion of FF toward the original baseline (Fig. 4c, bottom panel, 'initial shift'). Across five experiments in five birds in which AP5 was infused into RA, we observed similar effects on the expression of learned changes to FF. At baseline, there was a significant reduction in the CV of FF (mean reduction of 36.3 +/- 9.9%; $P < 0.01$, one-tailed paired t-test) but no significant effect on the mean FF (Fig. 4d, 'baseline'; $P = 0.8$, paired t-test). During initial learning, there was again a reduction in CV, but there was additionally a significant reversion of FF back towards baseline (Fig. 4d, 'initial shift'; $P < 0.05$, one-tailed paired t-test). The magnitude of this reversion (46.4 +/- 8.8%) was not significantly different from that observed across experiments in which lidocaine or muscimol were infused directly into LMAN (46.9 +/- 7.0%; $P = 0.9$, unpaired t-test).

The similarity of the reversion induced by inactivating LMAN and by blocking NMDA receptors in RA suggests that in both cases reversion occurs because of interference with LMAN-core's projections to RA, either at the level of LMAN (in the case of inactivation of LMAN) or the level of RA (in the case of NMDA receptor blockade in RA). Additionally, this finding indicates that LMAN-RA glutamatergic release contributes to the initial expression of learning (see Discussion).

Consolidation does not correspond with the establishment of lasting changes to song

The contributions of LMAN to the expression of learning gradually wane (Fig. 3e), indicating a process of consolidation in which the expression of learning is transferred to

other circuitry. Following this consolidation, inactivations of LMAN have only a small effect on the mean FF but still significantly reduce variability of FF, similar to the effects of inactivations at baseline. This similarity raises the question of whether consolidation establishes a new baseline for song structure. If this is the case, then consolidated changes to syllable structure might become engrained so that they are retained even without continued external reinforcement (e.g. Brashers-Krug et al. 1996; Joiner and Smith 2008; Shadmehr and Holcomb 1997). To test this possibility, we turned off WN after maintaining an extended, stable shift in FF and measured subsequent changes to the FF of the target syllable.

Following the cessation of WN reinforcement, birds consistently restored the FF of the target syllable back towards the original baseline in a spontaneous, self-driven recovery process. This is illustrated in Figure 5. Here, for 5 experiments, contingent reinforcement with WN was first used to drive and maintain a learned shift in FF over a period of 7 to 15 days. Based on our previous experiments (i.e. Fig. 3e), we expected that by the end of this period, the expression of learning would have consolidated so that it no longer greatly depended on glutamatergic transmission from LMAN. We directly confirmed this in two experiments by infusing either muscimol into LMAN (red triangle) or APV into RA (red square) on the last day of WN (Fig. 5, day -1). We then turned off WN and monitored subsequent changes to FF that occurred in the absence of external reinforcement (Fig. 5, 'WN off'). In each of the five experiments, FF systematically recovered towards the original baseline value over a period of several days. On average, by the third day following cessation of WN, FF had recovered 84.3% of the way back to the original baseline (Fig. 5, 'mean recovery'). These data indicate that, despite the apparent consolidation of learning following prolonged instruction with contingent WN, a new and lasting 'baseline' for FF is not established. Rather, there is a stable representation of the original baseline FF and the

avian nervous system restores FF back to this baseline via a self-driven process in the absence of continued external instruction.

LMAN contributes to the initial expression of song recovery to baseline

The recovery of FF to the initial pre-learning baseline indicates that birds retain a covert representation of the original baseline even following a period of maintained learning in which FF is held at a constant offset from baseline. This covert representation of the baseline FF raises two possible explanations for the effects of LMAN inactivations on the initial expression of learning reported above (Fig. 3). One possibility is that LMAN inactivations always reverse recent learning, while another possibility is that LMAN inactivations instead reverse changes to song structure that deviate from the original baseline. To distinguish between these possibilities, we inactivated LMAN during the initial recovery of FF back towards baseline following a period of maintained learning. If LMAN inactivation causes a reversal of differences from the original baseline, then inactivation during recovery should cause FF to shift towards baseline. Alternatively, if LMAN inactivation causes a reversal of recent learning, then inactivation during recovery should cause FF to shift *away* from baseline, towards the previously maintained value.

Interfering with input from LMAN to RA during recovery consistently shifted FF *away* from baseline and towards the previously maintained value. An example of this is shown in Figure 6a. In this experiment, reinforcement with WN was used to drive a shift in FF away from baseline and to maintain changes to FF over an 8 day period ('WN on'). As shown above (Fig. 4), infusion of AP5 during the initial shift away from baseline caused a reversion of FF back towards baseline (Fig. 6a, reinforcement day 3). The magnitude of this reversion gradually diminished so that on the last day of reinforcement there was little reversion

towards baseline (reinforcement day 8). Reinforcement with WN was then terminated, and the FF of the target syllable gradually recovered back towards the original baseline. On the second day of recovery, at a point when the FF had recovered 81% of the way back to baseline, AP5 infusion resulted in a large shift in FF away from the original baseline and back towards the previously maintained level of learning (recovery day 2). Such a significant shift *away* from baseline during recovery, and toward the level of previously consolidated learning, was observed in 2 experiments (in 2 birds) in which we tested the effects of LMAN inactivation during spontaneous, self-driven recovery (Fig. 6b, recovery, 'self-driven'; $P < 0.01$, permutation test relative to baseline effects). We additionally tested the effects of inactivating LMAN during recovery in 5 experiments (in 3 birds) in which recovery to baseline was not completely spontaneous but was instead propelled by switching the reinforcement contingency so that syllable renditions with FF closer to the original baseline escaped WN. In these cases too, LMAN inactivation caused FF to shift significantly away from baseline and back towards the previously maintained level of consolidated learning (Fig. 6b, recovery, 'WN driven'; $P < 0.001$, permutation test relative to baseline effects).

After FF had recovered to the original baseline there was a gradual 'reconsolidation' in the sense that the influence of LMAN inactivations on the mean FF progressively waned. For 6 of the 7 recovery experiments we tested the effects of inactivating LMAN (or infusing AP5 into RA) after song remained at baseline for at least 3 days (3-14 days at baseline for 5 experiments and 43 days at baseline for one experiment). Across these 6 experiments, after FF had been maintained at the original baseline, inactivating LMAN no longer caused a significant shift in mean FF (Fig. 6b, 'maintained recovery'; $P > 0.8$, permutation test).

These data indicate that inactivation of LMAN causes a reversion of recent learning, rather than a reversal of differences from the original baseline. Moreover, they indicate that

LMAN plays a similar role during learning in which FF is directed away from baseline with external reinforcement and during self-driven recovery without external reinforcement. In both cases, initial changes to song rely on LMAN for their expression, while later, maintained changes are subserved by mechanisms that do not require LMAN.

Discussion

In this study we address how the cortical analogue LMAN contributes to vocal learning in adult Bengalese finches. Consistent with Andalman and Fee (2009), we found that inactivation of LMAN reversed the initial expression of learning in an aversive reinforcement paradigm. We additionally found that blocking NMDA receptors in nucleus RA within the primary song motor pathway reduced the expression of initial learning in a manner that quantitatively matched the effects of inactivating LMAN (Figs. 3 and 4); this indicates that LMAN contributes to the initial expression of learning via direct glutamatergic projections from LMAN-core to RA and argues against effects mediated by the influence of LMAN on its other targets (Fig. 1). We found that successive inactivations of LMAN over a multi-day period of maintained learning had a gradually decreasing effect on the expression of learning (Fig. 3e), indicating that learning becomes consolidated in circuitry that can operate independently of LMAN. This rules out the possibility that consolidation in this paradigm is completed in a single night and instead suggests that consolidation may gradually develop online during singing. Even after this consolidation, song structure recovered to the original baseline following the termination of reinforcement. This indicates that this form of consolidation does not correspond with a lasting retention of learning (Fig. 5). Finally, we found that LMAN contributed to the initial expression of changes to song during this self-driven recovery process (Fig. 6), indicating that LMAN's role in the

expression of adult learning is not limited to aversive reinforcement learning but extends more broadly to other forms of adaptive plasticity.

Mechanisms by which LMAN contributes to the initial expression of adult learning

Both our study (Fig. 3) and prior work by Andalman and Fee (2009) demonstrate that the expression of initial learning in an adult vocal reinforcement paradigm can be reversed within a period of tens of minutes following the infusion of sodium channel blockers into LMAN. We additionally found that the expression of learning can be reversed by the infusion of muscimol into LMAN (Fig. 3), suggesting that this effect results from inactivation of cell bodies rather than inactivation of fibers passing through or near to LMAN. Our finding that blocking NMDA receptors within RA has a quantitatively similar effect on the expression of learning as inactivating LMAN (Fig. 4) has several additional implications regarding the circuitry and mechanisms by which LMAN activity contributes to the expression of adult vocal learning.

Implications for the possible pathways contributing to the initial expression of learning

We found at baseline that infusions of AP5 into RA reduced rendition-to-rendition variability of adult song, without otherwise disrupting song structure, in a similar manner to lesions or inactivations of LMAN (Fig. 4d, bottom panel; Andalman and Fee 2009; Kao and Brainard 2006; Kao et al. 2005; Stepanek and Doupe 2010). This suggests that infusions of AP5 into RA achieve a functional de-afferentation of RA from its glutamatergic LMAN inputs, without grossly disrupting transmission within the motor pathway (Mooney 1992; Mooney and Konishi 1991; Stark and Perkel 1999). Moreover, because LMAN and the other direct and indirect targets by which LMAN might influence song are spatially distinct from

RA (e.g. Fig. 1: Area X, medial striatum, Ad, mMAN, VP, VTA), it is unlikely that infusions of AP5 into RA act directly to alter transmission at these other targets. Here we find that inactivations of LMAN and infusions of AP5 into RA have quantitatively matched effects on the initial expression of learning (Figs. 3 and 4). Hence, the most parsimonious interpretation of our results is that LMAN contributes to the initial expression of learning via its direct glutamatergic projections to RA.

The finding that local blockade of NMDA receptors in RA can reverse the expression of recent learning is similar to previous findings in other systems in which local blockade of NMDA receptors also reversed the expression of recent learning (Feldman et al. 1996; Fendt 2001; Lee et al. 2001). One interpretation of these prior results is that during learning, local synaptic plasticity creates new local synapses that preferentially rely on NMDA receptor dependent transmission; according to this possibility, NMDA receptor blockade interferes with the expression of learning mediated by these new local synapses (c.f. Feldman et al, 1996). Our results suggest an alternative possibility; in those prior studies, NMDA receptor blockade could have interfered with the expression of learning mediated by long-range, NMDA receptor-dependent projections from other structures to the site of infusion, as appears to be the case in our experiments.

Implications for the possible role of neurotrophins in the initial expression of learning

Previous work has suggested that neurotrophin release from LMAN to RA might play a critical role in the initial expression of learned changes to song (Akutagawa and Konishi 1998; Johnson et al. 1997; Kittelberger and Mooney 2005; 1999). Neurotrophins are thought to be released from LMAN to RA (Akutagawa and Konishi 1998; Johnson et al. 1997), and in juvenile birds, structural and functional changes to RA following LMAN lesions arise in

part due to depletion of this source of neurotrophins (Johnson et al. 1997; Kittelberger and Mooney 1999). In adults, introducing the neurotrophin BDNF into RA can drive formation of new synapses from HVC to RA neurons and cause changes to song (Kittelberger and Mooney 2005). Though the timecourse of the effects of manipulating neurotrophin levels within RA remains unclear, in other systems alteration of neurotrophin levels can affect both electrophysiological and structural measures of synaptic connectivity on a timescale of minutes or even more rapidly (Collin et al. 2001; Kafitz et al. 1999; Kossel et al. 2001; Schuman 1999; Wardle and Poo 2003; Yang et al. 2002). Noteworthy in this respect are studies indicating that removal of neurotrophins can preferentially reverse weaker or more recently formed synapses within minutes (e.g. Berninger et al. 1999). These results suggest that effects of inactivating LMAN on the expression of learning that appear over a period of minutes, as in our experiments and in those of Andalman and Fee (2009), could arise by preventing neurotrophin release into RA; in this scenario, reduced neurotrophin levels might preferentially attenuate or silence newly modified synapses within the motor pathway that contribute to the expression of learning.

Our finding that infusion of AP5 into RA causes similar effects on the expression of learning as silencing LMAN (Fig. 4) argues against a mechanism that relies solely on neurotrophin release. Since the blockade of NMDA receptors in RA is thought to act post-synaptically on RA neurons, it should not alter release of neurotrophins from LMAN into RA. One caveat to this interpretation is that, in other systems, blockade of pre-synaptic NMDA receptors has been shown to affect glutamate release (Bardoni et al. 2004). It therefore remains possible that neurotrophin release into RA could be altered in this way, though such a mechanism has not been reported in the songbird. Likewise, even if AP5 infusion does not block neurotrophin release, possible contributions of neurotrophins to the

expression of learning might be prevented by interfering with postsynaptic activity via NMDA receptor blockade (McAllister et al. 1996). Hence, while our results indicate that glutamatergic transmission from LMAN to NMDA receptors in RA is essential for the initial expression of learning, they do not rule out the possibility that neurotrophin release from LMAN to RA also participates in the initial expression of learning or other stages of learning.

How might LMAN to RA glutamatergic transmission contribute to the initial expression of learning?

Our findings and prior results (Andalman and Fee 2009) are compatible with at least two conceptually distinct models whereby glutamatergic transmission from LMAN to RA could contribute to the initial expression of learning. According to an 'instructive' or 'biasing' model, patterned activity from LMAN directs detailed moment-by-moment changes to RA activity that are responsible for the initial expression of learning. In this model, silencing LMAN causes a reversion of recent learning by removing those structured patterns of activity that are responsible for directing the expression of learning. Consistent with this possibility, LMAN activity is temporally patterned during singing, suggesting that LMAN could provide biasing input to RA that implements changes to song structure at specific time points during song (Hessler and Doupe 1999; Kao et al. 2005; Kao et al. 2008; Leonardo 2004). Moreover, alterations in the pattern of LMAN activity elicited by microstimulation of LMAN in singing birds can acutely implement changes to song structure, including changes to the FF of individual syllables (Kao et al. 2005), demonstrating that alteration of LMAN activity patterns is sufficient to cause real-time changes to song structure.

According to an alternative, but not mutually exclusive, 'permissive' or 'gating' model, the plasticity that underlies the initial expression of learning resides in the motor

pathway itself, and input from LMAN to the motor pathway enables the expression of this plasticity but does not direct the specific changes to RA activity responsible for learning. In this model, silencing LMAN causes a reversion of recent learning by removing neural activity or trophic factors that are required for the expression of plasticity that resides within the motor pathway. Consistent with this possibility, LMAN is tonically active during singing (Hessler and Doupe, 1999; Leonardo, 2004; Kao et al., 2005; Kao et al., 2008). Therefore, silencing LMAN will remove excitatory drive to RA in a manner that is likely to alter the efficacy of HVC to RA (and RA to RA) synapses (Mooney and Konishi, 1991; Stark and Perkel, 1999). Moreover, theoretical work investigating how LMAN might influence patterns of activity within RA indicates that glutamate release from LMAN onto NMDA receptors in RA is well-positioned to modulate or 'gate' transmission at other glutamatergic synapses within RA (Kepecs and Raghavachari 2007). Hence, tonic glutamate release from LMAN might enable the expression of learning-related changes to the patterning of activity in RA without specifically implementing those changes. In this case, the initial expression of learning could rely on changes to connectivity in the motor pathway, without necessitating any learning-related changes to the inputs from LMAN (c.f. Mooney 2009b).

Time course of consolidation

Our results and those of Andalman and Fee (2009) indicate that learned changes to song structure eventually become consolidated in the sense that the effects of LMAN inactivation on the expression of learning wane over time. This consolidation of learning so that its expression becomes LMAN-independent implies that plasticity eventually develops outside LMAN. Andalman and Fee (2009) drove a trajectory of learning in which the fundamental frequency of a target syllable was constantly changing and therefore did not

directly assess how the contributions of LMAN to a fixed amount of learning change over time. However, their results indirectly suggest that consolidation is completed over a single night; when LMAN was inactivated during the day, FF tended to revert to the level sung that morning and no further. Here, we maintained learned changes to FF at a fixed value over a period of days so that we could directly determine the time course over which learning consolidates to become LMAN-independent. We found that consolidation in our experiments reflects a slow multi-day process and that even after 4 days over which FF was held at a fixed value, a significant amount of learning remained LMAN-dependent (Fig. 3e). This finding demonstrates that consolidation in our paradigm is not completed over a single night and instead indicates that consolidation may gradually accumulate online as birds are singing.

The LMAN-dependent component of learning that remains after several days of maintained learning could reflect ongoing but incomplete consolidation. Alternatively, some learning may remain LMAN-dependent, regardless of how long learned changes to FF are maintained at a constant value via external reinforcement. Results from our study (Fig. 5) demonstrate that birds retain a capacity and impetus to restore song back to its original baseline structure even after an extended period of maintained learning (c.f. Sober and Brainard 2009; Tumer and Brainard 2007). This suggests that during maintained learning, while FF remains at a constant value, there may be an underlying equilibrium between learning directed away from baseline by external reinforcement and learning directed towards baseline by the bird's own impetus to restore song to baseline. Under these circumstances, there may continue to be ongoing learning, even while FF remains constant. Such ongoing learning during stable behavior, which has been inferred in analogous studies of motor adaptation (Criscimagna-Hemminger and Shadmehr 2008; Sober and Brainard 2009; Tumer

and Brainard 2007), could explain LMAN's continuing influence on the expression of learning.

The consolidation of learning does not enable the retention of learning

We found that consolidation does not correspond with the establishment of a new stable baseline structure of adult song. Rather, there is some covert representation of the original baseline structure that can guide self-driven recovery of song once external reinforcement is removed (e.g. Fig. 5). Previous work suggests that this covert representation may be a stable perceptual target to which the bird restores his song via error corrective learning (Sober and Brainard 2009). The contributions of LMAN to self-driven song recovery (Fig. 6) additionally suggest that this recovery is an active error corrective process, rather than relaxation of the motor system to its prior state. It remains to be determined whether and under what conditions learned changes to adult song can become stabilized in the sense that they are maintained even after external instruction is removed.

Contributions of LMAN to song recovery

The effects of inactivating LMAN during song recovery allowed us to distinguish between two alternative explanations for the reversion toward baseline caused by LMAN inactivations (Figs. 3 and 4; Andalman and Fee, 2009). This reversion could occur because inactivating LMAN reverses recent learning, or because inactivating LMAN reveals a covert internal representation of the original baseline. Both possibilities (i.e. reversion of learning and return to baseline) are consistent with the finding that during learning in which FF is directed away from baseline, inactivations of LMAN cause a shift in FF towards baseline. However, these possibilities are explicitly distinguished by our experiments in which LMAN

is inactivated during recovery of FF to the original baseline after a period of reinforcement-driven learning. In this case, we found that inactivation of LMAN causes a shift in FF away from the original baseline (Fig. 6), inconsistent with the possibility that such inactivation somehow reveals the baseline representation. Rather, this finding indicates that both during reinforcement learning and during recovery, LMAN contributes to the initial expression of changes to song.

The effects of inactivating LMAN during self-driven recovery also indicate that avian basal ganglia circuitry contributes similarly to two distinct forms of learning. The initial learning in our paradigm and in Andalman and Fee (2009) relies on external reinforcement of syllable variants that have FF above (or below) a threshold value. Basal ganglia circuitry is widely thought to play a central role in this form of reinforcement learning (Doya 2000). In contrast, recovery to baseline following the cessation of reinforcement is a self-driven process that requires no external instruction. This recovery seems likely to reflect an error corrective learning process in which song is actively adjusted via auditory feedback to match a stably represented acoustic target (Sober and Brainard 2009). Though error corrective learning is often associated with cerebellar mechanisms (Doya 2000; Raymond et al. 1996), here we find that LMAN, the output nucleus of a basal ganglia circuit, contributes to the initial expression of self-driven recovery in a manner that parallels its contributions to reinforcement-driven learning. This generalization of LMAN's contributions to the expression of distinct forms of adult learning suggests that LMAN may also play a crucial role in the initial expression of changes to juvenile song during sensorimotor learning, in which auditory feedback guides developing vocalizations towards a stable acoustic target, or 'template'.

Variability and learning

Previous studies have found that lesioning or inactivating LMAN in adult birds reduces rendition-to-rendition variation in adult song (Hampton et al. 2009; Kao and Brainard 2006; Kao et al. 2005; Stepanek and Doupe 2010). Here, we similarly found that inactivation of LMAN and interruption of LMAN-RA glutamatergic transmission reduced rendition-to-rendition variation in acoustic structure of adult song across distinct stages of learning (Figs. 3 and 4). This finding is consistent with models of adult song learning in which variability introduced by LMAN-core to the motor pathway serves as a form of motor exploration that enables subsequent learning (Bottjer et al. 1984; Doya and Sejnowski 2000; Fiete et al. 2007; Jarvis et al. 1998; Kao et al. 2005; Miller et al. 2010; Olveczky et al. 2005). We found that LMAN's contributions to variability were maintained at a constant level even while LMAN's contributions to the expression of learning waned (Fig. 3e). This indicates that the nervous system may be continuously engaged in a process of motor exploration to optimize song structure.

Our findings support an emerging view that frontal cortical circuits associated with the basal ganglia can direct the initial expression of learning via their top-down influence on primary motor circuitry (Andalman and Fee 2009; Isoda and Hikosaka 2007; Miller and Cohen 2001; Narayanan and Laubach 2006) and that over time, control over the expression of learning gradually shifts to more posterior motor circuits (Sakai et al. 1998; Shadmehr and Holcomb 1997). We find that in the songbird, the cortical analogue LMAN is required for the initial expression of learning and contributes to behavioral variation important for learning. This raises the possibility that in mammals, the frontal cortical circuits that similarly contribute to the initial expression of learning might also contribute to the motor exploration that enables such learning. The relative simplicity of songbird neural circuitry

and the highly quantifiable nature of song may facilitate further elucidation of the precise mechanisms by which frontal cortical areas and basal ganglia circuitry contribute to motor learning and motor consolidation.

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Chapter 1: Figure 1

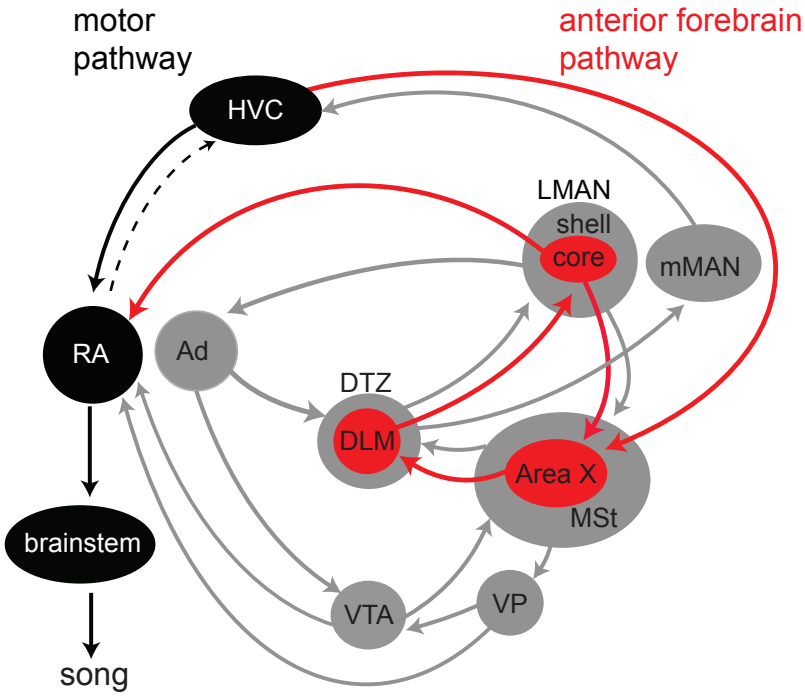

Figure 1. Neural circuitry contributing to song production and learning. Neural pathways that control song production and learning include a song motor pathway (black nuclei and arrows) that controls much of the moment-by-moment structure of song as well as a cortical-basal ganglia circuit, the anterior forebrain pathway (AFP; red nuclei and arrows), that plays a crucial role in juvenile song learning and adult vocal plasticity. The song motor pathway includes forebrain nuclei HVC and RA, analogous to vocal motor cortex. RA projects to brainstem structures that control the vocal musculature; changes in RA activity are likely to underlie learned changes to syllable structure (Leonardo and Fee 2005; Sober et al. 2008; Vu et al. 1994; Yu and Margoliash 1996). LMAN, a cortical analogue that is part of the AFP, plays a crucial role in juvenile and adult song plasticity. LMAN activity could influence RA during learning via a number of direct and indirect pathways. Neurons in LMAN-core make direct glutamatergic projections onto RA neurons and are also thought to release neurotrophins onto RA neurons. Neurons in LMAN-core also project to the basal ganglia homologue Area X, which is well-positioned to influence activity in neuromodulatory nuclei such as ventral pallidum (VP), a cholinergic nucleus projecting to RA and HVC, as well as the ventral tegmental area (VTA), a dopaminergic nucleus that also projects to RA and HVC (Appeltants et al. 2000; Appeltants et al. 2002; Gale and Perkel 2010; Gale et al. 2008; Li and Sakaguchi 1997). LMAN activity could also influence RA activity via indirect projections from LMAN-shell to the motor pathway. LMAN-shell, part of a distinct motor circuit that plays a functional role in song learning, projects both to Ad and the medial striatum ('MSt'), surrounding Area X (Bottjer and Altenau 2010; Iyengar et al. 1999). Figure adapted from Bottjer and Altenau (2010), and Gale et al. (2008).

Chapter 1: Figure 2

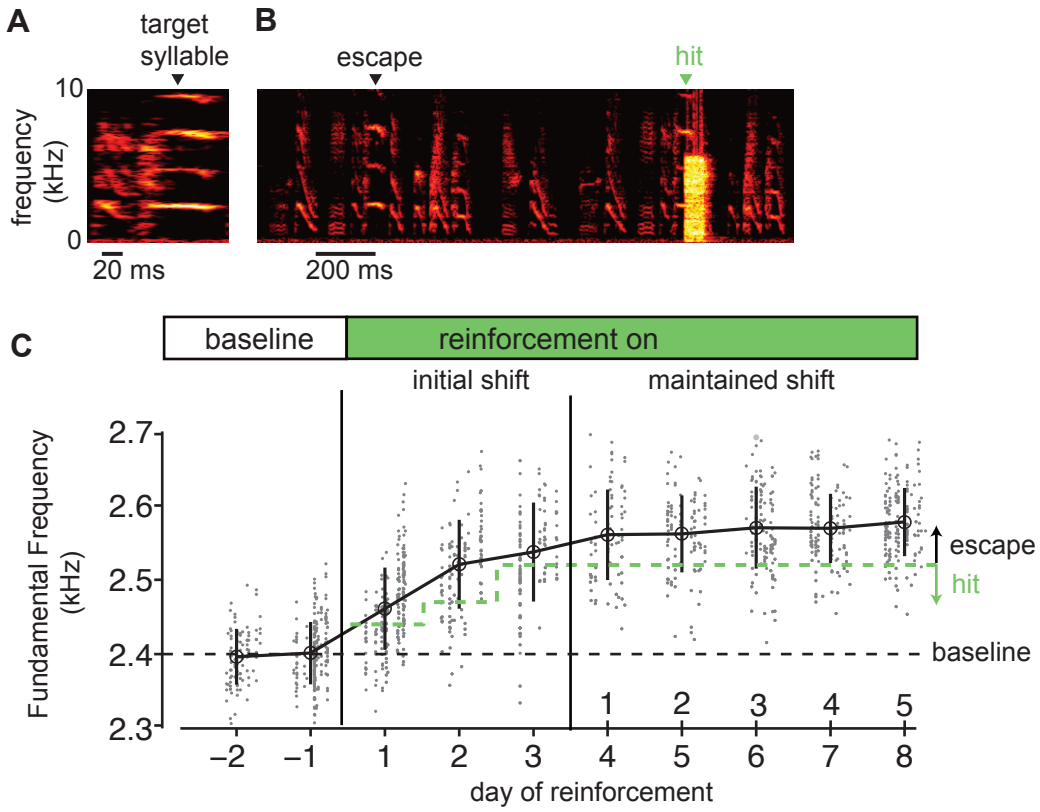

Figure 2: Example trajectory of changes to syllable structure driven via a reinforcement learning paradigm

A, Spectrogram of the syllable targeted for reinforcement learning. An automated system (Tumer and Brainard 2007), reliably detected a specific time point in the target syllable (inverted black triangle, 'target syllable'). B, On renditions of the target syllable with a fundamental frequency (FF) below a set threshold, no reinforcement was delivered ('escape'); on renditions of the target syllable with a FF above the threshold, an aversive reinforcement signal, a 60 ms white noise stimulus (WN), was played over the target syllable ('hit'). C, Example trajectory of learning. During the baseline period ('baseline', days -2 to -1), no reinforcement was delivered. The mean FF at baseline was 2400 Hz (open black circles and vertical lines indicate daily mean FF +/- 1 s.d.). During an initial learning period ('initial shift', days 1-3), WN was delivered to syllable renditions with FF below a set threshold (dashed green line). In response to this reinforcement, the values of FF of the target syllable (gray points) gradually increased. After two days of upward shift in FF, the threshold was fixed at 2520 Hz. In response to this stable reinforcement contingency, the FF of the target syllable stabilized at ~2560 Hz, a fixed offset of 160 Hz from baseline. This learned shift in FF was maintained for 5 consecutive days ('maintained shift', days 4-8). In this figure and subsequent figures, a random sample (10-15 %) of all songs are displayed and were used to measure syllable FF (see Methods).

Chapter 1: Figure 3

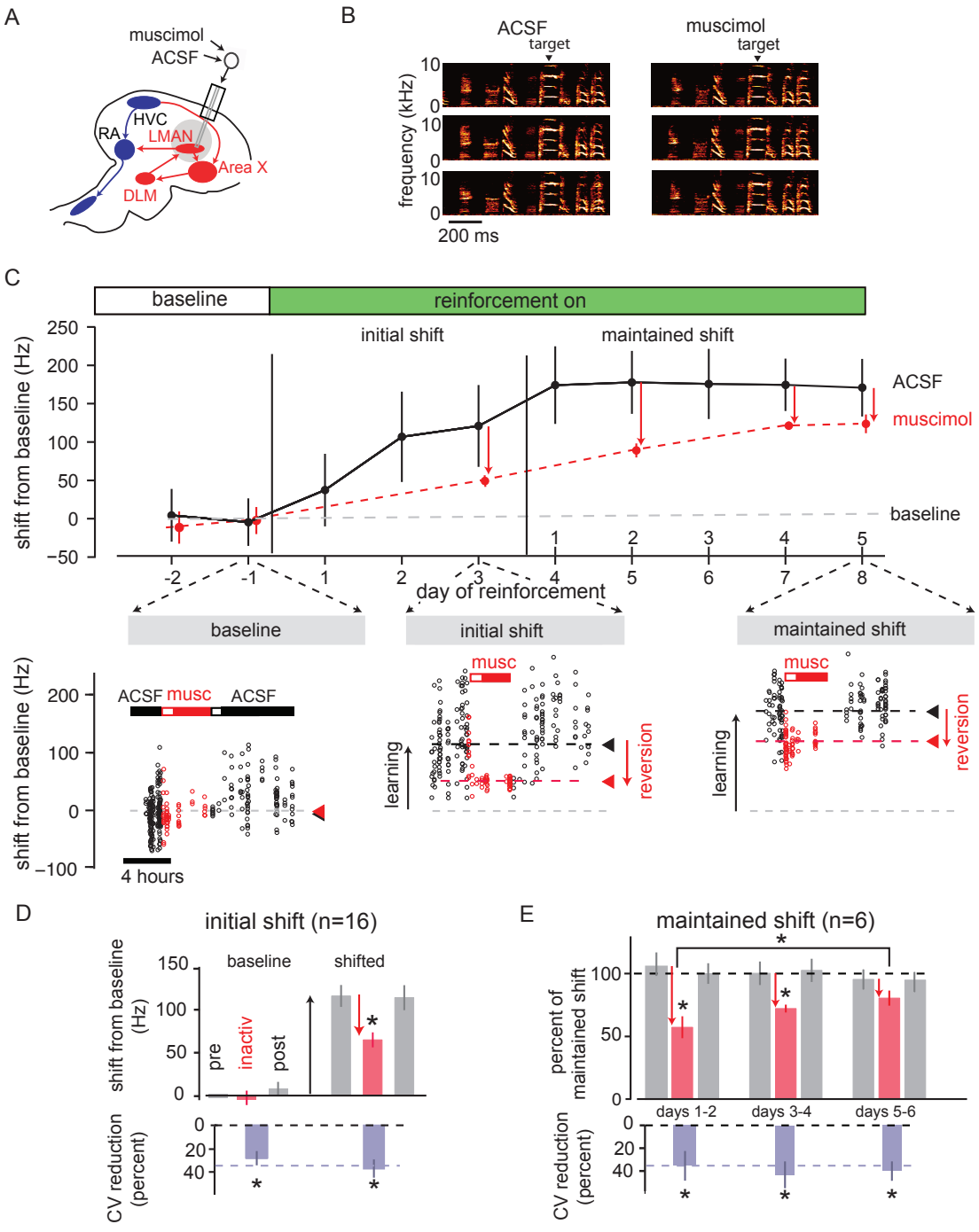

Figure 3. The expression of learned changes to syllable structure initially relies on LMAN activity and gradually consolidates to become LMAN-independent over multiple days.

Figure 3. The expression of learned changes to syllable structure initially relies on LMAN activity and gradually consolidates to become LMAN-independent over multiple days.

A, Experimental design. Dialysis probes (one hemisphere shown, sagittal section) were bilaterally implanted into LMAN; retrodialysis solution was switched from a control ACSF solution to muscimol, a GABAA agonist, or lidocaine, a Na+ channel blocker. B, Spectrograms illustrate preservation of the overall spectrotemporal structure of song following a switch from dialysis of ACSF to dialysis of muscimol. Also shown is the syllable targeted for learning in this experiment ('target'). C, Effects of muscimol retrodialysis during a trajectory of vocal learning. As in Fig. 2, WN reinforcement was used to drive a shift in FF of the target syllable during a period of initial learning ('initial shift', days 1-3) and then FF was maintained at a fixed offset from baseline ('maintained shift', days 4-8 of reinforcement, corresponding with days 1-5 of the maintained shift). Top panel shows mean values of FF during periods of ACSF dialysis (black circles and vertical lines indicate daily mean FF +/- 1 s.d.) as well as mean values of FF during periods of several hours of dialysis with 200 µM muscimol (red circles and vertical lines). Bottom panel shows expanded time axis with values of FF for individual syllable renditions on three specific days: baseline day -1, reinforcement day 3, and reinforcement day 8 (day 5 of maintained shift). Horizontal bars on bottom panel indicate time periods of drug infusions ('ACSF', black; 'musc', red): solid bars indicate the period included for analysis and open bars indicate periods excluded from analysis due to delayed onset of drug effects. Muscimol infusion on day 3 of learning caused a rapid downward reversion of FF towards the original baseline (downward red arrow, 'reversion'). Subsequent muscimol infusions on days 5, 7, and 8 of learning, while the FF was maintained at a learned offset of ~180 Hz from baseline, caused gradually decreasing reversions. D, Summary across all learning trajectories of the effects of inactivating LMAN during the initial shift period. All data are normalized so that positive values indicate shifts of FF in the direction of learning. At baseline, LMAN inactivation caused no significant change in mean FF compared to ACSF pre and post periods ('baseline'; n=16 experiments in 7 birds; red bars indicate mean +/- SEM for muscimol/lidocaine experiments; gray bars show mean +/- SEM for pre and post periods). In contrast, during the initial shift, inactivation of LMAN caused a significant reversion of mean FF towards baseline (mean reversion 46.9%, $P < 0.01$, one-tailed t-test). Inactivations of LMAN reduced rendition-to-rendition variability in FF during both the baseline and initial shift period (bottom panel, purple bars). For comparison, in this and subsequent figures, the dashed line shows the reduction in CV of FF (34%) reported following lesions of LMAN in adult Bengalese finches from a previous study (Hampton et al. 2009). E, Summary effects of disrupting LMAN activity across six learning trajectories (n=4 birds) in which learning was maintained at a stable offset from baseline for at least five days. Over this period, the LMAN-dependent component of learning gradually decreased from 45% to 15% while the LMAN-independent component of learning gradually increased. The reduction in variability caused by LMAN inactivations remained stable across these time periods (bottom panel, purple bars).

Chapter 1: Figure 4

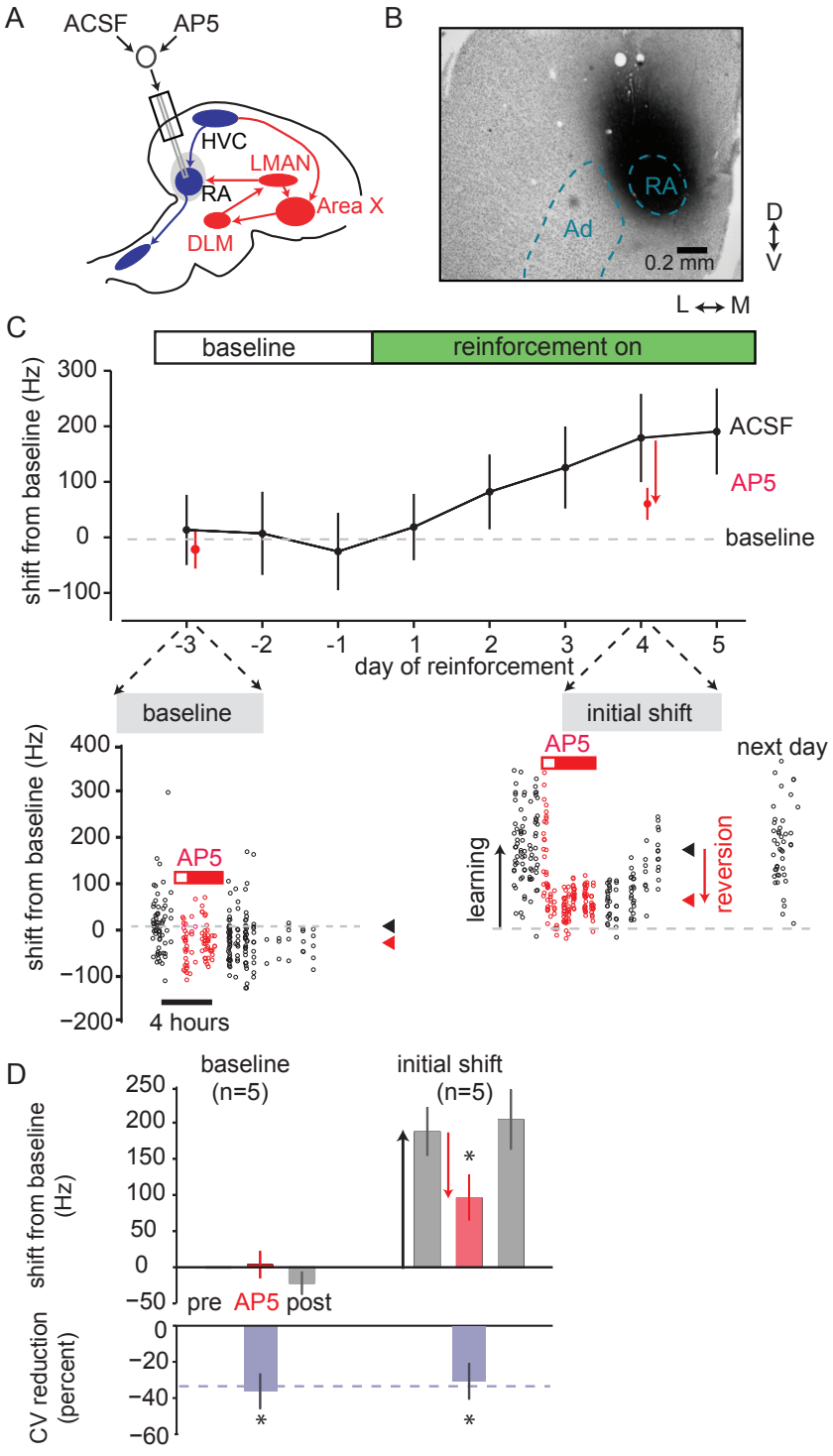

Figure 4. The initial expression of learned changes to syllable structure relies on NMDA receptor activation in RA.

Figure 4. The initial expression of learned changes to syllable structure relies on NMDA receptor activation in RA.

A, Experimental design. Dialysis probes were implanted bilaterally into RA (one hemisphere shown, sagittal section). DL-AP5, an NMDA-receptor antagonist, was dialyzed across the probes. B, Example of drug spread; coronal section. Dark biotin stain shows spread of biotinylated muscimol used to infer drug spread, which encompasses the entirety of RA but not Ad. C, Effects of dialysis of AP5 at baseline and during learning. Top panel shows daily mean values of FF during periods of ACSF infusion and interleaved periods of AP5 infusion. Bottom panel shows raw values of syllable FF on baseline day -3 and reinforcement day 4. At baseline, retrodialysis of AP5 caused little change to mean FF. In contrast, retrodialysis of AP5 on day 4 of reinforcement caused a rapid and large (119 Hz) reversion of learned changes to FF. D, Summary effects of AP5 infusion during the initial shift period ($n = 5$ experiments in 5 birds). AP5 dialysis at baseline caused no significant change in FF relative to pre and post ACSF periods ($P = 0.8$, paired t-test). In contrast, during the initial shift period, AP5 dialysis caused a significant reversion of FF towards the original baseline ($P < 0.05$, one-tailed paired t-test). During both periods, AP5 significantly reduced the rendition-to-rendition variability in FF ($P < 0.05$, one-tailed paired t-test). Conventions as in Figure 3.

Chapter 1: Figure 5

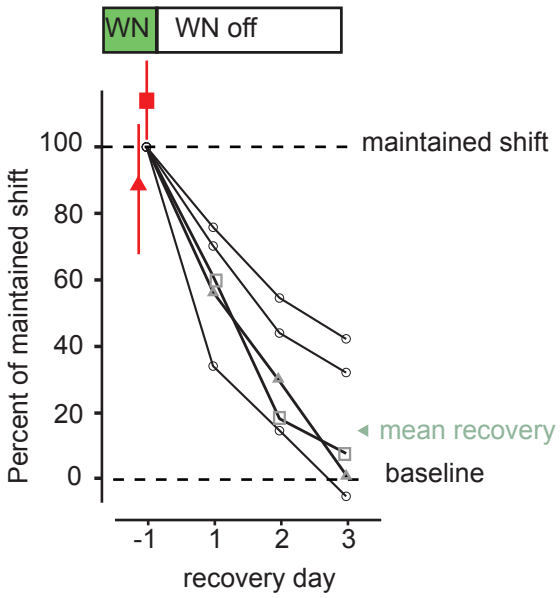

Figure 5. Syllable structure recovers to the original baseline following cessation of reinforcement even after learning has consolidated.

Trajectory of syllable FF for 5 birds over the last day (recovery day -1) of a maintained shift in FF driven via white noise ('WN') and over the following three days in which no WN was played ('WN off'; recovery days 1-3). All data are normalized relative to the magnitude of the shift in FF on the last day of the maintained shift (duration of maintained shifts ranged from 4-7 days). Two birds were equipped with retrodialysis probes, allowing confirmation that consolidation had occurred by the last day of the maintained shift, so that the expression of learning no longer depended on LMAN (red triangle, muscimol infusion in LMAN; red square, AP5 infusion in RA; error bars indicate +/- 1 s.d.). In all 5 birds, following the termination of reinforcement, syllable FF recovered back toward the original baseline over three days (recovery days 1-3; gray triangles and squares show recovery trajectories for birds in which consolidation was confirmed with muscimol and AP5 respectively). By day 3, birds had recovered on average 81% of the difference from the original baseline (green triangle, 'mean recovery').

Chapter 1: Figure 6

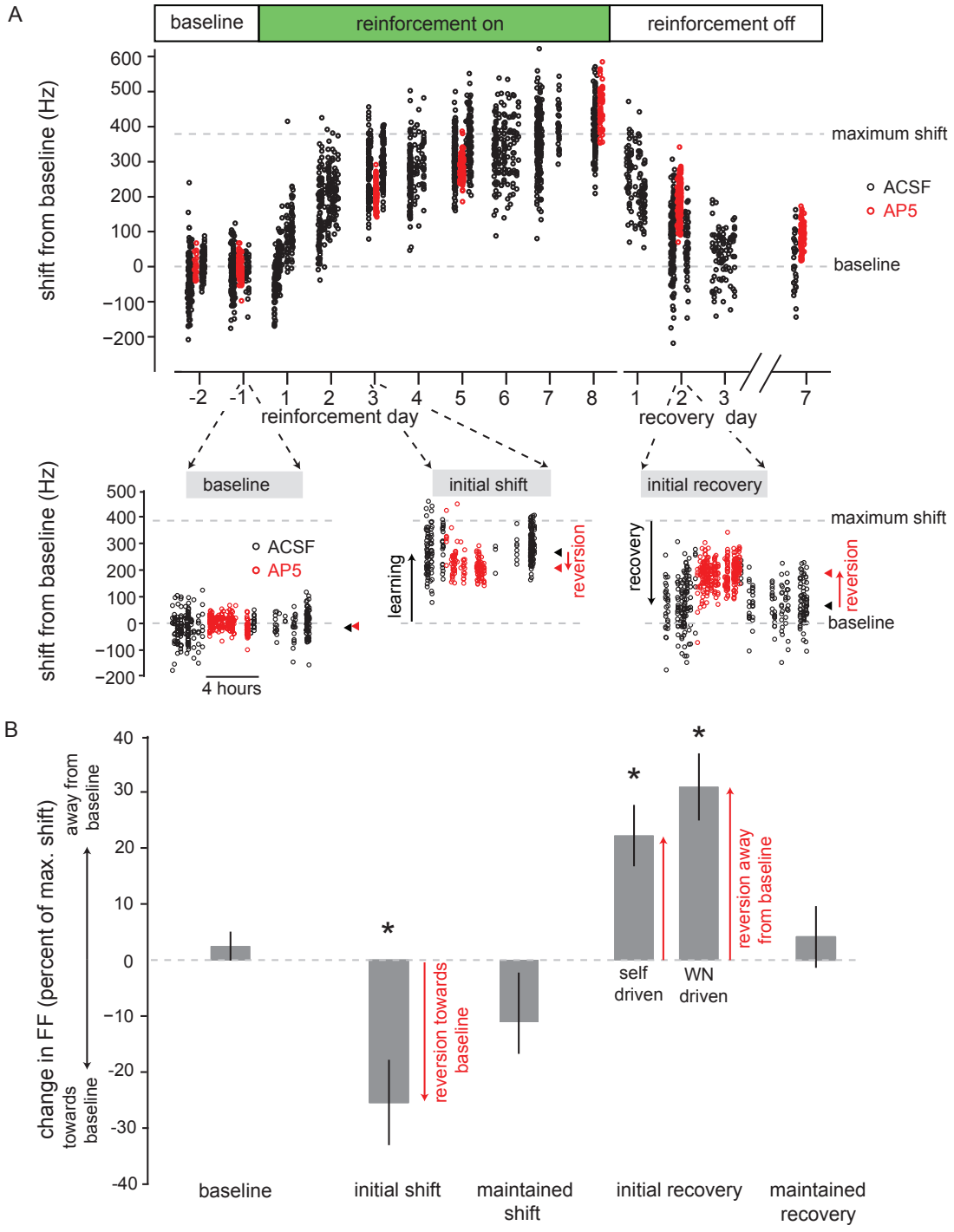

Figure 6. Contributions of LMAN to recovery of syllable structure.

Figure 6. Contributions of LMAN to recovery of syllable structure.

A, Example effects of AP5 dialysis into RA during self-driven recovery towards baseline. Top panel shows values of FF for individual syllable renditions. Bottom panel shows FF on an expanded time scale for baseline (day -1), initial shift (day 3 of reinforcement) and initial recovery (day 2 following termination of WN). During the initial shift, infusion of AP5 caused a reversion of FF towards baseline, as previously observed. During initial recovery, infusion of AP5 caused a reversion of FF towards the previously maintained level of learning, away from baseline. B, Summary effects of blocking LMAN input to RA, via infusion of muscimol in LMAN (n=6 experiments) or infusion of AP5 in RA (n=1 experiment), at 5 stages of learning and recovery. Effects are plotted as percent change in FF relative to the magnitude of the maintained shift in FF. Interfering with LMAN's input to RA caused a significant reversion towards baseline during the initial shift period (downward red arrow) and a significant reversion away from baseline during the initial recovery period (upward red arrow), both for experiments in which recovery was self-driven ('self driven', n=2 experiments; $P < 0.01$, permutation test with baseline effects) and in which recovery was propelled by WN ('WN driven', n=5 experiments; $P < 0.001$, permutation test). Error bars denote mean +/- SEM.

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Chapter 2: Variable sequencing is actively maintained in a well-learned motor skill

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Abstract

Rendition-to-rendition variation in the sequencing of actions occurs in many natural behaviors, including predator avoidance, foraging, and female courtship. Sequence variation has been suggested to confer benefits, raising the possibility that such variation does not reflect uncontrollable noise but instead is actively maintained. We tested this possibility for adult Bengalese finch song, a learned motor skill in which there is rendition-to-rendition variation in how discrete 'syllables' are sequenced. We found that the probabilities of transitions between syllables are ordinarily stable over many weeks (i.e. syllable 'a' might transition to 'b' with 70% probability and to 'c' with 30% probability). We then tested whether these probabilities could be modified, by delivering an aversive stimulus following one transition (e.g. 'a-b') but not the alternative (e.g. 'a-c'). Such differential reinforcement induced gradual, adaptive decreases in the probability of transitions targeted with the aversive stimulus and compensatory increases in the probability of alternative transitions. This indicates that the normal stability of transition probabilities does not result from hard-wired, immutable premotor circuitry. Rather, adult birds can monitor the consequences of producing different transitions and adaptively adjust sequencing. Despite this capacity for modification, when we terminated reinforcement, birds gradually restored transition probabilities to their baseline values. Therefore, a representation of baseline probabilities is retained during sequence modification, and the nervous system has the impetus to restore these values. Together, our results indicate that the statistics of sequence variation in a motor skill can reflect an end point of learning that is stably maintained via continual self-monitoring.

Introduction

Many complex behaviors are composed of distinct elements performed in a sequence. When such behaviors are practiced extensively, the sequencing of elements can become highly stereotyped (Immelmann, 1969; Schwartz, 1980; Cohen et al., 1990; Grafton et al., 2002). However, rendition-to-rendition variation in sequencing can persist even in well-practiced behaviors where individual elements have become highly stereotyped. For example, persistent variation in sequencing occurs in trained operant behaviors, in rodent and insect grooming, and in birdsong (Schwartz, 1980; Lefebvre, 1981; Berridge, 1990; Okanoya, 2004). Sequence variation has been hypothesized to aid in attracting mates, in evading predators, and in optimal foraging (Humphries and Driver, 1967; Real and Caraco, 1986; Searcy and Andersson, 1986). Thus, rather than reflecting a limitation in motor control, sequence variation may be a feature of learned behavior that is actively maintained.

Here we test this possibility in adult birdsong, a learned behavior in which the statistics of sequencing (e.g. the probabilities of transitions between distinct elements) can be monitored precisely. Birdsong, like speech, gradually progresses from variable 'babbling' to a highly stereotyped, 'crystallized' adult form (Marler and Tamura, 1964; Mooney, 2009). Production of the crystallized adult song involves two hierarchically distinct levels of motor control – over individual vocal elements, termed 'syllables', which have high acoustic stereotypy, and over the sequencing of those syllables (Vu et al., 1994; Yu and Margoliash, 1996; Tchernichovski et al., 2001; Fee et al., 2004; Ashmore et al., 2005; Horita et al., 2008; Fujimoto et al., 2011). In some species, such as the zebra finch, a single sequence of syllables ('a-b-c-d') is performed with little variation (Zann and Bamford, 1996). However, in other species, including the Bengalese finch, syllable sequencing can remain highly variable even in adult song (Okanoya, 2004; Sakata and Brainard, 2006; Jin, 2009). For

example, at a ‘branch point’ in Bengalese finch song, syllable ‘a’ might transition to ‘b’ 70% of the time and to ‘c’ 30% of the time (e.g. Fig. 1).

In this study we investigate the rules that govern the stability of sequencing in adult Bengalese finch song. We first demonstrate that the probabilities of transitions between syllables ordinarily remain stable over weeks. Such stability could reflect stochastic output of a hard-wired pattern generator, consistent with a view of adult song as a 'crystallized' behavior that is not subject to adaptive modification (Immelmann, 1969; Marler, 1970). However, recent experiments indicate that adult birds can modify the acoustic structure of individual syllables to reduce aversive feedback or to correct perceived errors in production. Moreover, birds restore the original structure of song in the absence of continued instruction (Tumer and Brainard, 2007; Sober and Brainard, 2009). Thus, for individual syllables, the normal stability of acoustic structure derives from an active process of maintenance, rather than a loss of plasticity.

Here we investigate whether adult birds exert analogous adaptive control over syllable sequencing. First, we test whether birds can adjust probabilities of transitions between syllables in a directed manner in response to differential reinforcement. Then, we test whether birds restore these probabilities to their baseline values following termination of reinforcement. Our results indicate that the stability of transition probabilities does not simply reflect hard-wiring of neural circuitry, but instead depends on a capacity for adaptive adjustment coupled with an impetus to actively maintain probabilities at specific values.

Methods

Subjects

17 adult (>150 d old; range 150-933 d) Bengalese finches (*Lonchura striata domestica*) were used in this study. All birds were bred in our colony and housed with their

parents until at least 60 d of age. During experiments, birds were isolated and housed individually in sound-attenuating chambers (Acoustic Systems) on a 14 hr on/10 hr off light cycle. All song recordings were of undirected song (i.e. no female was present). All procedures were performed in accordance with protocols approved by the University of California, San Francisco Institutional Animal Care and Use Committee.

We investigated two types of sequence variation in the adult Bengalese finch song - branch points, where one syllable can be followed by one of various distinct alternative syllables (Figs. 1-3) and repeats (Fig. 4), where one syllable was repeated a variable number of times. Our data sets derive from birds with songs that contained one or more instances of these forms of sequence variation.

Computerized control of sound recording and sound playback.

Song recording and delivery of auditory feedback were controlled using modified versions of EvTAF (Tumer and Brainard, 2007). Song was recorded continuously, in 8 ms segments. We detected specific syllable transitions in song by comparing the spectral structure of recorded sound segments to multiple, successive spectral templates (Tumer and Brainard, 2007; Warren et al., 2011). For instance, we detected the transition 'a-b' (as opposed to 'a-c' or 'x-b', where 'x' refers to any syllable other than 'a') through successive spectral detection of 'a' and 'b' within a time interval that ensured that they were performed consecutively. Sequences were differentially reinforced via playback of loud white noise ('WN'; duration 50-80 ms). Prior to initiating WN playback, song was recorded during a baseline period (2-4 days) used to define baseline transition probabilities.

For differential reinforcement of transitions at branch points (e.g. Figs. 2-3), we played WN over the latter syllable in the targeted transition and not over alternative, non-targeted transitions. For instance, if a transition from 'a' to 'b' was targeted with WN, WN

was played back over syllable ‘b’ when syllable ‘b’ followed syllable ‘a’. In 10/13 experiments targeting transitions at branch points, there were two distinct possible transitions (e.g. ‘a’ could transition to ‘b’ or ‘c’). In 3 experiments, there were three possible transitions (‘a’ could transition to ‘b’, ‘c’ or ‘d’); in these cases, one transition was targeted with WN and two were not. The mean latency from syllable onset to onset of WN delivery, across all experiments, was 24.5 +/- 2.4 ms. WN playback was not contingent on variation in syllable acoustic structure (i.e. all acoustic variants of the targeted syllable at the branch point elicited WN playback). WN playback was maintained for 4-11 days.

A *repeat* was defined as one instance in which one syllable was sung consecutively a variable number of times. *Repeat length* was defined as the number of times (in a given repeat) the syllable was sung consecutively. Variation in repeat length was differentially reinforced by playing WN over all syllables in a given repeat after a repeat length threshold was exceeded. For instance, if the repeat length threshold was set to a value of 2, no WN was played over the first or second syllable in the repeat, but WN was played over the third syllable and all subsequent syllables in the repeat. Differential reinforcement of repeat length via WN playback was maintained for 2-5 days.

In both the experiments involving branch points and repeats, WN was omitted in a random subset (5-10%) of 'catch' songs. This subset was used to measure transition probabilities and repeat length independently of any acute effects of disrupting auditory feedback (as described in Sakata and Brainard, 2006). Following termination of reinforcement, we monitored song for a period of at least 3 days.

Analysis

All analysis was performed using custom MATLAB software.

The probability of a specific syllable transition at a branch point was defined as the

fractional occurrence of that transition, relative to all possible transitions at that branch point (i.e. for a given branch point, all distinct transition probabilities summed to 1). Ninety-five percent confidence intervals for transition probabilities were calculated by random resampling (10,000 times with replacement) from the observed transitions (Efron and Tibshirani, 1993). To analyze the long-term stability of transition probabilities (Fig. 1d), we compared transition probabilities across 2 time periods (each of duration of 2-4 days) via a permutation test (Fisher, 1935); these periods were separated by 30-60 days.

The effects of differential reinforcement were assessed by comparing transition probabilities across three time periods: 1) 'baseline', a 2-4 day time period prior to WN onset, 2) 'white noise', the final day of WN delivery, and 3) 'post white noise', the third day following the termination of WN. The percentage change in transition probability induced by differential reinforcement was defined as $100*(\mathbf{p}_{bas}-\mathbf{p}_{WN})/(\mathbf{p}_{bas})$, where $\mathbf{p}_{bas}$ refers to the transition probability during the baseline period and $\mathbf{p}_{WN}$ refers to the transition probability on the final day of WN delivery. The percent recovery of transition probabilities following the termination of WN was defined as $100* (\mathbf{p}_{post}-\mathbf{p}_{WN})/(\mathbf{p}_{bas}-\mathbf{p}_{WN})$, where $\mathbf{p}_{post}$ refers to the transition probability on the third day following the termination of WN.

We calculated transition entropy (measured in bits) as a measure of the variability, or uncertainty, in the identity of the syllable produced at a branch point (Sakata and Brainard, 2006). For a branch point at which syllable 'a' can be followed by syllable 'b' or 'c' (with probabilities p_{a-b} and p_{a-c}), transition entropy is defined as $- p_{a-b}*\log_2 (p_{a-b}) - p_{a-c}*\log_2 (p_{a-c})$. For stereotyped transitions (i.e. $p_{a-b} = 1$ and $p_{a-c} = 0$), the entropy is zero bits. For transitions with equal probability ($p_{a-b} = p_{a-c} = 0.5$), the entropy is 1.0 bit, corresponding to maximal uncertainty about which syllable will be produced next. For the subset of experiments (9/13) in which transition entropy on the final day of WN was outside its baseline range (measured over the baseline period prior to onset of WN), we analyzed the extent to which transition

entropy was restored following modification during reinforcement.

To analyze the time course of changes to transition probabilities, we compared the cumulative magnitude of learning that had been reached by a particular day of reinforcement to the cumulative amount of learning reached by the final day of reinforcement. Significant differences from the final level of learning (i.e. 100%) were calculated via a t-test. Here, no correction was made for multiple comparisons to maintain a conservative standard for what constituted a non-significant difference from the final level of learning (all differences that were reported as non-significant also would have been non-significant, and by a wider margin, following a correction for multiple comparisons).

We measured the extent to which acoustic structure of syllables changed in response to reinforcement by measuring changes to the fundamental frequency (FF) of the syllables that elicited WN playback. In 11/13 experiments, the baseline acoustic structure of the syllable targeted with WN permitted reliable measurement of FF, which was calculated as described previously (Tumer and Brainard, 2007). We compared the magnitude of difference in mean syllable FF across two equivalent time intervals; one interval was within the baseline period (first and last day) in which no WN was delivered, and the second interval was between the last day of the baseline period and day 4 of WN delivery.

The long-term stability of repeat length was analyzed by comparing mean repeat length over two time intervals (each of duration 2 days), separated from each other by 25-35 days. The significance of differences in mean repeat length between baseline and the WN period, and baseline and the post-WN period, were determined via a t-test.

Results

Transition probabilities at branch points remain stable over weeks.

The song of an individual Bengalese finch (BF) is composed of a discrete set of ~5-

10 acoustically distinct vocal elements, termed 'syllables', which are produced in sequences characteristic of the individual. We refer to each distinct syllable in an individual bird's repertoire with a unique label (e.g. the syllables for the song illustrated in Figure 1a-c are labeled ‘a’, ‘b’, ‘c’ and so on) and focus in this study on the statistics that characterize the transitions between these categorically defined syllables. For BF song, some transitions between syllables are stereotyped. For example, syllable ‘a’ for the song depicted in Figure 1a-c was always followed by syllable ‘b’ (p_{a-b} = 1.00; 1194/1194 cases over 4 days). In other cases, transitions between syllables are variable. For example, syllable 'b' in this bird's song could be followed by either syllable ‘c’ (p_{b-c} = 0.68; 808/1194 cases), or syllable ‘d’ (p_{b-d} = 0.32; 386/1194 cases). We refer to syllables that can be followed by alternative transitions as 'branch points' and characterize the sequencing of syllables at branch points by quantifying the transition probabilities for each of the alternate possible transitions (which sum to 1.0).

We found that transition probabilities in adult BF song remained highly stable over time. An example is shown in Figure 1a-c for a branch point at which syllable 'b' could be followed by either syllable 'c' or 'd'. Transition probabilities were calculated for a random sample of songs on 4 consecutive days at the beginning of a two-month period and 4 consecutive days at the end of the period (Fig. 1c). None of the transition probabilities on these eight days was significantly different from the others (p_{b-c} for each of the 8 days = 0.68, 0.66, 0.70, 0.72, 0.71, 0.67, 0.70. 0.72; n.s. by permutation test across all pairs, Bonferroni corrected). For 9 branch points (from 9 birds), we similarly compared transition probabilities over two time periods separated by 30-60 days (Fig. 1d). Measured transition probabilities spanned a broad range, from 0.05 to 0.74. For each transition the probabilities measured at the beginning and end of this period were highly correlated (r = 0.80) and for 17/20 transitions there was no significant change in transition probability (P > 0.05, Permutation test across pairs, Bonferroni corrected). These data demonstrate that transition probabilities

can span a broad range of values and remain stable at those specific values over periods of months, consistent with prior studies, which have suggested stability of transition probabilities (Okanoya and Yamaguchi, 1997; Woolley and Rubel, 1997; Yamada and Okanoya, 2003; Hampton et al., 2009). Hence, Bengalese finch song is a stochastic behavior in which the probabilities of behavioral transitions ordinarily remain stable, at values that are unique to individual transitions.

Differential reinforcement of transitions at branch points induces rapid, adaptive modification of transition probabilities.

The stability over time of transition probabilities could indicate that adult birds have lost the capacity to change sequencing adaptively. If this were the case, the specific values of transition probabilities at branch points might be relatively immutable, reflecting 'hard-wiring' of the neural circuitry that controls sequencing. We tested whether syllable sequencing in adult song could be modified adaptively by using an automated system to differentially reinforce birds for producing some syllable sequences over others. Previous experiments have shown that playback of loud white noise (WN) that is contingent on the fundamental frequency (FF) of a syllable induces birds to modify syllable FF to 'escape' WN, indicating that WN is effective for aversive reinforcement (Tumer and Brainard, 2007; Charlesworth et al., 2011). Here we used WN to deliver differential reinforcement that was contingent on the sequence transition that had just occurred in song, and not on the acoustic structure of individual syllables (Methods). Hence, while previous experiments showed that differential reinforcement could shape the acoustic structure of an individual syllable, here we tested whether differential reinforcement could modify the normally stable probabilities of transitions between categorically distinct syllables.

Although transition probabilities normally remain stable, birds rapidly modified

transition probabilities in response to differential reinforcement. Figure 2a illustrates an experiment in which WN was played back over one of two alternative transitions at a branch point (the same branch point shown in Figure 1a-c). In this case, the transition from syllable 'b' to 'c' was targeted with WN while the alternate transition, from 'b' to 'd', was not. This reinforcement via contingent playback of WN over the b-c transition induced a significant reduction in the probability of that transition within the first day of WN (baseline $p_{b-c} = 0.68$, day 1 $p_{b-c} = 0.55$; $P < 0.05$, permutation test). The probability of the b-c transition decreased further over the subsequent three days, and on day 4 of reinforcement, p_{b-c} was 0.40. This decrease in the probability of b-c transitions resulted in a complementary increase in the probability of transitions to the alternative syllable 'd' (baseline $p_{b-d} = 0.32$, day 4 $p_{b-d} = 0.60$). Hence, in this case, the bird adaptively reduced exposure to WN by decreasing the probability of the targeted transition and increasing the probability of the alternate, non-targeted transition.

The probabilities of transitions at a branch point could be increased or decreased depending on which transition was targeted. For example, for the experiment described above, the probability of the b-c transition was systematically decreased over time when this transition was targeted with WN. However, for a later experiment from this same bird, the probability of the b-c transition was systematically increased when the alternate transition was targeted with WN. This is illustrated in Figure 2b. Here, when the transition from syllable 'b' to 'd' was targeted with WN, the probability of the b-d transition decreased (from a baseline value of $p_{b-d} = 0.32$ to a value on day 4 of $p_{b-d} = 0.15$), and the probability of the non-targeted b-c transition increased.

As illustrated in this example, transition probabilities at branch points always responded to differential reinforcement by shifting in an adaptive direction (reducing exposure to WN). Figure 2c summarizes the changes to transition probabilities at branch

points for 13 experiments (in 10 birds) in which one transition was targeted with WN. Over the period of reinforcement (4-11 days) the probability of targeted transitions (filled green points, 'WN') always decreased, and the probability of alternate transitions (gray points, 'no WN') increased in a compensatory fashion. The mean reduction in the probability of targeted transitions, measured on the last day of reinforcement, was 55.0 +/- 6.7 %, corresponding to a 55% reduction in the amount of white noise experienced by the birds. Hence, differential reinforcement with WN consistently elicited learning in the form of directed, adaptive changes to sequencing at branch points.

Learned changes to transition probabilities occurred rapidly and reached a stable level after ~4 days. This is suggested by the examples in Figure 2a-b, in which the majority of changes to transition probabilities occurred during the first two days of reinforcement with little apparent change thereafter. To define the time course of learning systematically, we examined the progression of changes to transition probabilities in 7 experiments in which reinforcement was maintained for at least 6 days (Fig. 2d). Changes to transition probabilities were normalized to the probabilities on the last day of reinforcement to examine the percentage of the final learning that had been completed by each day of reinforcement (see Methods). By day 4, the amount of learning that had been completed was 89.7 +/- 8.8%, which was slightly below but not significantly different from the amount of learning assessed on the last day of reinforcement (Fig. 2d; $P = 0.14$, one-tailed t-test comparing day 4 values with final values). By day 5, the amount of learning that had been completed was 98.2 +/- 9.1%, indistinguishable from its final value ($P = 0.58$, one tailed t-test). Hence, the probability of targeted transitions was reduced to a new value by the end of day 4, with little change thereafter.

While differential reinforcement reduced the probability of targeted transitions, it never caused the total elimination of targeted transitions. This can be seen in Figure 2c,

which plots the final transition probabilities after at least 4 days of reinforcement for all 13 experiments. In none of these cases did the final transition probability drop to zero. Moreover, in the subset of 4 experiments in which we maintained reinforcement for at least 9 days, transition probabilities achieved stable, non-zero values by day 6 that were not subsequently further reduced by the final day (P = 0.36, one-tailed paired t-test for change between day 6 and final day). These data indicate that while the probability of targeted transitions could be rapidly decreased, these transitions could not be readily eliminated.

Changes to sequencing of syllables occurred independently of changes to the acoustic structure of syllables. In these experiments, the playback of WN was contingent on which transition the bird produced at a branch point (i.e. 'a-b' versus 'a-c'), and not on the acoustic structure of any of the syllables surrounding the branch point. Under these conditions, in which playback of WN is not contingent on variation in the acoustic structure of targeted syllables, previous experiments suggest that acoustic structure will not change (Charlesworth et al., 2011). Consistent with this, we observed no change in syllable structure in experiments in which we drove changes to transition probabilities; for example, the magnitude of change in mean fundamental frequency (of the syllables that received WN playback) over the period of reinforcement was not significantly different from the magnitude of change in fundamental frequency that occurred over a comparable baseline period (mean change during baseline: 44.9 +/- 11.7 Hz; mean change during reinforcement: 25.2 +/- 7.6 Hz; paired t-test, P = 0.38; n=11).

These data demonstrate a previously unrecognized capacity of adult birds to make adaptive changes to the ordinarily stable sequencing of the syllables in their songs. As a corollary, they indicate that this stable sequencing does not simply reflect a loss of plasticity in the song premotor circuitry responsible for the control of syllable sequencing.

Transition probabilities recover to a baseline set point following termination of reinforcement

To determine whether the stable transition probabilities that are normally present in adult song are actively maintained, we assessed whether baseline transition probabilities were restored following termination of reinforcement. We found that following cessation of reinforcement, transition probabilities at branch points reliably recovered to their baseline values. An example of this recovery is shown in Figure 3a. Here, differential reinforcement over six days induced a large shift in transition probabilities (the probability of the targeted 'ab' transition was reduced from 0.67 at baseline to a stable value of 0.06 +/- 0.02 from days 4 to 6). After 6 days, the delivery of WN was terminated. Transition probabilities then gradually returned to their original baseline values, and by the third day of recovery the transition probabilities were not significantly different from their baseline values (by day 3 of recovery, $p_{a\text{-}b}$ was 0.64 versus a baseline value of 0.67). A similar recovery was observed across experiments in which transition probabilities were monitored after termination of reinforcement. On average, by the third day after termination of WN, transition probabilities recovered 94.8 +/- 2.6 % of the way back toward baseline (Fig. 3b; n=13). This recovery of the baseline values, in the absence of any instruction, indicates that a representation of baseline transition probabilities is retained by the nervous system even while overt transition probabilities are maintained at distinct values during the period of reinforcement. Moreover, this recovery indicates that the baseline transition probabilities represent stable set points that birds have both the capacity and impetus to restore.

A corollary of the finding that birds restore transition probabilities to baseline values is that they restore the transition entropy to its baseline value. Transition entropy expresses, in units of bits, the variability or uncertainty in the identity of the next syllable at a branch point (Sakata and Brainard, 2006; Methods). Stereotyped transitions have zero entropy (0

bits), while variable transitions with equally probable alternatives have maximal entropy (1 bit in cases with two equally probable alternatives). In a majority of experiments, transitions became more stereotyped (i.e. lower entropy) during reinforcement-driven sequence modification. This was the case for the experiment illustrated in Figure 3a, where the initial probabilities of 0.67 (a) : 0.33 (b) had an entropy of 0.92 bits. When transition probabilities were modified to 0.06 (a) : 0.94 (b), the entropy decreased to 0.33 bits. Transition entropy was modified to a value outside its baseline range in 9/13 experiments (Fig. 3c; Methods). In 7 of these cases, entropy decreased in response to differential reinforcement and in 2 of these cases the entropy increased. The examples in Figure 2 illustrate a branch point at which entropy at a single branch point could be either increased (Fig. 2a) or decreased (Fig. 2b). In all cases, the entropy returned towards its baseline value following termination of reinforcement. While this recovery of entropy to its baseline value follows directly from the recovery of transition probabilities, it illustrates a distinct point. These data show that the degree of variability at branch points (as quantified by entropy) can be both increased and decreased by targeted reinforcement, but that it ultimately is restored to a stable and specific level in the absence of reinforcement.

Mean repeat length can be adaptively modified in response to differential reinforcement yet is restored to its baseline value upon the termination of reinforcement

We next tested whether similar principles of modifiability and active maintenance apply for a second form of sequence variation in Bengalese finch song, in which one syllable is repeated consecutively a variable number of times. Figure 4a shows an example from a segment of song containing two instances in which a syllable was repeated consecutively. In the first instance, the syllable was repeated 7 times and in the second instance it was repeated 2 times. We refer to each of these instances as a 'repeat' and refer to the number of times a

syllable is produced within a repeat as the 'repeat length'; thus, in the example, there were two repeats, with repeat lengths of 7 and 2. For the songs produced over the course of a day, the repeat length varied from 1 to 9 with a standard deviation of 1.75 (Fig. 4b, 'Day 1', n=206 repeats).

As was the case for transition probabilities at branch points, mean repeat length remained remarkably stable over weeks. For the song depicted in Figure 4a, mean repeat length was 4.47 +/- 0.12 (s.e.m) on the first day of recorded song (Fig. 4b, 'day 1') and 4.46 +/- 0.12 one week later (Fig. 4b, 'day 8'). Such long-term stability in repeat length was consistent across the population of repeated syllables that we examined. For 7 repeated syllables (n= 5 birds), there was no significant change in mean repeat length over an interval of 25-35 days (mean absolute change was 0.14 syllables per repeat, $p = 0.53$, t-test, $n = 7$).

While repeat length remained stable under control conditions, birds adaptively modified repeat length in response to differential reinforcement with WN. To differentially reinforce repeats, we delivered WN within each repeat for those syllables that were produced after a threshold was exceeded (Methods). For example, for the experiment illustrated in Figure 4a-e, the threshold was set to 2; hence, the first and second consecutive renditions of the syllable did not elicit WN playback, but all subsequent consecutive renditions of the syllable did elicit WN playback (e.g. Fig. 4c). This differential reinforcement induced a significant reduction in mean repeat length, from 4.46 +/- 0.12 to 2.27 +/- 0.04 over 4 days (Fig. 4d-e, $P<1e-85$, rank sum test). Accordingly, the percentage of repeats with length less than 3, which avoided WN playback altogether, increased from 19% to 83% (Fig. 4d). After cessation of reinforcement with WN, mean repeat length gradually recovered to its original value (Fig. 4e).

Such a capacity for both adaptive modification and restoration of mean repeat length was consistent across experiments (Fig. 4f; 9 experiments in 7 birds). On average, mean

repeat length was reduced by 1.24 +/- 0.18 by the final day of reinforcement (5.43 before vs. 4.19, P < 1e-4, t-test). This reduction in repeat length was adaptive in that it corresponded to a 47% reduction in the amount of WN playback for the repeated syllables. Following cessation of reinforcement, mean repeat length recovered to its original value (Fig. 4f, 'post WN'; 5.43 before vs. 5.44 after, P = 0.96, t-test). These results demonstrate that birds have the capacity to adaptively modify repeat length in response to reinforcement, and that, as for transition probabilities at branch points, there is a stable underlying set point for repeat length that is restored following the termination of reinforcement.

Discussion

Adaptive modification of the statistics of syllable sequencing

Our results demonstrate that adult songbirds, with normally stable songs, have a previously unrecognized capacity to modify syllable sequencing adaptively. The modifications of both transition probabilities and repeat length were adaptive, in that they reduced the amount of aversive white noise (WN) delivered. Moreover, these modifications were local and specific; only sequence transitions targeted with WN were modified and no changes to syllable acoustic structure occurred. These adaptive modifications to sequencing contrast with changes to sequencing induced in prior studies, in which disruptive experimental manipulations (such as deafening or non-contingent perturbation of feedback) caused non-adaptive and generalized deterioration of syllable sequencing and syllable structure (Woolley and Rubel, 1997; Leonardo and Konishi, 1999; Yamada and Okanoya, 2003; Thompson and Johnson, 2006; Mooney, 2009; Miller et al., 2010a). The changes to sequencing induced here, which persisted over a period of days and were present on 'catch' songs without altered feedback, are also distinct from acute or 'online' switches in sequencing caused by perturbation of feedback or presentation of a female (Sossinka and Bohner, 1980;

Sakata and Brainard, 2006; Sakata et al., 2008). Therefore, in contrast to prior studies, our findings demonstrate that adult birds with 'crystallized' song retain a capacity for directed modification of syllable sequencing.

Our results also reveal that similar 'learning rules' govern changes to hierarchically distinct aspects of song structure. Previous studies have shown that the acoustic structure (fundamental frequency) of an individual syllable of adult song can be modified by differentially reinforcing subtle rendition-to-rendition variation in how that syllable is produced (Tumer and Brainard, 2007). Here, we demonstrate that similar principles govern changes to the probability of transitioning between distinct syllables in response to differential reinforcement. Both forms of adaptive adult plasticity proceed on a timescale of hours to days and are rapidly reversed following the termination of reinforcement. However, despite these phenomenological similarities, the neural substrates that subserve the control and modification of syllable acoustic structure versus syllable sequencing are thought to be quite distinct. Each categorically different syllable of a bird's song (i.e. 'a', 'b', 'c'…) is produced by a different ensemble of neurons in the motor cortical analogs HVC and RA (Hahnloser et al., 2002; Fee et al., 2004; Leonardo and Fee, 2005; Sober et al., 2008; Wohlgemuth et al., 2010; Fujimoto et al., 2011). In contrast, variation in fundamental frequency across renditions of an individual syllable is correlated with variation in the firing rate of neurons that are active within an RA ensemble (Sober et al., 2008). Hence, while learning that alters the mean fundamental frequency of a syllable could reflect modulation in the firing rates of already active RA neurons, learning that alters syllable sequencing is likely to reflect gross changes in which neurons are active within the entire recurrent circuitry, including nucleus HVC, that participates in the sequencing of syllables (Hahnloser et al., 2002; Fee et al., 2004; Ashmore et al., 2005; Jin, 2009; Fujimoto et al., 2011).

The capacity that we observe for modification of syllable sequencing without

modifying syllable structure further supports the view that song is controlled at two hierarchically distinct levels (Vu et al., 1994; Yu and Margoliash, 1996; Tchernichovski et al., 2001; Hahnloser et al., 2002; Ashmore et al., 2005; Horita et al., 2008). In mammalian systems, movement similarly is thought to be controlled at hierarchically distinct levels (Lashley, 1951; Koechlin et al., 2003; Desmurget and Turner, 2010). Theoretical studies have suggested that hierarchical algorithms of reinforcement learning, which divide different aspects of learning into independent subroutines, can enhance the speed and generalization of learning (Botvinick et al., 2009; Ribas-Fernandes et al., 2011). Our results indicate that learning in adult birdsong may serve as a biological instantiation of this strategy.

Variation is a stable end point of learning.

While our results demonstrate that that the statistics of sequencing can be modified in adult song, they also reveal an underlying drive to maintain those statistics at a stable set point. Following the termination of reinforcement, birds restored both transition probabilities and repeat length to their pre-learning baseline values. This self-driven recovery indicates both that the nervous system maintains a stable representation of baseline sequencing statistics and also that it has the capacity and impetus to restore those baseline statistics in the absence of externally imposed instruction. The presence of an underlying impetus to maintain transition probabilities at their baseline values is also supported by the observation that targeted transitions were reduced but never completely eliminated; this maintenance of non-zero transition probabilities despite persistent targeting with WN may result from a dynamic equilibrium between opposing drives to restore transition probabilities to their baseline values and to avoid WN, analogous to competing processes described in human motor learning (Criscimagna-Hemminger and Shadmehr, 2008; Warren et al., 2011).

A corollary of the finding that birds maintain probabilities of transitions at set values

is that they also maintain the entropy of transitions at set values. The transition entropy is lowest when sequences are least variable (i.e. when the transition probability is 1.0), and highest when sequences are most variable (i.e. when transition probabilities are 0.5:0.5). For every experiment in which transition entropy was driven lower by differential reinforcement (reflecting a reduced magnitude of sequence variation), entropy was increased back towards baseline levels following the termination of reinforcement. This increase in the magnitude of sequence variation (as quantified via entropy) contrasts with prior studies, in which recovery from reversible manipulations was always associated with a decrease in sequence variation (Okanoya and Yamaguchi, 1997; Woolley and Rubel, 1997; Leonardo and Konishi, 1999; Yamada and Okanoya, 2003). Hence, while studies of motor learning often emphasize that rendition-to-rendition variation in behavior is minimized via extensive practice (Schwartz, 1980; Cohen et al., 1990; Grafton et al., 2002), our findings demonstrate that for birdsong there is a drive to maintain the level of variation above the minimum possible value. Thus, persistent variation in the sequencing of well-learned motor skills can reflect a stably maintained end point of learning, rather than a limitation in motor control.

What might be the adaptive value of maintained sequence variation?

The observation that variation in sequencing is actively maintained in adult song raises the question of whether such variation serves a useful function. Rendition-to-rendition variation in sequencing has previously been hypothesized to play an adaptive role in diverse behaviors, ranging from courtship, to predator avoidance, to optimal foraging of resources (Humphries and Driver, 1967; Real and Caraco, 1986; Searcy and Andersson, 1986). Previous studies in songbirds, including the Bengalese finch, have shown that females prefer songs with greater sequence complexity (Searcy and Andersson, 1986; Searcy and Nowicki, 1998; Morisaka et al., 2008), supporting the hypothesis that complexity in song, and variation

in sequencing, may have evolved via sexual selection. Therefore, the capacity to maintain a significant amount of sequence variation in Bengalese finch song (as evident in Fig. 3) could be adaptive, as it enables the construction of a complex and varying courtship repertoire out of a small number of distinct acoustic elements.

How might sequence variation be maintained?

Our results raise the question of how the statistics of sequencing are stably encoded by the nervous system. Prior modeling work has suggested that transition probabilities at branch points are highly sensitive to the relative synaptic 'weights' between neural assemblies in premotor areas (Yamashita et al., 2008; Jin, 2009). Our finding that sequencing statistics can be modified by differential reinforcement indicates that relevant synaptic weights and other features of the circuitry that control transition probabilities are modifiable. Over time, even in the absence of external perturbation, structural changes in the nervous system (e.g. modifications to synaptic weights) would likely cause transition probabilities to drift in the absence of error correction (Holtmaat et al., 2005; Elhilali et al., 2007; Kasai et al., 2010). Therefore, the stable sequencing statistics that we observe may depend on an active process of error correction in which deviations from baseline statistics are detected and corrected. Such error correction has been demonstrated for maintenance of the fundamental frequency of individual syllables of adult song (Sober and Brainard, 2009). In this context, the plasticity that we have demonstrated for syllable sequencing in adult song might function normally to enable the maintenance of stable sequencing statistics rather than to enable modification of those statistics.

General conclusions

We investigated the control of sequencing in a specific well-learned motor skill, adult

birdsong, but similar principles are likely to apply to other vertebrate behaviors. The quantifiable nature of syllable sequencing, coupled with accessible underlying neural substrates, makes songbirds an especially suitable model for defining general principles whereby variable transitions are generated and maintained by the nervous system. Our results demonstrate that the songbird nervous system can produce stochastic behaviors in which the probabilities of transitions from one action to distinct alternative actions are stably maintained at fixed values. Analogous variable transitions between distinct actions or brain states are observed in a wide range of other species, in both behavior and perception (Wheatstone, 1838; Kornmeier and Bach, 2005; Buzsaki, 2010; Miller and Katz, 2010; Miller et al., 2010b). The ability to control and modify such transitions is thought to contribute not only to perception and movement but also to complex cognitive abilities such as reasoning, planning, and recollection (Rabinovich et al., 2008; Deco et al., 2009; Buzsaki, 2010); correspondingly, dysfunction in transitions between actions or brain states may contribute both to movement disorders and to psychiatric illnesses such as bipolar disorder and schizophrenia, which are associated with abnormalities in the transitions between cognitive states (Miller et al., 2010b; Ngo et al., 2011). Our findings for birdsong raise the possibility that for other variable behaviors, the statistics of transitions between such brain states may be governed by processes that both enable and constrain the adaptive adjustment of sequencing.

Chapter 2: Figure 1

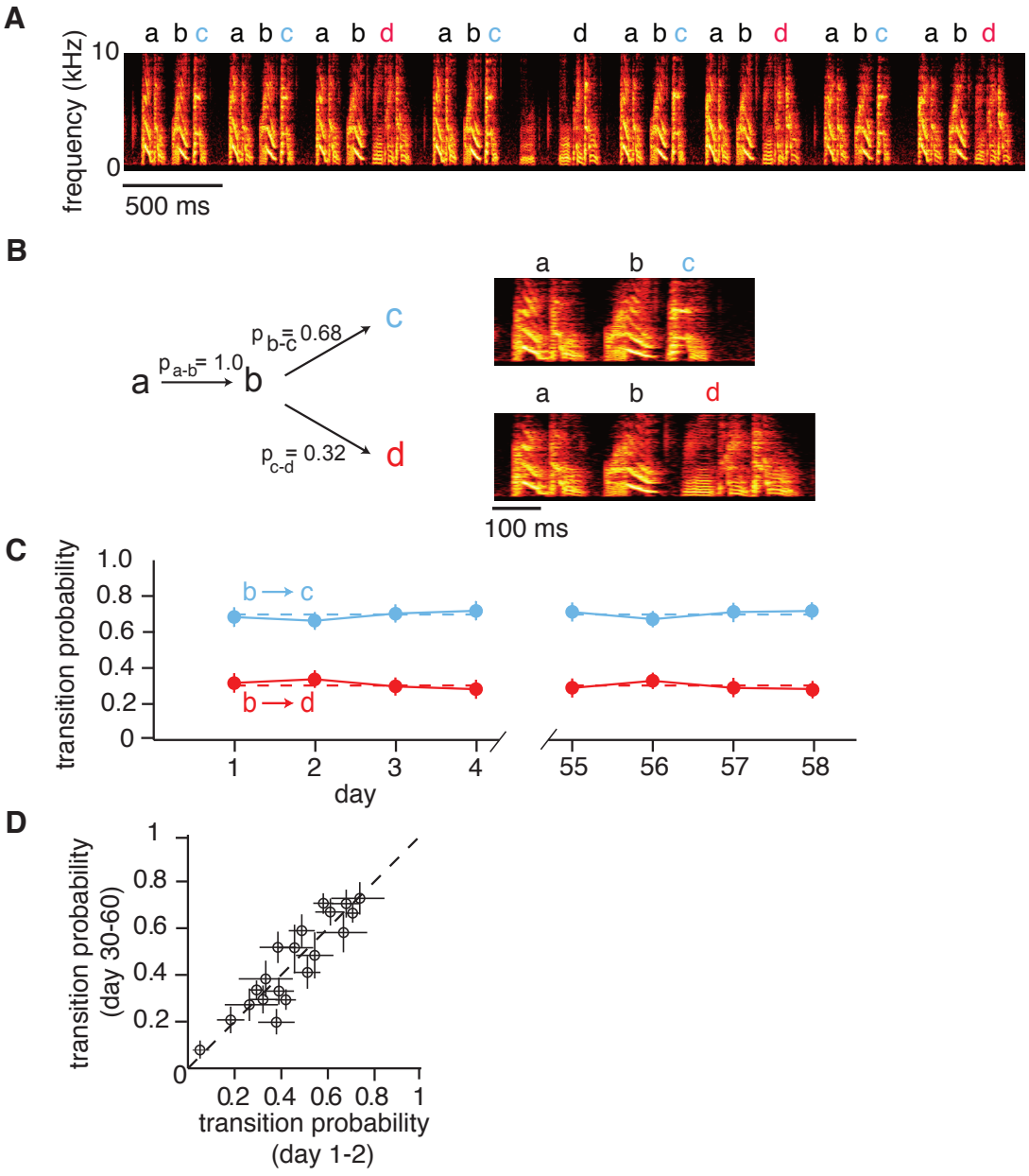

Figure 1. Transition probabilities at branch points remain stable over weeks.
A. Spectrogram of Bengalese finch song illustrating the sequencing of the acoustically distinct syllables 'a', 'b', 'c' and 'd'. Syllable 'a' always transitioned to syllable 'b'. In contrast, syllable 'b' was a branch point at which a transition could occur to either syllable 'c' or syllable 'd'. B. Schematic (left) and expanded spectrograms (right) for the branch point at syllable 'b'. For this branch point the probability of transition to syllable 'c' was 68% (pb-c = 0.68) and the probability of transition to syllable 'd' was 32% (pb-d = 0.32) measured over a 4 day period. C. Transition probabilities of b-c (blue) and b-d (red) transitions over 8 days separated over two months (days 1, 2, 3, 4 and 55, 56, 57, 58). None of the probabilities was significantly different from the others. D. Summary of 20 transition probabilities for 9 branch points (from 9 birds). Transition probabilities were measured over two-day time periods separated by 30-60 days.

Chapter 2: Figure 2

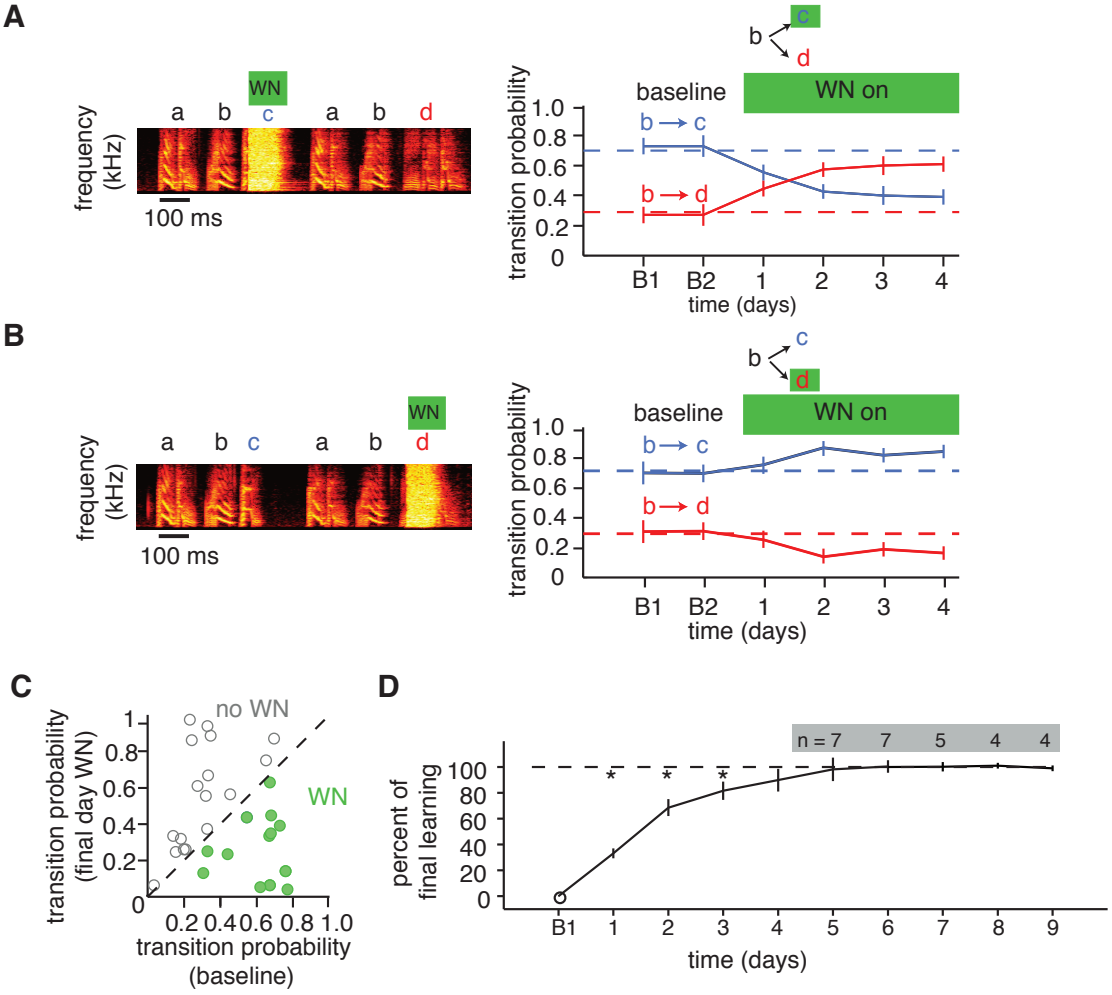

Figure 2. Differential reinforcement of sequence transitions induces adaptive modification of transition probabilities

A. Left panel: Example spectrogram from an experiment in which transitions from syllable 'b' to syllable 'c', but not transitions to syllable 'd', elicited playback of WN. Right panel: Daily transition probabilities of both possible transitions over 2 days at baseline (days B1 and B2) and over 4 days of WN (days 1-4). B. Left panel: Example spectrogram from a later experiment in the same bird in which transitions to syllable 'd', but not transitions to syllable 'c', elicited playback of WN. Right panel: Daily transition probabilities, as in 2A, right panel. C. Comparison of transition probabilities (n=13 experiments in 10 birds) at baseline and on last day of WN (duration of WN playback 4-11 days). Filled green circles indicate transitions targeted with WN; open gray circles indicate transitions not targeted with WN. D. Trajectory of learning, normalized to the amount of learning on the final day of reinforcement. The number of experiments contributing to each point were days 1-6: 7, day 7: 5, days 8-9: 4. The cumulative learning completed on days 1-3 was significantly less than the final amount of learning, as calculated on the last day of reinforcement (asterisks indicate $P < 0.05$; t-test). The cumulative learning completed on day 4 (89.7 +/- 8.8 percent), as well as subsequent days, was not significantly different from the final amount of learning.

Chapter 2: Figure 3

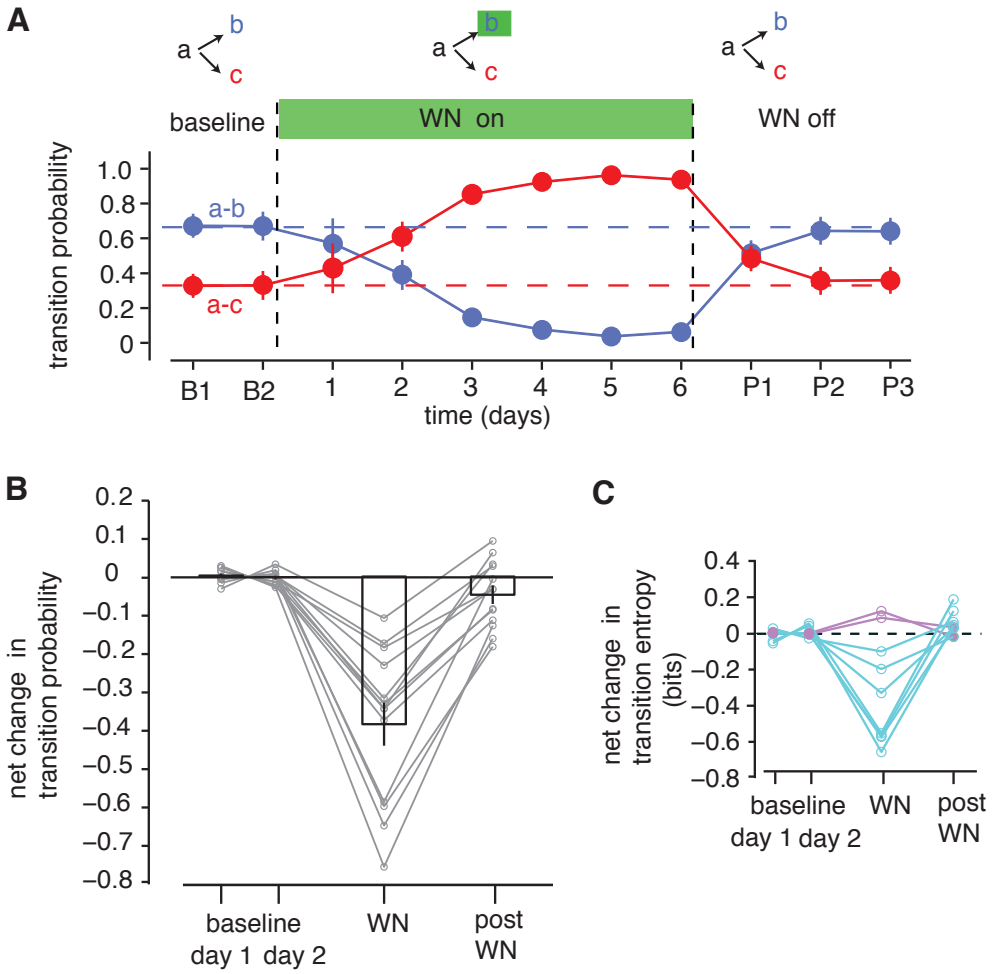

Figure 3. Transition probabilities recover to a baseline set point following termination of reinforcement.

A. Example trajectory of probabilities of two transitions, a-b (blue) and a-c (red), over two days at baseline ('baseline'), six days of WN targeting the a-b transition ('WN on'), and three days following the cessation of WN ('WN off '). B. Summary data for 13 experiments, showing differences in transition probabilities between two consecutive days at baseline ('baseline – day 1 and day 2'), the final day of WN ('WN') and the third day following the termination of WN ('post WN'). C. Net changes in transition entropy (bits) at baseline, in response to WN, and following termination of WN (same labels and time periods as B). Data shown are from the 9/13 experiments in which transition entropy, during WN period, was modified to a value outside the range of values measured at baseline (Methods). Cyan lines and circles show experiments (n=7) in which transition entropy was reduced during WN period (reflecting more stereotyped transitions). Purple lines and circles show experiments (n=2) in which transition entropy was increased during WN period. Baseline values of transition entropy were recovered following termination of WN, both for cases in which transition entropy increased and for cases in which it decreased during WN period.

Chapter 2: Figure 4

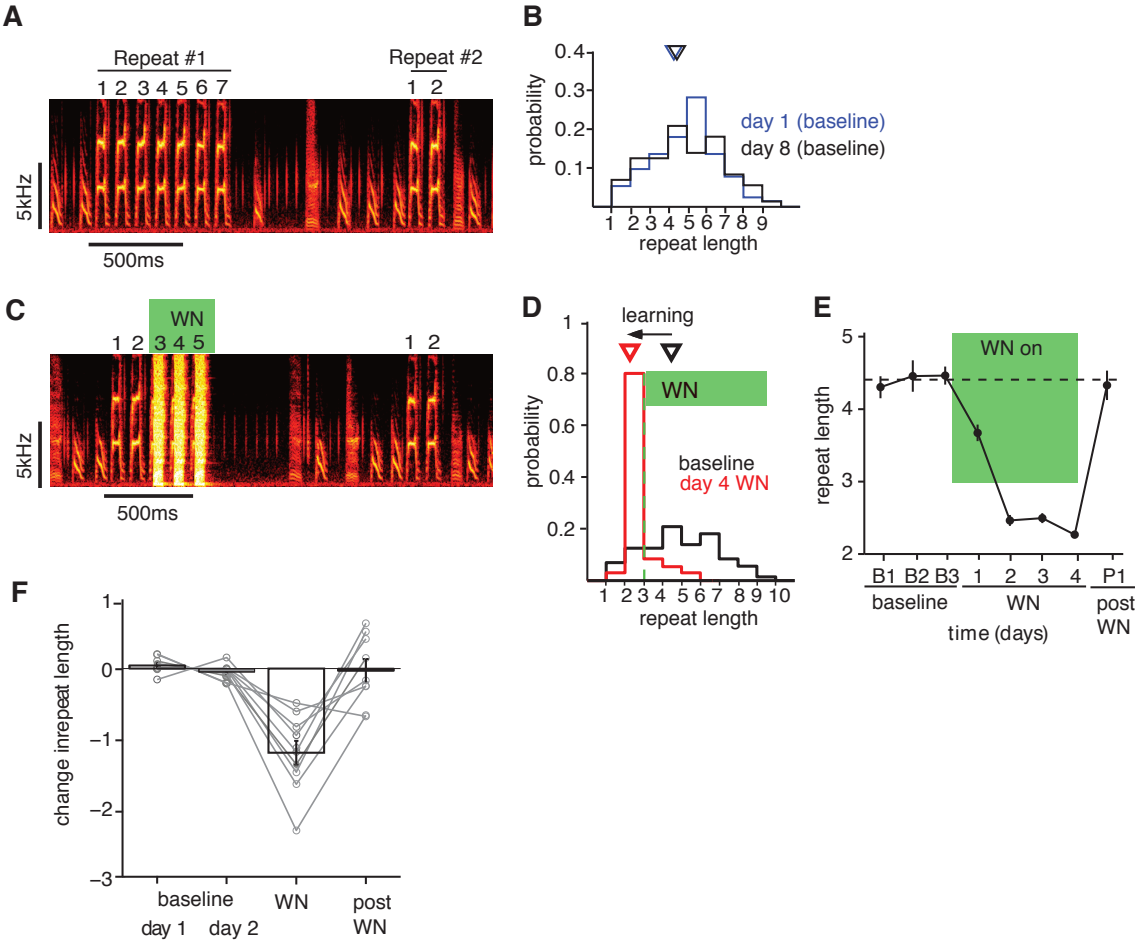

Figure 4. Mean repeat length is adaptively modified in response to differential reinforcement.

A. Spectrogram of song showing two 'repeats', in which one syllable is produced consecutively a variable number of times. The first repeat ('Repeat #1') has a 'repeat length' of 7 and the second (Repeat #2) has a 'repeat length' of 2. B. Probability distributions of repeat length for the repeated syllable shown in panel A. Distributions are shown for 2 distinct days at baseline, prior to WN playback ('day 1' and 'day 8'.) Inverted triangles indicate mean repeat lengths for each day. C. Example of differential reinforcement of repeat length with WN. To aversively reinforce repeats with longer repeat length, WN was played back over syllables produced after a repeat length threshold of 2 was exceeded. D. Differential reinforcement induced an adaptive reduction in mean repeat length from 4.46 +/- 0.12 ('baseline') to 2.27 +/- 0.04 ('day 4 WN'). E. Daily values of mean repeat length over 3 consecutive days at baseline (B1-B3), 4 days of WN (days 1-4) and the first day following the termination of reinforcement (P1). Error bars indicate +/- s.e.m. F. Summary data for 9 experiments showing changes in mean repeat length over two days at baseline ('baseline – day 1 and day 2'), on the last day of reinforcement ('WN'), and following the termination of reinforcement

Chapter 2 References

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Chapter 3: Differential contributions of an avian cortical-basal ganglia circuit to hierarchically distinct forms of vocal learning

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Abstract

Birdsong, like speech, is a learned behavior that requires precise control at two hierarchically distinct levels. At one level of control, distinct vocalizations, termed syllables, are performed with high acoustic precision; at another level of control, syllables are sequenced according to a specific syntax. Though basal ganglia circuitry has been shown to play a critical role in motor learning, the extent to which the adaptive modification of individual actions and the modification of action sequencing depend on the same basal ganglia circuits is not well understood. Here we address this question in adult Bengalese finches. We first tested how lesions of LMAN, the cortical outflow nucleus of the avian basal ganglia circuit, affected birds' respective capacities to adaptively modify – in response to aversive reinforcement – the fundamental frequency of individual syllables and the transition probabilities between syllables. We found that lesions of LMAN prevented birds from learning adaptive changes to syllable structure; however, the same lesions did not significantly affect birds' capacity to adaptively modify syllable sequencing. We then tested whether the expression of newly learned changes to syllable sequencing depended on output from basal ganglia circuitry to the motor pathway (as has shown to be the case for the expression of newly learned changes to syllable structure). We found that transient pharmacological interference with LMAN's output to the motor pathway did not affect the expression of learned changes to syllable sequencing. Together, these results indicate that

avian basal ganglia circuitry is not obligatory for all forms of vocal learning in songbirds but instead can contribute differentially to hierarchically distinct types of learning.

Introduction

Birdsong is an ideal behavior to test how basal ganglia circuits contribute to hierarchically distinct aspects of motor learning. One reason for this is that adult songbirds have a capacity to adaptively modify, in an independent fashion, both the execution of individual vocal gestures as well as how these gestures are sequenced (Tumer and Brainard, 2007; Warren et al., 2012). A second reason is that in songbirds, a cortical-basal ganglia circuit dedicated to song can be monitored and manipulated with ease (Bottjer et al., 1984; Brainard and Doupe, 2000; Kao et al., 2005; Olveczky et al., 2005; Andalman and Fee, 2009; Warren et al., 2011).

In learned adult song, individual vocal gestures, termed syllables, are performed with high acoustic stereotypy and are sequenced according to specific transition probabilities. In some species, including the zebra finch, these transition probabilities have a value of 1.0, meaning transitions between syllables are stereotyped. In other species, however, including the Bengalese finch, transitions between syllables can be variable (i.e. from one syllable 'a', termed a branch point, transitions occur to syllable 'b' with probability of 0.7 and to syllable 'c' with probability of 0.3). While both the acoustic structure of individual syllables and the transition probabilities between syllables are ordinarily maintained at stable values in adult birds, both song parameters are modifiable. Syllable acoustic structure and syllable sequencing are modified as part of the non-adaptive, global deterioration of song that is induced by deleterious experimental manipulations such as deafening (Nordeen and Nordeen, 1992; Woolley and Rubel, 1997; Leonardo and Konishi, 1999; Thompson and Johnson, 2006). In addition, both parameters of song can be modified adaptively. Adult birds can

adaptively shift either the fundamental frequency of individual syllables or the probabilities of transitioning between syllables to reduce the amount of playback of loud white noise, an aversive reinforcer (Tumer and Brainard, 2007; Warren et al., 2012).

Here we test how an avian basal ganglia circuit, termed the anterior forebrain pathway (AFP), which is generally implicated in vocal plasticity, contributes to both forms of adaptive song modification. The AFP is one of two neural pathways, along with a primary motor pathway, that converge to control song production (Fig. 1). The motor pathway is required for song production, and is thought to transmit the core motor commands that drive the vocal muscles to pattern ongoing song output (Yu and Margoliash, 1996; Fee et al., 2004; Sober et al., 2008). The AFP, on the other hand, is not required for song production but is required for various forms of vocal plasticity (Bottjer et al., 1984; Williams and Mehta, 1999; Brainard and Doupe, 2000; Thompson et al., 2007). In juvenile birds, lesions of LMAN cause song to stabilize prematurely in an unlearned state (Bottjer et al., 1984). In adult birds, lesions of LMAN prevent the onset of vocal deterioration (of both syllable structure and syllable sequencing) that ordinarily occurs following deleterious manipulations such as deafening (Williams and Mehta, 1999; Brainard and Doupe, 2000; Thompson et al., 2007). Additionally, lesioning or inactivating LMAN at times when song is highly variable (e.g. during juvenile song learning or during experimentally-induced adult song deterioration) – induces an abrupt stabilization of song, reducing variation in syllable sequencing and syllable acoustic structure (Olveczky et al., 2005; Nordeen and Nordeen, 2010; Kojima and Doupe, 2011). Together, these studies are consistent with a view that vocal plasticity depends on avian basal ganglia circuitry.

Recent studies provide direct evidence that LMAN contributes to the adaptive modification of syllable acoustic structure, though the extent to which LMAN contributions to the adaptive modification of syllable sequencing is unclear. Pharmacological inactivations

of LMAN partially reverse the expression of newly learned changes to syllable acoustic structure (Andalman and Fee, 2009; Warren et al., 2011). During the first days of learning, these inactivations cause syllable fundamental frequency to revert from a learned value toward the original baseline value. However, over multiple days, learning gradually becomes expressed in a LMAN-independent manner. These results indicate that LMAN exerts an initial adaptive premotor bias that supports production of newly learned changes to syllable structure but that over time, plasticity occurs in the LMAN-independent motor pathway that enables the motor pathway to produce adaptive changes to syllable structure on its own (Andalman and Fee, 2009; Warren et al., 2011). Whether or not LMAN contributes in an analogous manner to the adaptive modification of syllable sequencing is unclear. On the one hand, LMAN's role in non-adaptive sequence deterioration suggests LMAN may be required for adaptive changes to sequencing (Brainard and Doupe, 2000; Olveczky et al., 2005; Thompson et al., 2007; Nordeen and Nordeen, 2010). On the other hand, lesions of LMAN do not affect baseline transition probabilities at branch points in Bengalese finches, suggesting that LMAN does not play a premotor role in influencing transition probabilities (Hampton et al., 2009). Here we test, via both lesions of LMAN and pharmacological interference with LMAN's transmission to RA, the respective contributions of the AFP to both modifications of syllable structure and syllable sequencing.

Methods

Subjects

Fourteen adult (>150 d old) Bengalese finches (*Lonchura striata domestica*) were used in this study. All birds were bred in our colony and housed with their parents until at least 60 d of age. During experiments, birds were isolated and housed individually in sound-attenuating chambers (Acoustic Systems) on a 14 hr on/10 hr off light cycle. All song

recordings were of undirected song (i.e. no female was present). All procedures were performed in accordance with protocols approved by the University of California, San Francisco Institutional Animal Care and Use Committee.

Computerized control of song recording and delivery of reinforcement.

We drove changes to syllable structure and syllable sequencing via an aversive reinforcement learning paradigm, described previously, in which 50-80ms bursts of loud white noise (WN) were used as aversive reinforcers (Tumer and Brainard, 2007; Charlesworth et al., 2011; Warren et al., 2011).

We drove changes to syllable FF before and after lesions of LMAN (i.e. Fig. 2b-f) in a total of 13 birds. A threshold for reinforcement was set according to the baseline variation in fundamental frequency (FF) over two days prior to the initiation of reinforcement. The threshold for reinforcement was set so that WN was played back over all syllable variants with a FF above (or below) the threshold. The threshold was set so that 70% of the variants (as measured at baseline) would have received WN. In 5/13 birds, we differentially reinforced both syllable FF and syllable sequencing before and after lesions of LMAN (i.e. Fig. 2c-f, Fig. 2h-j). In 4 of these 5 birds, changes to transition probabilities were induced at a branch point by playing WN when a targeted transition occurred, but not when alternative, non-targeted transitions occurred (Warren et al., 2012). In 2/5 birds, we drove changes to transition probabilities within a repeat by playing back WN when a repeat length threshold was exceeded (Warren et al., 2012). In the 5 birds for which we differentially reinforced both syllable acoustic structure and syllable sequencing, reinforcement was maintained for 1-2 days with LMAN intact and for 2 days with LMAN lesioned. In the 8 birds in which only syllable acoustic structure was differentially reinforced, reinforcement was maintained for 6 days (Fig. 2g).

Electrolytic lesions of LMAN

Bilateral electrolytic lesions were stereotaxically targeted at LMAN, with five penetrations per side and one or two current injections per penetration (50 or 100 μA for 60s). Removal of LMAN was confirmed by histology at the end of experiments, using anti-CGRP to stain for LMAN and mMAN, the medial magnocellular nucleus of the anterior nidopallium (Brainard and Doupe, 2001). The total amount of LMAN that was lesioned per bird averaged 77% (range from 45% to 97%). Nucleus mMAN remained intact in all birds. There was no correlation between estimated lesion size per bird and either the measured reduction in the variability of FF or any measures of learning. Lesions were verified histologically (Methods), and also caused a significant reduction in variability (33.8% +/- 12.1%), not significantly different from the magnitude of variability reduction reported previously for LMAN lesions and inactivations in Bengalese finches (Hampton et al., 2009; Warren et al., 2011).

Reversible blockade of LMAN-RA synaptic transmission by retrodialysis

Methods for implantation of dialysis probes and delivery of drug have been described previously (Warren et al., 2011). We switched from dialysis of ACSF to dialysis of DL-AP5 (2-5mM) for a period of 4 hours every 1-2 days. Dialysis of DL-AP5 was performed during a baseline period, with no reinforcement, and during the first 4 days of reinforcement.

Retrodialysis of DL-AP5 was performed on 9 birds. We drove changes to syllable FF during retrodialysis in 5 birds and to syllable sequencing in 6 birds.

Analysis

All analyses of song were on a 5-10% percent subset of songs chosen randomly for analysis. Analysis was conducted with custom software in MATLAB.

Adaptive modification of syllable FF

Fundamental frequency was calculated as described previously (Tumer and Brainard, 2007). We compared mean FF at baseline and following differential reinforcement (e.g. Fig. 2c-e) by comparing mean FF on the final day at baseline and the final day of reinforcement. For each day, FF was calculated over the second half (last 7 hours) of the day. In retrodialysis experiments (e.g. Fig. 3d), we compared the expression of learning (net change in FF from baseline) over a 4 hr control period prior to dialysis with AP5 with the expression of learning during AP5 dialysis (c.f. Warren et al. 2011).

Adaptive modification of syllable sequencing

The effects of differential reinforcement on transition probabilities were calculated over the same experiment days as syllable FF.

Results

LMAN is required for the adaptive modification of syllable structure but not syllable sequencing

We tested how LMAN, the cortical outflow nucleus of an avian basal ganglia circuit (Fig. 1), contributes both to the adaptive modification of syllable acoustic structure as well as to the adaptive modification of syllable sequencing (Tumer and Brainard, 2007; Warren et al., 2012). We addressed this question in Bengalese finch song, which consists of a sequence of highly stereotyped vocal elements, termed syllables. An example of Bengalese finch song is shown in Figure 2a. In this song, several transitions between syllables were stereotyped. For example, syllable 'a' was always repeated exactly 3 times (Fig. 2a, 'a_1, a_2, a_3'). However, as is typical in Bengalese finch song, other transitions between syllables were

variable. For example, following the third consecutive rendition of syllable 'a' ('a_3'), transitions occurred to syllable 'b' with a probability of 0.7 and to 'c' with probability of 0.3.

In adult birds, both the acoustic structure of individual syllables and the statistics of syllable sequencing are ordinarily highly stable over many weeks; however, birds can modify both syllable structure and sequencing in response to differential reinforcement. An example of the adaptive modification of syllable fundamental frequency (FF), induced with LMAN intact, is shown in Figure 2b-c. Here, to drive an upward shift in the FF of syllable 'd_1', we selectively played back WN over the subset of renditions of syllable 'd_1' in which the FF was below an experimentally-set threshold. In response to this differential reinforcement, the FF of the targeted syllable was shifted upwards, in one day, by 84 Hz, from 3331 HZ to 3415 Hz (Fig. 2c, 'baseline', days b1 and b2 ; Fig. 2c, 'WN', day 1). This shift was adaptive, in that it reduced the amount of WN played back over song.

In the same bird, with LMAN still intact, we then induced, via differential reinforcement, adaptive changes to the probability of a targeted sequence transition. The logic of this differential reinforcement is illustrated in Figure 2g-h. Here, WN was delivered following transitions from syllable 'a_3' to syllable 'b' but not following transitions from syllable 'a_3' to syllable 'c'. This sequence-contingent differential reinforcement caused a large decrease in the probability of the 'a_3-b' transition, which was targeted with WN, and a complementary increase in the probability of the alternative 'a_3-c' transition, from its baseline value 0.3 to a value of 0.92 (Fig. 2g-h).

We induced similar shifts to both syllable FF and syllable transition probabilities in 5 birds with LMAN intact. (Fig. 2e and Fig. 2j, 'LMAN intact'; mean change in FF: 51.2 +/- 9.3 Hz, mean change in transition probability: 0.33 +/- 0.07, n=5 birds). These data establish for these individual birds the magnitude and rapidity with which both types of learning could be driven with LMAN intact.

We then tested the same birds' capacity to modify syllable sequencing and syllable acoustic structure following bilateral, electrolytic lesions of LMAN (Methods). We found that lesions of LMAN prevented the modification of syllable acoustic structure, even when reinforcement was maintained for multiple days, but did not prevent the adaptive modification of syllable sequencing. An example is shown in Figure 2d (for the same bird for which modification to syllable structure and syllable sequencing was shown with LMAN intact). In this example, following lesions of LMAN, there was no significant adaptive shift in FF over two days of differential reinforcement (Fig. 2d, baseline mean 3346; mean, second half of day 2, 3319 Hz). The net change in syllable FF, of 27 Hz, was in the non-adaptive direction, meaning the bird slightly increased the fraction of syllables that received WN (in a non-adaptive manner). Across 5 birds, for which we similarly maintained reinforcement for two days, the average change in FF was not significantly different from zero (Fig. 2e, 'LMAN lesioned', mean change in FF: 5.4 Hz +/- 8.2 Hz in the non-adaptive direction). Therefore, for at least two days of maintained differential reinforcement, lesions of LMAN prevented adaptive modification of syllable acoustic structure.

In a separate set of birds, we further tested whether or not LMAN lesions prevented modification of syllable acoustic structure over longer periods of time. Prior findings indicate that modification of syllable FF involves both a fast, LMAN-dependent component of learning as well as a slow, LMAN-independent component of learning that develops over multiple days (Andalman and Fee, 2009; Warren et al., 2011). This raises the possibility that after LMAN is lesioned, a slow, LMAN-independent component of learning could develop over longer time periods than the two-day period examined in Figure 2e. We found that this was not the case. In 8 birds in which learning was measured before and after LMAN lesions (Fig. 2f), rapid adaptive learning occurred with LMAN intact. FF was significantly shifted from baseline during the first day of WN exposure ($p < 0.05$; Bonferroni corrected), and by

the third day, the mean adaptive shift in FF was 77 Hz. Following lesions of LMAN, contingent exposure to WN no longer caused any learning even after prolonged exposure to WN. After 6 days of WN exposure, the mean shift of FF in the adaptive direction was not significantly different from zero (7.8 Hz +/- 7.0 Hz). These results indicate that LMAN is required for any learning of changes to syllable acoustic structure, including the slow component of learning that ordinarily develops in the motor pathway when LMAN is intact.

Though lesions of LMAN prevented birds from modifying syllable structure adaptively, they did not prevent adaptive modification of syllable sequencing. An example is shown in Figure 2i. Here, we played back WN selectively following the 'a_3-b_1' transition (as in Fig. 2h). In response, the probability of this sequence transition was adaptively decreased, and the probability of the alternative, non-targeted 'a_3-c' sequence transition was adaptively increased. The net change in transition probability closely matched the change induced while LMAN was intact (net change in transition probability, post lesion = 0.58; net change in transition probability, pre lesion = 0.62). Across birds, the changes to transition probability induced by aversive reinforcement following lesions of LMAN were not significantly from those observed when LMAN was intact (Fig. 2j; net change post lesion = 0.28 +/- 0.07 ; net change pre lesion = 0.33 +/- 0.06; P = 0.64, paired t-test). Hence, lesions of LMAN that completely prevented learning of changes to syllable structure had no significant effect on learning of changes to syllable sequencing.

Together, these results demonstrate that similar principles of differential reinforcement can be used to drive adaptive changes to syllable sequencing and syllable structure, though only changes to syllable structure are prevented by removing LMAN, the cortical outflow nucleus of the basal ganglia circuit.

Transient interference with LMAN's output to the motor pathway does not affect the expression of newly learned changes to syllable sequencing

We next tested how LMAN contributes to the expression of newly learned modifications to syllable sequencing. Our observation that learning of syllable sequencing can occur without LMAN (i.e. Fig. 2i-j) does not rule out the possibility that ordinarily, when LMAN is intact, LMAN contributes to the expression of sequence learning. That is, the modification of transition probabilities when LMAN is lesioned could engage distinct neural substrates than those engaged when LMAN is intact. Such 'context-dependent' contributions of neural circuitry have been observed in other forms of learning (Fortis-Santiago et al., 2009; Ke et al., 2009)

We tested LMAN's contribution to the expression of learned changes to sequencing by transiently interfering with LMAN's output to the motor pathway. We exploited a pharmacological distinction between glutamatergic inputs to RA from LMAN and alternative glutamatergic inputs to RA from HVC. The inputs to RA from LMAN are predominantly mediated by NMDA receptors, while inputs to HVC to RA are mediated by a mixture of NMDA and AMPA receptors (Mooney and Konishi, 1991; Stark and Perkel, 1999). Therefore, blocking NMDA receptors in RA (with AP5) can selectively block LMAN inputs to RA (Olveczky et al., 2005; Warren et al., 2011).

As previously reported, blocking LMAN's input to RA pharmacologically reversed the expression of newly learned changes to syllable acoustic structure (Warren et al., 2011). An example is shown in Figure 3b. Here, at baseline, infusion of AP5 into RA caused no significant change in FF (Fig. 3b; day b1). However, infusion of AP5 into RA on the third day of adaptive modification of FF caused a partial reversion of learned changes to FF (Fig. 3b). Across 5 birds, there was a 47% reversion in the expression of adaptive learned changes to FF during AP5 infusion (Fig. 3d, P=0.01 t-test, n=9 infusions), comparable to that

observed in previous experiments that examined the contributions of LMAN to the expression of learned changes to syllable FF (Andalman and Fee, 2009; Warren et al., 2011).

We found, however, that infusions of AP5 into RA did not reverse the expression of newly learned changes to syllable sequencing. An example is shown in Figure 3c (for the same bird depicted in Fig. 3b). Here, an increase in the probability of a targeted transition (the alternative transition was targeted with WN) was induced over three days of reinforcement. Infusion of AP5 into RA did not cause a significant change to transition probability at baseline, or when transition probabilities were adaptively modified (Fig. 3c, days b1, 2 and 3). Similarly, across 6 birds in which we performed similar experiments, dialysis of AP5 into RA had no significant effect on the expression of learned changes to syllable sequencing (Fig 3e, n=15 infusions, P=0.13, t-test). The distinct effects of blocking AFP output on the expression of learned changes to syllable structure and syllable sequencing could not be attributed to differences in the efficacy of the AP5 infusions, as assessed by the reduction in the coefficient of variation of FF (Fig. 3e, 36.3 +/- 9.9% for experiments reinforcing syllable structure, 35.9 +/- 3.6% for experiments reinforcing sequence transitions). Moreover, in two cases, we drove changes to syllable structure and syllable sequence in the same individual (c.f. Fig. 3b-c), using the same probes and drug concentrations.

Discussion

We found that the anterior forebrain pathway (AFP), an avian cortical-basal ganglia circuit, contributed differently to hierarchically distinct aspects of vocal learning. Lesions of the AFP's cortical outflow nucleus LMAN prevented the adaptive modification of syllable acoustic structure but did not affect the adaptive modification of syllable sequencing. Moreover, transient blockade of AFP output reversed the expression of recently learned

changes to syllable structure but did not affect the expression of learned changes to syllable sequencing.

LMAN is required for the adaptive modification of syllable acoustic structure

Lesions of LMAN prevented the adaptive modification of syllable acoustic structure (Fig. 2b-f). No modification of fundamental frequency (FF) occurred, even when reinforcement was maintained for six consecutive days (Fig. 2f). This finding is consistent with prior studies in which LMAN contributes to vocal plasticity as well as with prior studies that demonstrated a specific role for LMAN in the adaptive modification of syllable structure (Bottjer et al., 1984; Williams and Mehta, 1999; Brainard and Doupe, 2000; Thompson et al., 2007; Andalman and Fee, 2009; Warren et al., 2011).

Our findings rule out a possibility, raised by prior studies, that a slow component of learning might develop gradually in the motor pathway in the absence of LMAN. Prior studies showed that the modification of syllable structure involves two distinct components – a fast component of learning that occurs in the AFP and a slow component of learning that occurs in the motor pathway (Andalman and Fee, 2009; Warren et al., 2011). In other forms of learning, analogous changes in the neural substrate required for learning have been attributed both to the parallel operation of circuits that independently undergo plasticity at different time scales (Lee and Schweighofer, 2009; Yin et al., 2009) and to the serial transfer of learning from one substrate to another (Pasupathy and Miller, 2005; Ohyama et al., 2006). If a parallel model obtained in songbirds, a slow component of learning should have developed in the motor pathway in the absence of LMAN input. On the other hand, if a serial model obtained, no slow component of learning should have developed without input from LMAN. Since we observed no evidence for a slow component of FF learning following lesions of LMAN (Fig. 2f), our findings support a serial model of consolidation, in which

fast, LMAN-dependent learning causes the slower learning that develops in the motor pathway.

LMAN is not required for the adaptive modification of syllable sequencing

We found that LMAN was required for neither the adaptive modification of syllable sequencing nor the expression of newly learned modifications to syllable sequencing. These findings therefore indicate that despite the underlying similarities of reinforcement-driven changes to syllable structure and syllable sequencing (e.g. a similar aversive reinforcement stimulus and similar dynamics of learning), LMAN contributes differentially to the two forms of learning.

Taken together with prior studies, our finding that LMAN is not required for adaptive modification of syllable sequencing suggests that distinct neural mechanisms underlie the non-adaptive changes to sequencing that occur when song deteriorates and the adaptive changes to sequencing induced by differential reinforcement. Prior studies have shown that lesions of LMAN prevent the deterioration of syllable sequencing that occurs (concurrently with deterioration of syllable acoustic structure) following deleterious manipulations such as deafening (Brainard and Doupe, 2000; Thompson and Johnson, 2006). One possibility that could explain these prior findings is that deleterious manipulations might generate a global error signal, causing LMAN to actively introduce aberrant neural activity patterns throughout song. These aberrant neural activity patterns could in turn disrupt both syllable acoustic structure (via perturbation of RA activity) and syllable sequencing (via recurrent pathways from RA that perturb HVC activity) (Ashmore et al., 2005; Wang et al., 2008). Lesions of LMAN therefore might prevent the degradation of sequencing by preventing the introduction of these aberrant signals. Analogously, the abrupt stabilization of syllable sequencing that is induced by silencing LMAN could be due to the removal of these aberrant signals (Olveczky

et al., 2005; Nordeen and Nordeen, 2010). This view that basal ganglia circuits can switch to a mode in which they produce highly aberrant output could also potentially explain deficits that are observed in the initiation and sequencing of movements in cases of dysfunction of the striatum (the input nucleus to the basal ganglia), such as Parkinson's or Huntington's disease (DeLong and Wichmann, 2007; Desmurget and Turner, 2010). These movement deficits, which are relieved by pallidotomies that silence the output of the basal ganglia, could be a result of aberrant basal ganglia output, analogous to what we postulate might occur in songbirds.

Variation and reinforcement-driven learning

Together with prior findings, our findings support a model of reinforcement learning in which the capacity to produce behavioral variation is linked to the capacity to effect behavioral change. The AFP (via LMAN) contributes both to the baseline rendition-to-rendition variation in syllable acoustic structure and is necessary for the adaptive modification of syllable acoustic structure (Kao et al., 2005; Olveczky et al., 2005; Andalman and Fee, 2009; Warren et al., 2011). Analogously, the AFP does not contribute to the baseline rendition-to-rendition variation in syllable sequencing and is not necessary for the learning of changes to syllable sequencing (Hampton et al., 2009; Fig. 2-3). Together, these findings suggest that there is a tight correspondence between a neural circuit's influence on baseline behavioral variation and the circuit's capacity to adaptively change behavior in response to differential reinforcement (Charlesworth et al., 2011).

Possible circuitry underlying adaptive modification of syllable sequencing

Our results raise the question of what neural substrates are required for the adaptive modification of syllable sequencing. One possibility is that in songbirds, parallel cortico-

thalamic basal ganglia loops control learning of syllable structure and sequencing, with the AFP contributing to syllable structure and a distinct basal ganglia loop contributing to syllable sequencing. In mammals, parallel basal ganglia loops have been shown to perform functionally segregated roles (Alexander et al., 1986) and this possibility has similarly been suggested for songbirds (Horita et al., 2008; Bottjer and Altenau, 2010). One candidate parallel pathway that could contribute to sequencing in songbirds includes a projection from mMAN (a frontal cortical area) to nucleus HVC, in which neural activity is correlated with syllable sequencing (Foster et al., 1997; Horita et al., 2008; Bottjer and Altenau, 2010). A distinct possibility is that in songbirds, basal ganglia circuitry is not crucial to sequence learning and that the key sites of neural plasticity are within HVC or within other inputs to HVC (e.g. Uva or NIF). Although mammalian basal ganglia circuits have long been assumed to contribute to action selection, and therefore to engage in sequence control and sequence learning (Mink, 1996; Shmuelof and Krakauer, 2011), a recent study in primates demonstrated specific effects of silencing the output of a basal ganglia circuit on the execution of movements but not on the sequencing of movements (Desmurget and Turner, 2010). While neurons in the striatum (the input nucleus to the basal ganglia) encode start/stop signals and sequence order (Mushiake and Strick, 1995; Jin and Costa, 2010), these signals could be efference copies of motor output that do not contribute causally to motor output. Our study highlights the importance of causal tests of basal ganglia circuits' contributions to forms of motor learning in which the learning of action sequencing can be disambiguated from the learning of action execution.

Chapter 3: Figure 1

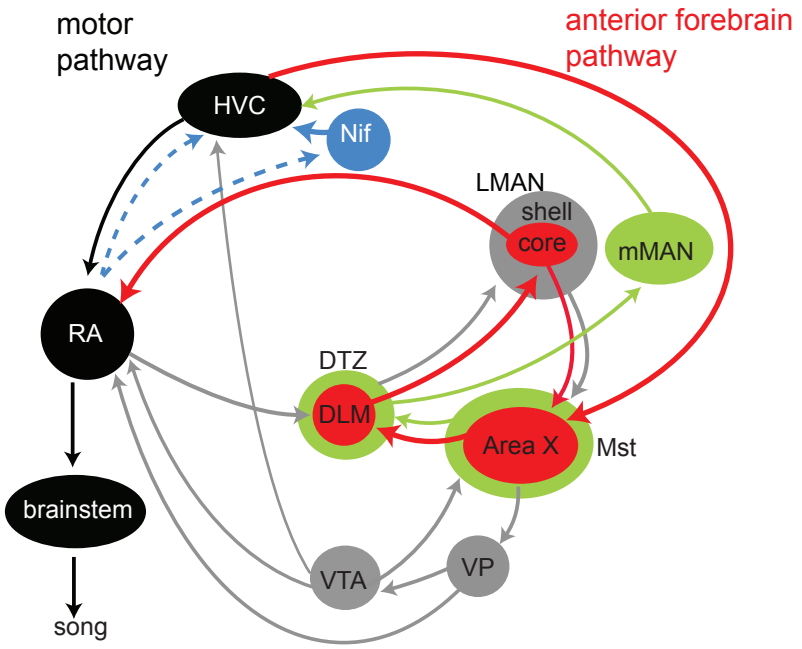

Figure 1. Neural circuitry underlying the production of birdsong
Neural pathways that control song production and learning include a motor pathway (black nuclei and arrows), which controls the moment-by-moment structure of song, as well as a cortico-thalamic basal ganglia circuit, the anterior forebrain pathway (red nuclei and arrows), which plays a crucial role in juvenile song learning and adult vocal plasticity. The song motor pathway includes nuclei HVC and RA, analogous to vocal premotor and motor cortex respectively. Neural activity in RA is correlated with the ongoing acoustic structure of song (Vu et al., 1994; Sober et al., 2008), while neural activity in HVC is correlated with syllable sequencing (Hahnloser et al., 2002; Fujimoto et al., 2011). LMAN, a cortical analog, projects to RA and is a principal outflow nucleus of the basal ganglia circuit. Other components of the AFP are the basal ganglia homolog Area X and the thalamic nucleus DLM. Various recurrent pathways project to HVC and could presumably allow the AFP to influence syllable sequencing by indirectly affecting HVC activity. These include: (1) an alternative cortico-thalamic basal ganglia loop from the AFP (green nuclei and arrows), in which a cortical nucleus mMAN projects to HVC, (2) recurrent pathways through the brainstem that project to Nif and HVC (blue nuclei and arrows), and (3) pathways through midbrain dopaminergic nucleus VTA, which projects to RA and HVC (gray nuclei and arrows)

Chapter 3: Figure 2

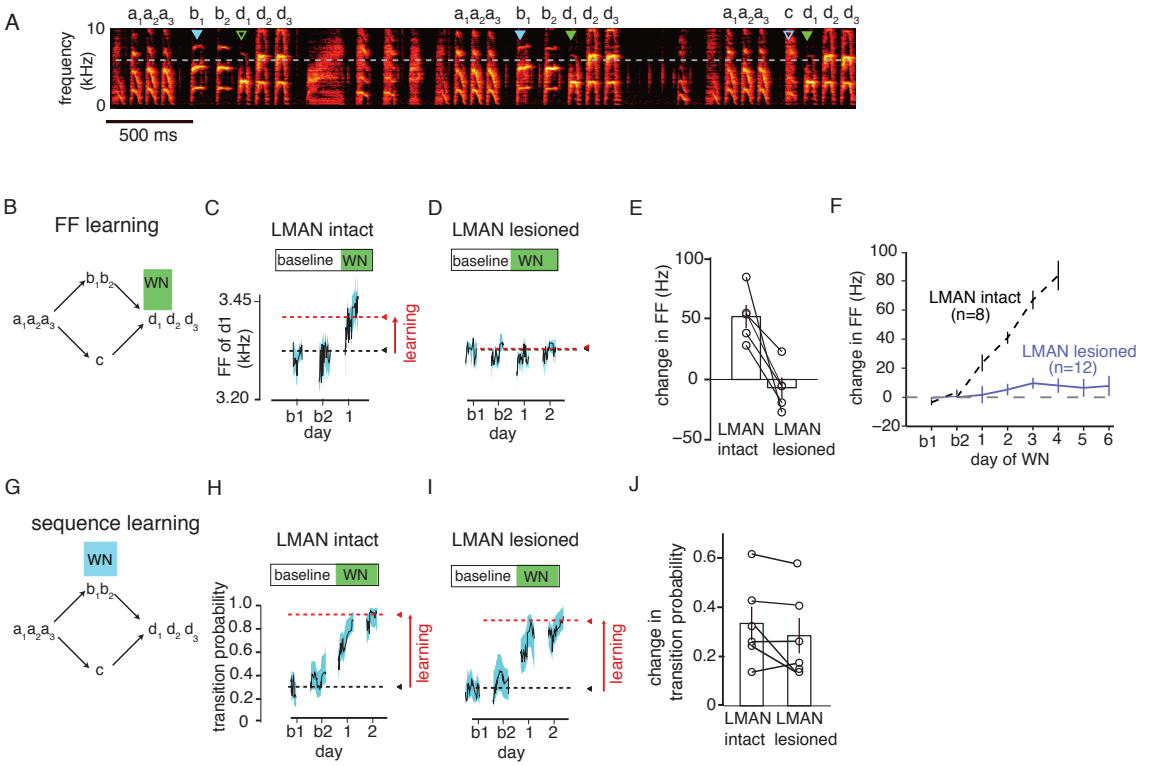

Figure 2. LMAN is required for the adaptive modification of syllable structure but not syllable sequencing

A. Spectrogram from an adult Bengalese finch with 4 distinct syllables: syllable a (a1, a2 and a3), syllable b, (b1 and b2), syllable c, and syllable d, (d1, d2 and d3). Several syllable transitions were stereotyped (e.g. b2-d) while other syllable transitions were variable (e.g. a3 could transition either to b1 or to c). B. Schematic of differential reinforcement of syllable acoustic structure. We drove an upward shift in the FF of syllable d1 by playing back WN over variants of d1 with a value of FF lower than an experimentally set threshold (Filled green triangles in panel A denote lower FF variants that elicited WN playback; open green triangle indicate higher FF variants that were detected but did not elicit WN playback). C. Example of adaptive modification of FF. Syllable FF had a stable mean value during two days at baseline ('baseline'; black solid line denotes running average of syllable FF; blue shaded area 95% confidence intervals; black triangle, mean FF at baseline) but initiation of WN playback induced a rapid and large change in FF ('WN'; red triangle, mean FF at end of day 1). D. Example effects of LMAN lesions on the capacity for adaptive modification of syllable FF. Playback of WN for two days induced no significant change to the FF of the targeted syllable. E. Summary data, from 5 birds, showing significant changes to FF induced with LMAN intact and no adaptive changes following LMAN lesions. F. The effects of LMAN lesions on the modification of syllable FF for prolonged periods of reinforcement. Here, reinforcement was maintained for 6 consecutive days. No adaptive changes to FF were observed following lesions, even after the 6th day of reinforcement. G. Differential reinforcement of syllable sequencing. We played back WN following a3-b1 transitions but not a3 -c transitions. H. Modification of transition probabilities with LMAN intact. A rapid increase was induced in the transition probability of the a3-c transition, which did not elicit WN playback. I. With LMAN lesioned, differential reinforcement induced an increase in transition probability of the a3 -c transition that was not significantly different from the increase induced with LMAN intact. J. Summary of changes to transition probabilities induced when LMAN was intact ('LMAN intact') and when LMAN was lesioned ('LMAN lesioned'). The magnitude of learning was not significantly affected by lesions.

Chapter 3: Figure 3

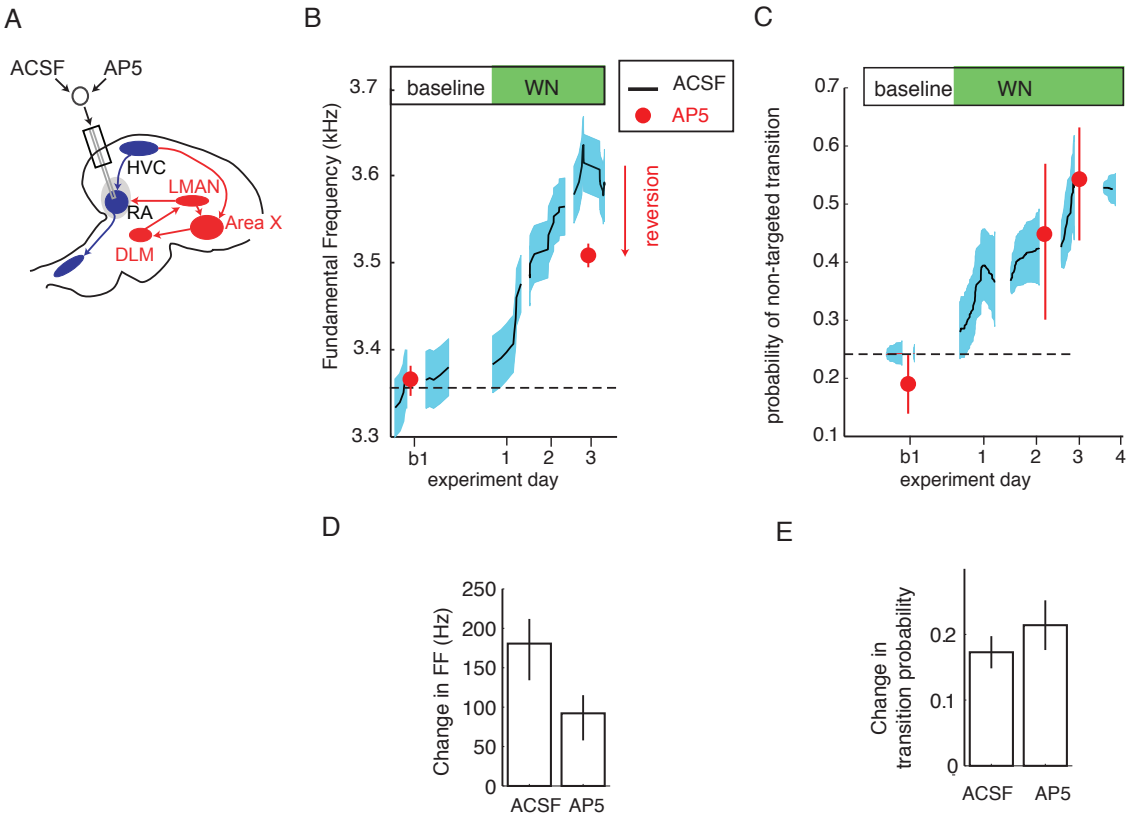

Figure 3 LMAN is required for the expression of learned changes to syllable structure but not the expression of learned changes to syllable sequencing.

A. Experimental design. Dialysis probes were implanted bilaterally into RA (One hemisphere shown, saggital section). DL-AP5, an NMDA-receptor antagonist, was dialyzed across the probes to interfere with LMAN-RA glutamatergic transmission. B. Example effects of AP5 dialysis on the expression of learned changes to syllable structure. At baseline, dialysis of AP5 into RA did not significantly affect mean FF ('day b1'). However, dialysis of AP5 on the third day of reinforcement caused a significant reversion of FF, toward the original baseline ('day 3', red arrow, 'reversion'). C. Example effects of AP5 dialysis on the expression of learned changes to syllable sequencing. Dialysis of AP5 into RA at baseline, and on days 2 and 3 of reinforcement caused no significant change in the transition probabilities. D. Summary effects of AP5 dialysis into RA on the expression of FF learning (n=9 infusions in 5 birds). On average, there was a 47% reversion in the expression of learned changes to FF. E. Summary effects of AP5 dialysis on the expression of sequence learning (n=15 infusions in 6 birds, 2/6 birds also in Fig. 3d). No significant change in transition probabilities was observed, and the net change in transition probabilities induced by AP5 infusions was away from baseline.

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