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The contribution of LMAN, the output of an avian basal ganglia-forebrain circuit to
song variability and adaptive plasticity in the adult Bengalese finch

by

Cara M. Hampton

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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of the

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Dedication and Acknowledgements:

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I dedicate this thesis to my grandfather General Rex Hampton, who's crazy idea it was in the first place for me to join the world of scientific research ("do something important for the world, now") and would have given anything to see me here today.

A portion of this thesis has been co-authored by Jon T. Sakata and previously published in the Journal of Neurophysiology:

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The contribution of LMAN, the output of a basal ganglia-forebrain circuit, to song variability and adaptive plasticity in the adult Bengalese finch.

Cara M. Hampton

ABSTRACT

The capacity for change in adult behavior and in the adult brain is much greater than previously thought. In songbirds, adult behavior continues to change measurably even after song is well-learned and stable. Whether adult song change serves any function, however, is under debate. LMAN, the output of a basal-ganglia forebrain pathway in the song system, has been broadly implicated in adult song change including adult song variability and sensory feedback plasticity. For adult song variability, previous studies have shown that loss of LMAN by lesion or inactivation reduces variability in syllable structure. These findings, however, have been limited due to the low variability present in the most common songbird species of study, the zebra finch. For adult plasticity, it has been shown that LMAN can prevent degradation in song before auditory feedback manipulations. Although this suggests active regulation of adult song change, it is unclear whether the role of LMAN is adaptive under these circumstances. Here, we have investigated the role of LMAN in the adult Bengalese finch in both adult song variability and adult song plasticity. We first find that adult Bengalese finch retains and modulates a significant amount of variability in song, including variability in syllable structure and variability in syllable sequencing. We then show that LMAN is involved in the regulation of one specific aspect of song variability: syllable structure. Finally, using a new behavioral paradigm for adult plasticity, we extend these findings to demonstrate that LMAN is involved in adaptive recovery of song following injury. Taken together, these data suggest that LMAN in the adult Bengalese finch retains a strong capacity for actively driving adult song change, and that its function remains adaptive long after learning is complete.

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INTRODUCTION

Classically, it was believed that adult behaviors, once learned, remained fixed or stereotyped as did the neural regulation of such behaviors. Henceforth expressions such as “you can’t teach an old dog new tricks”. Several studies over the last few decades have revealed that remarkable changes are possible in the adult brain, and often coincide with changes in behavior (reviewed in Buonomano and Merzenich 1998; Nottebohm 2004). In songbirds, changes in adult behavior are well documented. Moment-by-moment changes in adult behavior are expressed as variability from one song to the next. Long-term changes in adult behavior, or song plasticity, have been also been observed (Nordeen and Nordeen 1992; Williams and McKibben 1992; Leonardo and Konishi 1999; Hough and Volman 2002; Woolley and Rubel 2002; Cynx and Gell 2004; Zevin et al. 2004; Kao and Brainard 2006; Sakata and Brainard 2006; Roy and Mooney 2007; Tumer and Brainard 2007; Sakata et al. 2008). Interestingly, LMAN (the lateral nucleus of the anterior nidopallium), a forebrain region of the songbird brain has been implicated in both variability and plasticity of adult birdsong (Williams and Mehta 1999, Brainard and Doupe 2000, Kao et al 2005, Kao and Brainard 2006; Scott et al. 2007). It is unclear, however, what aspects of song variability are regulated by LMAN or whether changes to adult song serve any adaptive purpose. We investigated the role of LMAN in adult song variability and sensory feedback plasticity in the adult Bengalese finch, a species that has been suggested to have a greater capacity for adult song change than the more commonly studied zebra finch.

As in the zebra finch, song learning in the Bengalese finch occurs during a critical period in development. Song is composed of individual acoustic elements ‘syllables’ that occur in a particular sequence or set of sequences. Once learned, song is stable, yet retains a measurable amount of variability. Song variability in the adult zebra finch is limited to syllable structure, while song variability in the adult Bengalese finch is present

for both syllable structure and syllable sequencing (Okanoya 2004). In [Chapter 1](#), we describe features of adult Bengalese finch song, and one way in which the variability of those features is actively regulated: by social context. Variability is decreased when a male sings to a female (female-directed song) versus singing alone (undirected song) (Sossinka and Bohner, 1980, Kao et al. 2005, Kao and Brainard 2006). We show that in the adult Bengalese finch, not just variability in syllable structure, but also variability in syllable sequencing, and a number of other features are modulated by social context on a moment-by-moment basis.

The extent to which the variability of different song features can be regulated by LMAN is unknown. In the zebra finch, moment-by-moment changes in syllable structure require LMAN. It is unclear, however, whether LMAN could be involved in moment-by-moment changes in syllable sequencing. In juvenile zebra finches, LMAN lesions reduce the variability of syllable structure and variability of syllable sequencing, so it is plausible that LMAN could have a similar role in adults. However, adult zebra finches do not retain variability in syllable sequencing, so it could not previously be addressed. In [Chapter 2](#), we investigate the role of LMAN in song variability for both syllable structure and syllable sequencing in the adult Bengalese finch. To our surprise, and contrary to what was found in studies of juvenile zebra finches, we found variability in syllable sequencing was *not* reduced by LMAN lesions in the adult Bengalese finch, while variability in syllable structure was, suggesting that the regulation of adult song variability may be more specific than previously thought and may involve multiple distinct neural pathways.

LMAN has also been implicated in long-term changes to adult song behavior, adult plasticity. In previous studies, long-term changes to song behavior were induced by long term sensory manipulations, either deafening or removing the input to the vocal organ, the syrinx. In both cases, song degraded slowly and permanently over several weeks (Nordeen and Nordeen 1992, Williams and McKibben 1992). It has been shown

that LMAN lesions prior to such manipulations prevent song from degrading (Williams and Mehta 1999, Brainard and Doupe 2000). These experiments were the first evidence that LMAN had an active role in adult song behavior because LMAN lesions prevented song changes. However, it is unclear whether the role of LMAN was adaptive under these circumstances, or just contributing to song degradation due to permanent sensory deprivation. We hypothesized that adult changes to song were part of an adaptive plasticity process, such that, given the opportunity for song recovery, LMAN might contribute to the process of recovery as well as song change. In [Chapter 3](#), we introduce a new behavioral paradigm for adult plasticity, reversible ts nerve crush (tsNC). We first find that song degrades following tsNC and subsequently recovers. Then, by lesioning LMAN after song degradation was induced, we find that song recovery is prevented. These data demonstrate that the function of LMAN can be adaptive in adult songbirds.

Taken together, these data suggest that the adult Bengalese finch retains a strong capacity for actively driving variability and adaptive plasticity into adulthood, and that LMAN is specifically involved in the regulation of these phenomena.

CHAPTER 1.

Behavioral variability in the adult Bengalese finch: social context modulation of syllable structure and syllable sequencing.

Social modulation of sequence and syllable variability in adult birdsong

Jon T. Sakata*, Cara M. Hampton, and Michael S. Brainard

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ABSTRACT

Birdsong is a learned motor skill that is performed with a high degree of stereotypy in adult birds. Nevertheless, even in species where song 'crystallizes' in a form that remains stable over time, there is residual variability. Such variability in well-learned skills is often construed as uncontrolled and irrelevant biological 'noise'. However, studies in the zebra finch indicate that variability in one song feature – the structure of individual syllables – is actively regulated and may serve a function. When male zebra finches sing alone ('undirected song') variability in syllable structure is elevated relative to when they sing to females in a courtship context ('female-directed song'). This elevated variability is actively introduced to premotor structures controlling syllable production by a forebrain-basal ganglia circuit. Here, we test whether social modulation of song variability extends to syllable sequencing, a hierarchically distinct feature of song organization controlled by separate neural substrates from syllable structure. We use Bengalese finches as a model species because, unlike zebra finches, they typically retain substantial moment-by-moment variability in the sequencing of syllables in crystallized adult song. We first show social modulation of previously studied song features, including syllable structure and song tempo. We then demonstrate that variability in syllable sequencing is rapidly and reversibly modulated by social context,

with greater variability present in undirected song. These data indicate that the nervous system exerts active control over variability at multiple levels of song organization and support the hypothesis that such variability in otherwise stable adult song serves a function.

INTRODUCTION

Variability is inherent to the expression of all behaviors, but its function and regulation have received little attention. In the context of well-learned skills, such variability is often construed as biological 'noise' that is below the threshold for behavioral importance or that the nervous system is unable to eliminate. However, theoretical studies suggest that trial-by-trial behavioral variability potentially plays an important role in learning: variation in motor output could reflect active 'motor exploration' required for the reinforcement of motor commands that produce more desired outcomes and the weakening of motor commands that produce less desired outcomes (Doya and Sejnowski 2000; Sutton and Barto 1998). Understanding whether and how variability is regulated can lend insight into the importance of behavioral variability and its neural sources.

In many species of songbird, adult song, once learned, is extremely stereotyped from one rendition to the next, and there may be little detectable change in acoustic structure over periods of months or even years (reviewed in Brainard and Doupe 2002; Doupe and Kuhl 1999; Tchernichovski et al. 2001). Hence, adult 'crystallized' song provides an example of an extremely well-learned motor skill. Nevertheless, there is small but measurable residual variation in adult song. Recent studies in the zebra finch have provided support for the hypothesis that residual variability in adult song might reflect a form of 'motor exploration' in which the nervous system actively generates

variation as a part of vocal practice required to continuously maintain and optimize song (Jarvis et al. 1998; Kao and Brainard 2006; Kao et al. 2005; Ölveczky et al. 2005).

Support for the idea that a component of adult song variability might be actively generated as part of vocal practice derives exclusively from behavioral and neural investigations of zebra finch song. At a behavioral level, it has been observed that adult male zebra finches subtly alter the variability of their songs between social contexts in which males sing alone ('undirected', or UD song) and those in which they sing to a female ('female-directed', or FD song)(Kao and Brainard 2006; Kao et al. 2005). The acoustic structure of individual song elements, or 'syllables', is consistently more variable from one rendition to the next for UD song than for FD song. At a neural level, several lines of evidence suggest that the excess variability present in UD song may be actively generated by the anterior forebrain pathway (AFP; Kao and Brainard 2006; Kao et al. 2005; Ölveczky et al. 2005), an avian homologue of a cortical-basal ganglia circuit (Perkel 2004; Reiner et al. 2004). This circuit specifically projects to song premotor nuclei and is required for normal song learning and adult song plasticity (Bottjer et al. 1984; reviewed in Brainard 2004; Brainard and Doupe 2000; Scharff and Nottebohm 1991; Williams and Mehta 1999). Neural activity within the AFP is greater and more variable for UD song than FD song, suggesting that signals arising from the AFP might actively drive variability in UD song (Hessler and Doupe 1999; Jarvis et al. 1998; Kao et al. 2005). Artificial manipulation of activity arising from the AFP further supports this possibility. Lesions of the lateral nucleus of the anterior nidopallium (LMAN), the output nucleus of the AFP, prevent the context-dependent modulation of syllable variability by reducing the variability present in UD song to the level present in FD song (Kao and Brainard 2006; Ölveczky et al. 2005). Conversely, introduction of variable neural activity into LMAN, by microstimulation during singing, can drive increased variability in syllable structure (Kao et al. 2005). Hence, signals from LMAN are both necessary and sufficient

to account for the increased variability in syllable structure in UD song relative to FD song.

Collectively, these data are consistent with a model in which, despite the great stereotypy of adult song, there is nevertheless a subtle but active regulation of song variability. More specifically, it has been hypothesized that brain and behavior switch between a state of vocal 'practice' (during UD song), in which variation is actively introduced to pre-motor song structures, to a state of 'performance' (during FD song), in which neural sources of variability are reduced in order to emphasize features of the current 'best' song as part of effective courtship (Jarvis et al. 1998; Kao and Brainard 2006). This 'practice versus performance' hypothesis has been systematically investigated only in the zebra finch, and derives almost exclusively from measurements of the modulation of the acoustic structure of individual song syllables. The active regulation of syllable sequencing, a hierarchically distinct aspect of song organization from syllable structure, has not been systematically investigated, in part because the sequencing of syllables in adult zebra finch song is often invariant (Kao and Brainard 2006; Zevin et al. 2004; but see also two birds in Sossinka and Böhner 1980). Current models of song production suggest that separate components of song premotor circuitry contribute differentially to the sequencing versus structure of syllables (reviewed in Fee et al. 2004; Hahnloser et al. 2002; Margoliash 1997; Vu et al. 1994; Yu and Margoliash 1996), and, consequently, syllable sequencing may be subject to different forms of regulation from syllable structure.

Here we take advantage of the intrinsic variability of syllable sequencing in another songbird, the Bengalese finch, to investigate whether syllable sequencing is subject to active modulation in adult songbirds. Bengalese finches, like zebra finches, produce an adult song that remains stable over time (Clayton 1987; Immelmann 1969). However, unlike zebra finch song, the songs of adult Bengalese finches retain a

significant degree of moment-by-moment variability in the sequencing of syllables (reviewed in Okanoya 2004). Hence, it is possible to quantify the variability of syllable sequencing in adult Bengalese finch song as well as the modulation of that variability in response to alterations of social context. We report that, in addition to the forms of song modulation previously observed in the zebra finch, there is a rapid social modulation of syllable sequencing in adult Bengalese finch song. The nature of this modulation is analogous to that previously reported for syllable structure, with greater variability in sequencing present in UD song than in FD song. Hence, even in the crystallized songs of adult birds, there is nevertheless a capacity for active and rapid modulation of multiple, hierarchically distinct song features.

MATERIALS AND METHODS

Animals: Adult Bengalese finch males (n=19; range: 4-18 months; median=6 months) were raised in our colony. Birds were housed with their parents and siblings until at least 60 days of age, then housed with other males on a 14L:10D photoperiod. Birds were isolated and housed individually in a sound-attenuating chamber (Acoustic Systems, Austin, Texas) for at least one day prior to testing, and food and water were provided ad libitum. All procedures were performed in accordance with established animal care protocols approved by the University of California, San Francisco Institutional Animal Care and Use Committee (IACUC).

Data collection: Sound was recorded using an omnidirectional microphone (Countryman Associates, Inc, Menlo Park, CA) positioned above the male's cage. A computerized, song-activated recording system was used to detect and digitize song (Observer, A. Leonardo, Caltech; C. Roddey, UCSF; digitized at 32 kHz; or Sound Analysis Pro v.1.04 (http://ofer.sci.ccny.cuny.edu/html/sound_analysis.html); digitized at 44.1 kHz).

Recorded songs were digitally filtered at 0.3-8 kHz for off-line analysis using software written in the Matlab programming language (The MathWorks, Natick, MA).

'Undirected' (UD) songs are produced in isolation, whereas 'female-directed' (FD) songs are produced during courtship interactions with females. During the day of the experiment, FD song was elicited by placing a cage with a female adjacent to the experimental male's cage. As in the zebra finch, FD songs of Bengalese finches are readily distinguishable from UD songs because they are produced after a male approaches or faces another individual, are accompanied by a courtship dance (e.g., pivoting body from side to side), and are associated with the fluffing of the male's plumage (Morris 1954; Zann 1996). Behavior of birds was monitored by experimenters either remotely by video camera or through a small observation window in the sound boxes, and only songs that were accompanied by at least two of the above behaviors were categorized as FD songs. When FD songs were produced, they were almost always produced within 15 seconds after the introduction of the female. Females were removed after <2 min regardless of whether the males produced FD song. This design ensured that FD songs in our study reflected those that were elicited at a short latency following exposure to a female (Hessler and Doupe 1999; Kao and Brainard 2006). The median interval between exposures to females was 5 min (range: 2-19 min). This allowed for the collection of UD song between exposures to females. Because UD and FD songs were interleaved in this manner, we could compare songs that were temporally proximal to each other, and, hence, our results emphasize rapid changes to song by social context. Although our experiment was designed to collect UD and FD song in an interleaved manner, males did not always sing UD song between exposures to a female. Therefore, up to 30 minutes of UD songs were also recorded before the first exposure and after the last exposure to a female.

Song parameters and definitions: An example of a Bengalese finch song is provided in Fig. 1. For purposes of description and analysis, we use the term 'syllable' to refer to individual acoustic elements of Bengalese finch song that are separated from each other by at least 5 ms of silence (Okanoya and Yamaguchi 1997). Syllables that are simple in structure and repeated at the beginning of song are referred to as 'introductory notes' (e.g., syllable 'i' in Fig. 1). Within song, syllables are organized into stereotyped or variable sequences, and syllables that are followed by variable transitions are referred to as 'branch points' (e.g., syllable 'a' in Fig. 1). Some syllables are repeated a variable number of times and are referred to as 'repeats' (e.g., syllable 'f' in Fig. 1). Following amplitude-based syllable segmentation (Matlab), we labeled syllables manually based on visual inspection of spectrograms and analyzed syllable and sequence differences across social contexts.

To analyze changes to the variability of syllable structure, we compared the fundamental frequency (FF) of syllables that had distinct and stable harmonic structure (see Kao et al. 2005). For each syllable we calculated the autocorrelation of a segment of the sound waveform. We focused on these syllables because the calculation of FF is less sensitive to changes in syllable duration (e.g., relative to syllables with frequency sweeps). The FF was defined as the distance, in Hz, between the zero-offset peak and the highest peak in the autocorrelation function. Each example of a syllable was visually screened to ensure that only examples devoid of sound artifacts that could affect FF calculation (e.g., sound of movement, female calls in background) were used in the analysis. To improve the resolution of frequency estimates, we performed a parabolic interpolation of the peak of the autocorrelation function (de Cheveigné and Kawahara 2002). We found that the FF of syllables varied from rendition to rendition and characterized this variation using the coefficient of variation ($CV: 100 * \sigma / \mu$).

To quantify changes to the variability of syllable sequencing, we analyzed changes in the probability of sequence transitions at branch points across UD and FD songs. An example of a branch point is presented in Fig. 1 in which the syllable 'a' could be followed by syllables 'b', 'c' or 'l'. We analyzed the probability of different syllable transitions immediately following a specific sequence of syllables (first-order transitions). Typically, there are 2-5 first order transitions at branch points. For each branch point, this variability was quantified as the transition entropy:

$$\text{transition entropy} = \sum p_i \log_2(p_i)$$

where the sum is over all possible transitions, and p_i is the probability of the i th transition across all songs (Gil and Slater 2000; Sakata and Brainard 2006). Branch points with transitions that are more variable (i.e., closer to uniform probability) have higher transition entropy scores. Branch point sequences in which the dominant transition occurred >95% of the time were not considered branch points. Instances in which song was terminated immediately following the branch point were not included in the calculation of entropy. Repeated syllables can also be construed as branch points (i.e., repeat syllable or transition to a different syllable), but we analyzed the entropy of repeats separately from other branch points. Context-dependent sequence changes within stereotyped sequences were not observed.

Additionally, we analyzed context-dependent differences in the number of introductory notes, song length, and song tempo, all features previously reported to be modulated by social context in zebra finches (Cooper and Goller 2006; Kao and Brainard 2006; Sossinka and Böhner 1980). We counted the number of introductory notes preceding each song by starting at the first introductory note prior to the first (non-introductory) syllable of the song and then counting backward in time until there was >500 ms of silence (Kao and Brainard 2006). If there were more than one type of introductory note, all were counted in the analysis. The song of one male did not have

introductory notes and, hence, was not included in this analysis. Song length was defined as the interval from the onset of the first (non-introductory) syllable to the offset of the last syllable of the song. We defined the end of a song as the offset of a syllable that was followed by >500 ms of silence. For comparisons of song tempo across social contexts, we measured the duration of matched sequences of syllables that occurred commonly. The interval from the onset of the first syllable to the onset of the last syllable in the sequence was computed. Onsets were selected as boundaries because the change in amplitude is sharper and less variable for onsets than for offsets, allowing for a more accurate estimate of duration.

Data analysis: Before all analyses, the distributions of data for all behavioral parameters were assessed for violations of normality using the Shapiro-Wilk W test, and only when distributions did not violate normality were parametric tests used. For comparisons within an individual, t-tests were used to assess whether song parameters were significantly different between UD and FD song (see examples in Results and Figures).

The songs of many birds contained multiple distinct examples of a measured song feature (FF, transition entropy, song tempo). Songs from a single Bengalese finch could contain multiple distinct syllables with flat acoustic structure for FF measurement, multiple unique branch points, and multiple distinct sequences in which song tempo could be measured. Data for each unique example were recorded (e.g., FF for each unique syllable in a bird), and for each example we calculated the percent change using the following formula:

$$\text{percent change} = 100 * (\mu_{\text{FD}} - \mu_{\text{UD}}) / \mu_{\text{UD}}$$

where μ_{FD} and μ_{UD} refer to the sample means for FD and UD songs, respectively. To avoid pseudoreplication in population analyses of social context, we computed the

weighted average of the percent changes for males with multiple examples of a specific feature. The weighted average was computed using the following equation:

$$\text{percent change per male} = \sum \Delta_i * n_i / \sum n$$

where Δ_i represents the percent change of the i th example of a feature, n_i represents the sample size for i th example, and $\sum n$ represents the sum of n 's across all examples of that feature. For example, for a single male with two examples of branch points in his song, if there was a 4% decrease in entropy at a branch point that was sung 100 times across both contexts and a 2% decrease in entropy at a branch point that was produced 300 times across both contexts, the overall percent change in entropy would be a decrease of 2.5% ($4\% * 100 / 400 + 2\% * 300 / 400 = 2.5\%$). Thereafter, we tested whether the mean of weighted differences was significantly different than zero using a t-test or Wilcoxon signed-ranks test.

Paired t-tests were used to analyze differences in introductory notes and song length between UD and FD songs.

For all tests we set $\alpha = 0.05$ (two-tailed). Analyses were done using JMP 5.0.1 (SAS Institute, Cary, NC) for the MacIntosh.

RESULTS

Organization of Bengalese finch song:

As in many songbird species, songs of the Bengalese finch consist of both simple and complex song elements called 'syllables' whose structure and sequencing are learned. Upon reaching sexual maturation, the song of an adult Bengalese finch is considered to be 'crystallized' because the composition of song remains stable across time (Clayton 1987; Immelmann 1969; Okanoya and Yamaguchi 1997; Woolley and Rubel 1997). Song is typically preceded by low amplitude, spectrally simple song

elements called introductory notes, and song itself is composed of a number of spectrally distinct syllables (typically 5-10) arranged into both stereotyped and variable sequences (Figs. 1a,b).

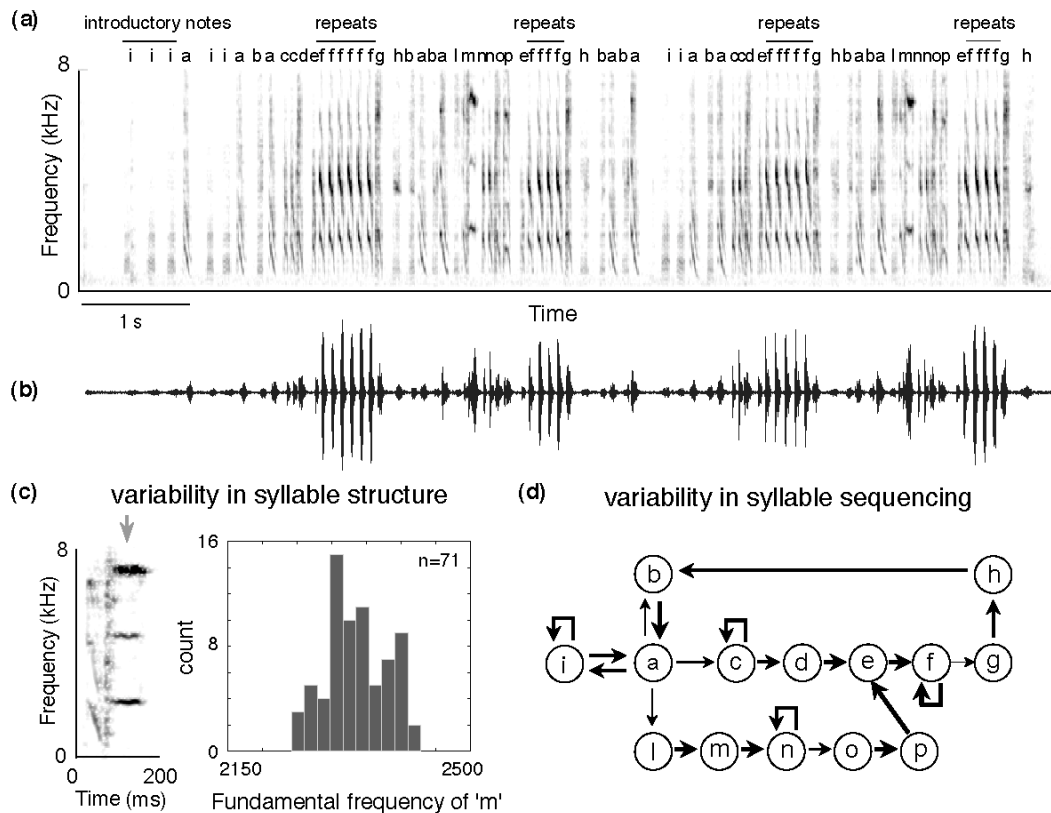


Figure 1. Organization of Bengalese finch song. (a) Spectrogram of an example of an undirected song from a Bengalese finch male. Power (grey scale) is plotted as a function of frequency and time. Above the spectrogram are distinct labels for each syllable to aid in sequence analysis. The song element 'i' is an introductory note, which predominantly occurs prior to song initiation but also can be produced within song. There are 13 distinct syllables for this male, and the syllable 'f' is repeated a variable number of times within song. (b) Oscillogram showing amplitude of the sound trace for the song in (a). (c) On the left is a spectrogram of the syllable 'm' [from song in (a)], and to the right is a histogram depicting the variation in the fundamental frequency of the syllable across renditions of undirected song. (d) Transition diagram describing the variability in syllable sequencing for undirected songs from this male. Thicker arrows represent more prevalent transitions. Syllables such as 'a', 'c', 'f', and 'n' are branch points where the transitions following the syllable are variable, as opposed to syllables 'b', 'd', 'e', 'g', 'h', 'l', 'm', 'o' and 'p' where the transitions are stereotyped.

In the example provided in Figure 1, there was one type of introductory note ('i') followed by 13 distinct syllables. Introductory notes can also be interspersed within song; in this

example, the introductory note 'i' could be produced following the syllables 'a' and 'g'. The acoustic structure of syllables varies from rendition-to-rendition, and we characterized this variability by measuring the distribution of FFs of syllables with flat harmonic structure (Fig. 1c). The sequencing of syllables also varies from rendition to rendition, and we characterized this variability by measuring the probability of each possible transition over a large set of songs. This sequence variability can be illustrated using a 'transition diagram' (Fig. 1d), where each node corresponds to a unique syllable and the thickness of arrows connecting each node reflects the probability of transitions between syllables. For this example, transitions from some syllables were stereotyped (e.g., syllables, 'b', 'd', 'e', 'g', 'h', 'l', 'm', 'o' and 'p'), whereas transitions from other syllables were variable (e.g., syllables 'a', 'c', 'f', and 'n'). Sequences followed by variable transitions are referred to as 'branch points'. For example, the syllable 'a' is a branch point that could be followed by the syllables 'b', 'c', and 'l'. The syllable 'f' was repeated a variable number of times (4-6 times in this example), and we refer to these types of syllables as 'repeats'. These types of variability in syllable structure and sequencing make adult Bengalese finches a useful model to study the modulation of vocal motor behavior.

Variability of syllable structure:

In order to test the generality of previous observations in the zebra finch and to confirm that our manipulation of social context was effective in altering song, we first examined changes to the variability with which individual syllables were produced in the Bengalese finch. In the adult zebra finch, the structure of syllables is less variable during songs produced to females relative to songs produced in isolation (Kao and Brainard 2006; Kao et al. 2005). Here we similarly found a dramatic reduction in the variability of syllable structure during FD song in the Bengalese finch. We quantified the variability in

syllable structure using the coefficient of variation (CV) for FF of individual syllables (see Methods).

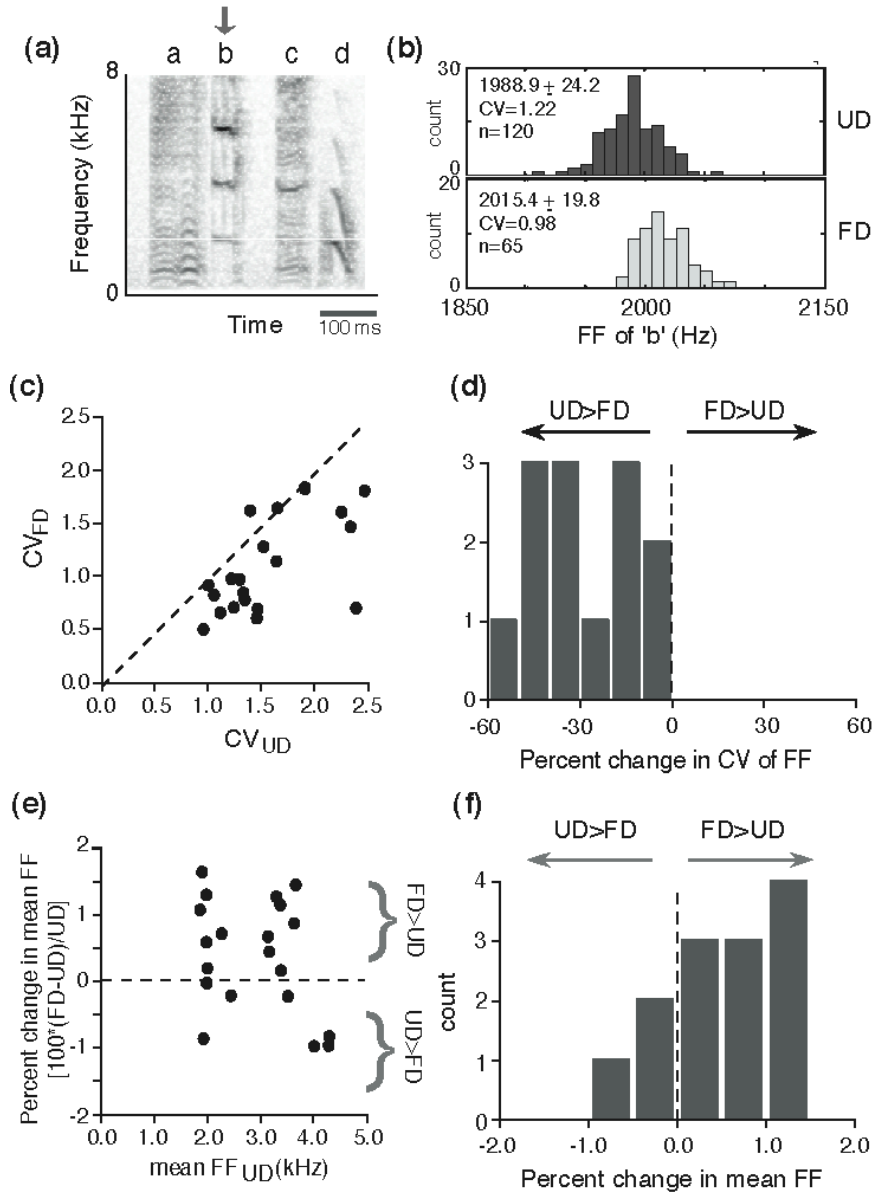


Figure 2. Effect of social context on syllable structure in the Bengalese finch. (a) Spectrogram of song with distinct labels for each syllable above. Arrow above the letter 'b' highlights an example from one Bengalese finch of the type of syllables with flat harmonic structure measured in the analysis of fundamental frequency (FF). Scale bar = 100 ms. (b) Distribution of FF values for the syllable 'b' produced during undirected (UD: dark bars) and female-directed (FD: grey bars) songs (data as means $\pm$ SD). Relative to UD song, the coefficient of variation (CV) was lower and the mean FF was significantly higher (t-test: $t_{183}=7.54$, $P<0.0001$) when this male produced FD song. (c) Plot showing that the CV of FF was lower during FD song than UD song for 18 of the 20 syllables measured. (d) Distribution of percent change values (per male; $n=13$) for CV of FF. The mean was significantly less than zero (t-test: $t_{12}=6.00$, $P<0.0001$). (e) Plot depicting how mean FF changed across social contexts for all 20 syllables measured in 13 males. On the x-axis is the mean FF value produced during UD song, and on the y-axis is the percent difference in mean FF $[100 * (FD - UD) / UD]$.

$(FF_{FD}-FF_{UD}) / FF_{UD}$]. (f) Distribution of percent change values (per male) for mean FF. The mean was significantly greater than zero (t-test: $t_{12}=2.34$, $P=0.0371$).

Figure 2a provides an example from an adult Bengalese finch of the type of syllable with clear harmonic structure that we measured; there were 20 such syllables in the songs of 13 (of 19) Bengalese finches included in our study. In Fig. 2b are plotted histograms of FF for the syllable depicted in Fig. 2a, during UD (top) and FD (bottom) song. The range of FF produced in FD song was reduced relative to UD song in this example, and the decrease in the CV of FF from 1.22 to 0.98 from UD to FD song reflects this change. Overall, a decrease in CV was observed for 18 of the 20 syllables measured (Fig. 2c), and, across males, this decrease was significant (mean + SEM: $-28.45 \pm 4.7\%$; t-test: $t_{12}=6.00$, $P<0.0001$; Fig. 2d). This decrease in CV was driven predominantly by a decrease in the standard deviation of FF from UD to FD song (mean + SEM: $-22.1 \pm 5.1\%$ t-test: $t_{12}=4.28$, $P=0.0011$).

In contrast to the zebra finch, where mean FF does not significantly change across social contexts (Kao and Brainard 2006; Kao et al. 2005), we found that there was an overall trend for FF to increase from UD to FD song. In the example provided in Fig. 2b, there was a significant increase in FF during FD song relative to UD song (t-test: $t_{183}=7.54$, $P<0.0001$). Overall, mean FF was greater during the production of FD song for 14 out of 20 measured syllables, and this difference was significant for 10 of the 14 examples (t-test, $P<0.05$). Across individuals, FF was significantly increased during FD song (mean + SEM: $0.46 \pm 0.2\%$; t-test: $t_{12}=2.34$, $P=0.0371$; Fig. 2e,f).

Variability of syllable sequencing: branch points

Branch points provide an opportunity to investigate whether variability in syllable sequencing is actively regulated. By definition, branch points reflect the locations within Bengalese finch song at which syllable transitions are variable (Fig. 1). Here we

measured how this variability at branch points is influenced by social context. In our dataset there were 35 examples of branch points in the songs of 16 Bengalese finches.

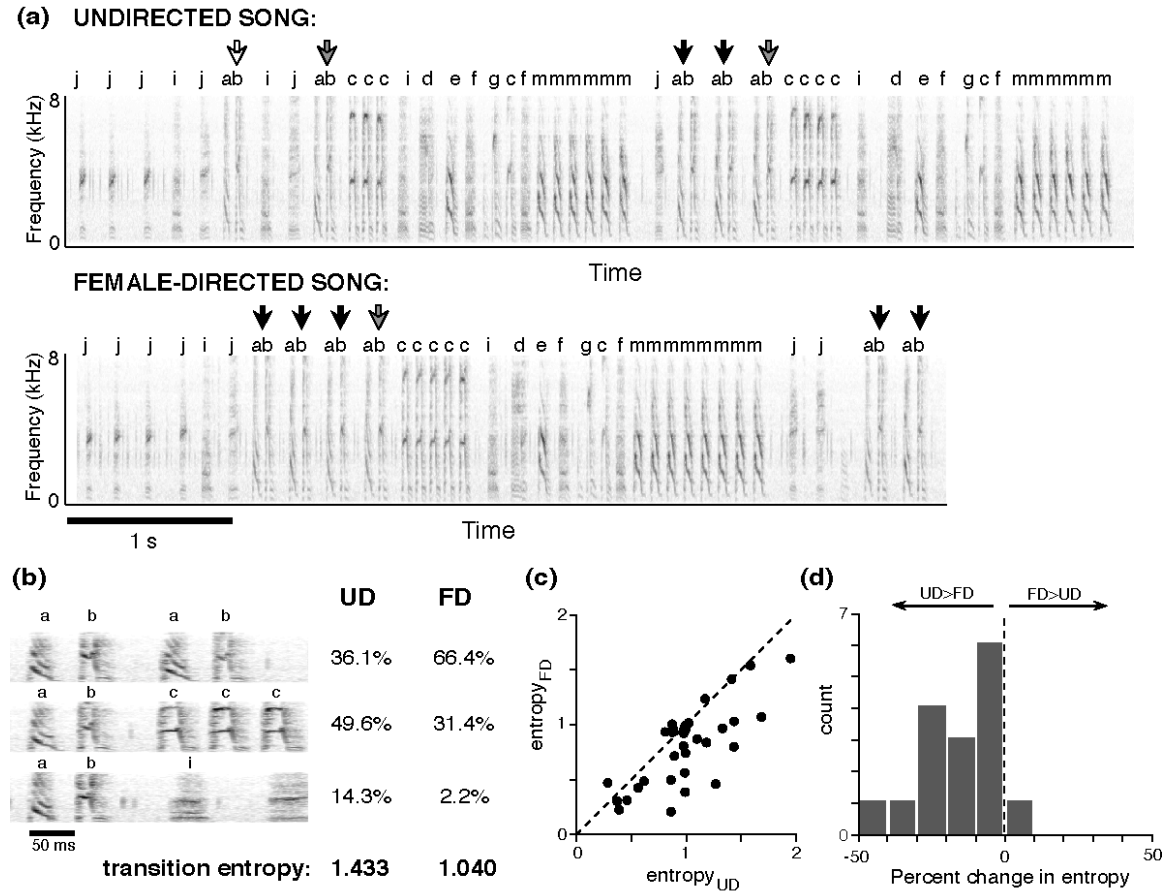


Figure 3. Effect of social context on transition entropy at 'branch points' for Bengalese finch song. (a) An example of the effect of social context on transition entropy. On top is a spectrogram of an example of undirected (UD) song, and on the bottom is an example of female-directed (FD) song. Arrows highlight instances of the branch point sequence 'ab'. Black arrows indicate transitions from 'ab' to 'ab'; grey arrows indicate transitions from 'ab' to the repeated syllable 'c'; and white arrows indicate transitions from 'ab' to 'i'. Scale bar: 1 second. (b) Transition probabilities during UD and FD songs for this male. On the left are spectrograms of the three distinct transitions following the sequence 'ab'. On the right is a summary of the percent of times the male transitioned to each of these possible sequences for UD and FD song, as well as the transition entropies for the two contexts. In this example, transition entropy was reduced by ~30%, reflecting more stereotyped sequence transitions during female-directed song. (c) Transition entropies for the 35 branch points analyzed in 16 males. Values for 28 of the 35 branch points fall below the line of unity, indicating that overall transition entropy was reduced during FD song relative to UD song. (d) Distribution of percent change values (per male) for transition entropy. The mean was significantly less than zero (mean $\pm$ SEM: $-16.7 \pm 3.7\%$; t-test: $t_{15}=4.53$, $P=0.0004$).

Figure 3a illustrates each occurrence of the branch point 'ab' in one UD song (top) and one FD song (bottom) from an individual bird. For this branch point, FD song had more transitions to 'ab' and fewer transitions to 'ccc' (as well as fewer transitions to 'i' that sometimes occurred but are not shown here) than did UD song. The probability of each type of transition at this branch point is quantified in Fig. 3b. Transitions from 'ab' to the sequences 'ab', 'ccc', and 'i' were produced, 36.1%, 49.6%, and 14.3% of the time during UD song (n=252 transitions in 40 songs) and 66.4%, 31.4%, and 2.2% of the time during FD song (n=223 transitions in 33 songs). For each branch point, we characterized the variability in sequence transitions by quantifying transition entropy (Gil and Slater 2000; Sakata and Brainard 2006); stereotyped sequences have entropy scores of zero, and increased variability translates into increased entropy (see Methods). This example illustrates a case in which transition entropy was reduced by 27.4% during FD song relative to UD song. The reduced entropy indicates that the variability of sequencing at this branch point decreased during the production of FD song. Across the 35 examples of branch points, there was a decrease in transition entropy from UD to FD song in 28 cases (Fig. 3c), and across individuals, the mean decrease in entropy from UD to FD song was significant (mean + SEM: -16.7 + 3.7%; t-test: $t_{15}=4.53$, $P=0.0004$; Fig. 3d).

In principle, decreases in transition entropy could reflect the consequence of birds consistently 'choosing' some types of transition over others at branch points. To examine this possibility, we analyzed whether social context had systematic influences for three types of transitions.

First, strings of repeated syllables are present in Bengalese finch song, and in songbird species such as the canary, such repeats can play an important role in social interactions (Kreutzer et al. 1999; Vallet and Kreutzer 1995; Vallet et al. 1998). Therefore, we asked whether UD and FD songs differed in the prevalence of transitions to strings of repeats at branch points. In the example provided in Fig. 3a, the sequence

'ab' could be followed by repeats of the syllable 'c' or by other transitions. In this case, the prevalence of transitions to the repeated syllable 'c' decreased from 49.6% during UD song to 31.9% during FD song. Across the ten examples (in eight males) of branch points with repeats as possible transitions, there was not a consistent difference between UD and FD songs in the prevalence of transitions to repeats and, consequently, no quantitative difference between social contexts in the probability of such transitions (paired t-test: $t_9=0.06$, $P=0.9543$).

Second, a fixed sequence of syllables is sometimes repeated following the branch point (e.g., transition from 'ab' to 'ab' in Fig. 3a). In our dataset there were 14 examples (in eight males) of branch points with such transitions, and in ten cases the frequency with which sequences were repeated was higher for FD song. Correspondingly, the proportion of times a sequence was repeated at these branch points was significantly greater during the production of FD song relative to UD song (paired t-test: $t_{13}=3.05$, $P=0.0093$).

Third, introductory notes can be produced within a song, and such syllables can potentially follow branch points. For example, in Fig. 3a, one of the potential transitions is to the introductory note 'i', and for this branch point, the prevalence of transitions to syllable 'i' was reduced from 14.3% during UD song to 2.2% during FD song. Across the 11 examples (in seven males) of branch points with introductory notes as potential transitions, there were ten cases in which the prevalence of transitions to introductory notes was reduced during FD song relative to UD song, and this reduction was significant (paired t-test: $t_{10}=2.56$, $P=0.0285$). This suggests that birds could bias transitions away from introductory notes during the production of FD song.

In summary, changes in the prevalence of transitions to introductory notes and the prevalence with which sequences were repeated at branch points contributed to the overall change in transition entropy across social contexts.

Variability of syllable sequencing: repeats

Repeated syllables can be considered nodes with variable sequence transitions, and, consequently, we applied the same analysis of transition entropy to repeats.

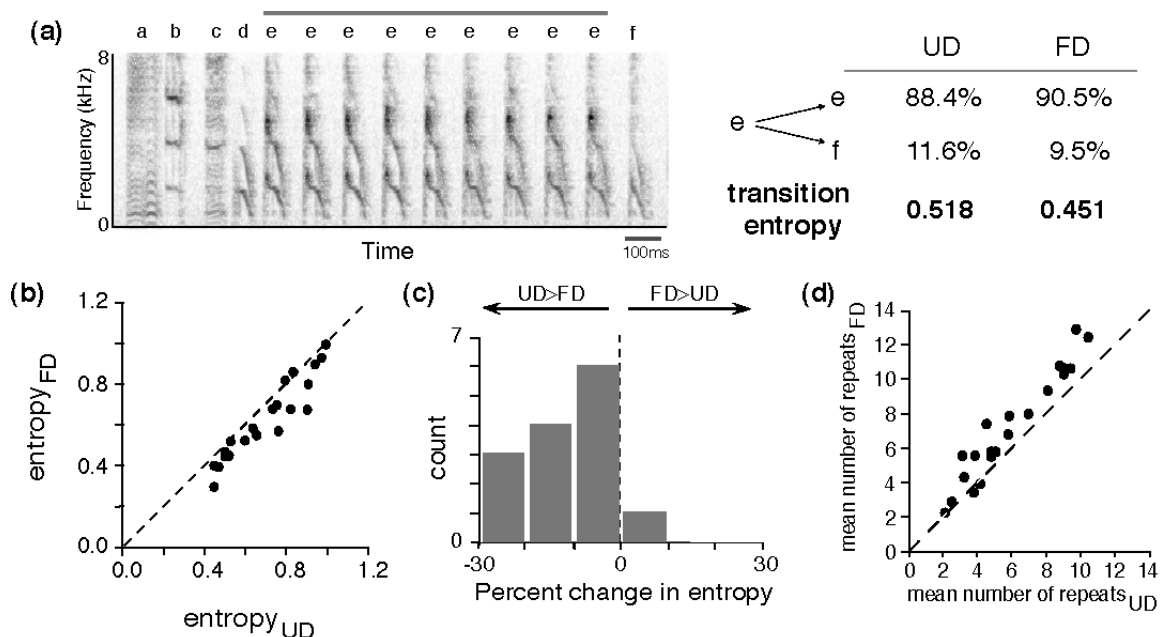


Figure 4. Effect of social context on transition entropy for repeated syllables (non-introductory notes) in the Bengalese finch. (a) Example of how the probability of repeating a syllable increased from undirected (UD) to female-directed (FD) song. On the left is a spectrogram of a string of repeated syllables, and on the right are the percent of transitions to 'e' and 'f' from the syllable 'e' during UD and FD song. (b) Transition entropy for UD and FD songs for the 21 repeated syllables in 14 males. For 19 of the 21 repeats, transition entropy was lower for FD songs than UD songs. (c) Distribution of percent change values (per male) for transition entropy for repeats. The mean was significantly less than zero (mean + SEM: $-12.2 \pm 2.0\%$; t-test: $t_{13}=6.13$, $P<0.001$). (d) Mean repeat numbers of the 21 repeated syllables for UD and FD songs. Overall, mean repeat numbers were ~25% higher for FD songs.

In the example provided in Fig. 4a, the syllable 'e' could be followed by either the syllable 'e' or 'f'. During UD song, the bird transitioned to 'e' and 'f', respectively, 88.4% and 11.6% of the time, whereas during FD the bird transitioned to 'e' and 'f', respectively, 90.5% and 9.5% of the time. This change in transition probability translated into a ~13% decrease in transition entropy from 0.518 to 0.451. For 19 of 21 repeated syllables in the songs of 14 Bengalese finches, transition entropy decreased from UD to FD song (Fig.

4b), and across all individuals, entropy was significantly reduced during FD song (Fig. 4c; mean + SEM: $-12.2 \pm 2.0\%$; t-test: $t_{13}=6.13$, $P<0.001$). This decrease in entropy was caused by a reliable increase in the mean number of times a syllable was repeated from UD to FD song (Fig. 4d). Therefore, the effect of social context on the sequencing of repeated syllables is congruent with the effect on sequencing at branch points.

Modulation of other song features:

Context-dependent modulations of song unrelated to variability have also been noted in other songbirds (Cooper and Goller 2006; Eens 1993; Kao and Brainard 2006; Sossinka and Böhner 1980). Therefore, we examined changes to three other song features— the number of introductory notes, song length, and song tempo.

In the zebra finch, the number of introductory notes preceding the onset of song is significantly higher when males sing to females than when they sing in isolation (Kao and Brainard 2006; Sossinka and Böhner 1980). We found that the number of introductory notes preceding song was also significantly higher for Bengalese finch FD song (paired t-test: $t_{17}=2.14$, $P=0.0469$). On average, there were 16.9% more introductory notes preceding FD song than UD song.

Songs produced in the presence of a female have been found to be significantly longer than those produced in isolation (Eens 1993; Kao and Brainard 2006; Sossinka and Böhner 1980). However, we did not find a significant difference in song length (exclusive of introductory notes) between UD and FD songs in the Bengalese finch (paired t-test: $t_{18}=0.86$, $P=0.3996$).

Songs produced to females are faster than songs produced in isolation in zebra finches (Cooper and Goller 2006; Kao and Brainard 2006; Sossinka and Böhner 1980), and we found a similar increase in tempo from UD to FD song in Bengalese finches.

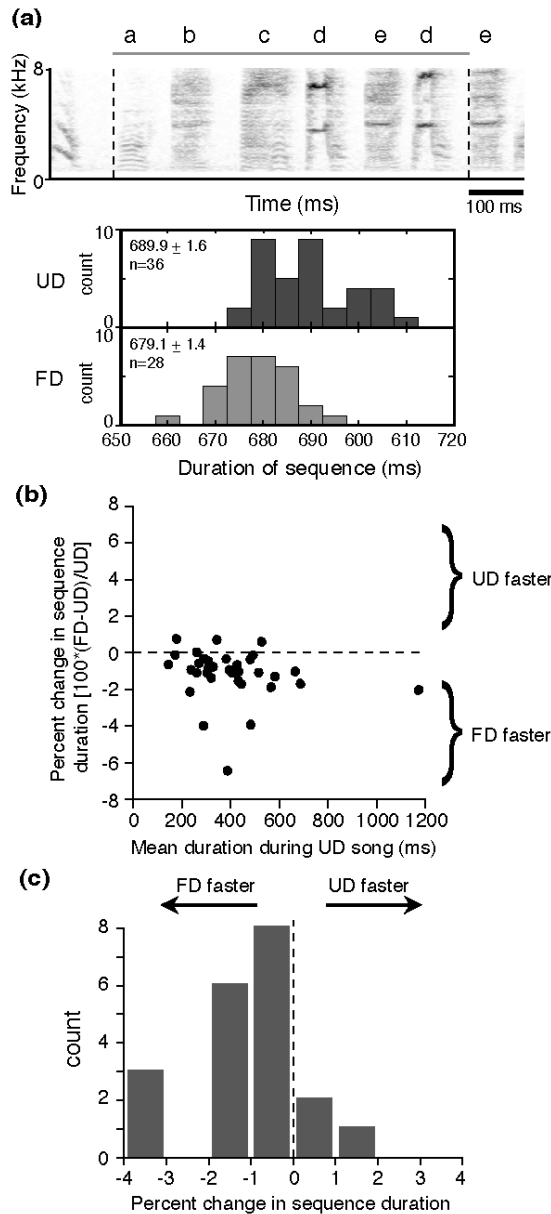


Figure 5. Effect of social context on song tempo in the Bengalese finch. (a) Top: Spectrogram of a sequence measured for song tempo analysis with unique labels above. For this individual, we measured the interval from the onset of the first syllable 'a' of the sequence 'abcde' to the onset of the last syllable 'e'. Bottom: Histograms summarizing the duration of the sequence 'abcde' for undirected (UD: black bars) and female-directed (FD: grey bars) song. Sequence durations were significantly lower for FD songs than UD songs (t-test: $t_{195}=6.29$, $P<0.0001$). Presented in the figure are the means $\pm$ SEM. (b) Data for all 33 sequences measured in 19 males. Plotted on the x-axis is the mean duration of the sequence during UD song, and on the y-axis is the percent change in sequence duration $[100 * (duration_{FD} - duration_{UD}) / duration_{UD}]$. For 29 of the 33 sequences measured, sequence duration was shorter during FD song than UD song, indicating that song was faster when males sang to females. (c) Distribution of percent change values (per male) for tempo. The mean was significantly less than zero (Wilcoxon signed-ranks test: $T=92.0$, $N=19$, $P<0.001$).

Figure 5a depicts an example where the tempo of a fixed sequence was significantly faster during FD song than UD song. In this example, the interval from the onset of the first syllable 'a' of the sequence 'abcde' to the onset of the last syllable 'e' was significantly reduced during FD song relative to UD song (1.56% reduction; t-test: $t_{62}=4.90$, $P<0.0001$; Fig. 5a). Across all males, this effect was very reliable, with mean sequence durations being lower for FD song than UD song in 29 of the 33 sequences

measured (Fig. 5b). Overall, sequence durations were $1.31 \pm 0.3\%$ (mean $\pm$ S.E.) shorter during FD song than UD song, a difference that was statistically significant and comparable to tempo differences observed in zebra finches (Cooper and Goller 2006; Kao and Brainard 2006; Sossinka and Böhner 1980)(Fig. 5c; Wilcoxon signed-ranks test: $T=92.0$, $N=19$, $P<0.001$).

Rapidity of song modulation:

We collected interleaved bouts of UD and FD song, which allowed us to examine how rapidly the structure of song changed between contexts. Assessing the degree to which songs changed rapidly or gradually across the course of the experiment lends insight into the types of mechanisms controlling this social modulation. In four birds we plotted, as a function of time, three features that could be analyzed for each rendition: repeat number, FF, and song tempo. As in all birds examined, these birds produced FD songs quickly (<1 min) following the introduction of a female.

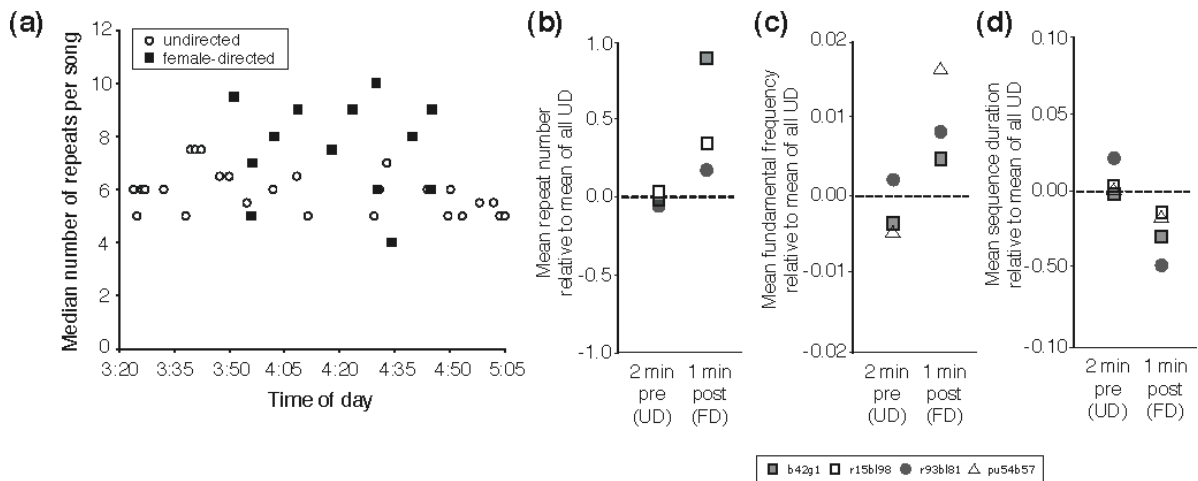


Figure 6. Rapidity of changes to song organization. (a) Plot depicting the median repeat number (per song) across the experimental period for a male whose repeat numbers were significantly elevated during female-directed song (undirected (UD) = open circles; female-directed (FD) = filled squares; t-test: $P<0.05$). On the x-axis is the time of day at which the song was produced. This highlights the interleaved collection of UD and FD songs and depicts the rapidity with which social context affected repeat number. An increase in repeat number translates into a decrease in transition entropy. (b) The average number of repeats for FD songs produced in the first minute following the introduction of the female (1 min post) was elevated relative to repeat numbers for UD songs produced during the preceding two minutes (2 min pre). Female-directed songs are usually produced within tens of seconds following the introduction of a female. Plotted are the

averages during these periods, divided by the average values across all UD songs for each male, for three syllables that showed significant changes in three males (in all cases $P < 0.05$). (c) Average fundamental frequency (FF) during FD songs produced in the first minute following the introduction of the female (1 min post) was elevated relative to FF for UD songs produced during the preceding two minutes (2 min pre). Plotted are the average FFs during these periods, divided by the average values across all UD songs for each male, for three syllables that showed significant changes in three males (in all instances $P < 0.05$). (d) The average duration of measured sequences for FD songs produced in the first minute following the introduction of the female (1 min post) was reduced relative to durations for UD songs produced during the preceding two minutes (2 min pre). Plotted are the averages during these periods, normalized by the mean across all UD songs for each male, for four sequences that showed significant changes in four males (in all instances $P < 0.05$).

In Fig. 6a we depict an example and plot the median number of times a syllable was repeated for each song produced during the experimental period. Two things are evident from this figure. First, the number of times a syllable was repeated during a particular song was generally higher during FD song than UD song, even when UD and FD songs were temporally proximal to each other. Second, although there was variability across songs in repeat number, there was no trend for a systematic change in repeat number across the experimental period. From data like these we also calculated mean repeat numbers for UD songs produced within the two minutes prior to the introduction of the female and for FD songs produced within a minute after the introduction of the female (Fig. 6b); the values are normalized to the mean repeat number across all UD songs. Plotted are the means for three repeats that were significantly increased during FD song relative to UD song (t-tests, $P < 0.05$; Fig. 6b). Similar data for FF and song tempo are summarized in Figs. 6c,d. Because FD songs were produced within a minute of exposure to a female, these data highlight the rapidity with which social context affects song organization.

DISCUSSION

The display of all behaviors, even well-learned behaviors, is variable. One prevalent view is that this variability is uncontrollable biological noise that is unimportant to the organism. Alternatively, a component of such variability might enable motor

learning or assist in the active maintenance of motor performance (Doya and Sejnowski 2000; Kao and Brainard 2006; Kao et al. 2005; Ölveczky et al. 2005; Sutton and Barto 1998). Indeed, it has recently been demonstrated that the residual variability in adult song can be used to guide vocal plasticity in a songbird (Tumer and Brainard 2007). While the precise function of behavioral variability remains unknown, some experimental studies indicate that variability is actively regulated, suggesting that variability could serve a function. For example, in the zebra finch, the acoustic structure of vocal motor elements (i.e., 'syllables') is less variable when males sing to females ('female-directed', or FD song) than when males sing in isolation ('undirected', or UD song)(Kao and Brainard 2006; Kao et al. 2005). For adult zebra finches, syllable sequencing is usually stereotyped, and there is limited evidence for social modulation of sequence (Kao and Brainard 2006; Zevin et al. 2004; but see two birds in Sossinka and Böhner 1980). Here we studied the social modulation of sequencing in the Bengalese finch, a species with a high degree of variability in syllable sequencing in adult 'crystallized' song. We found that the variability of syllable sequencing is rapidly modulated by social context with less variable sequencing produced during FD songs than UD songs (Figs. 3,4, & 6). Moreover, as in the zebra finch, we found that the variability of syllable structure was also rapidly modulated by social context, with reduced variability in acoustic structure produced during FD song (Fig. 2). These observations indicate that variability in both syllable sequence and structure is actively and rapidly regulated by the central nervous system and is consistent with the idea that control of variability could serve a function.

Because song variability is greater when males sing in isolation, it has been suggested that UD song represents a state of motor practice in which motor space is 'explored', whereas FD song reflects a state of motor performance of the 'best' renditions of learned song (Jarvis et al. 1998; Kao and Brainard 2006; Kao et al. 2005). This motor exploration provides greater opportunity for vocal plasticity by differential

reinforcement of motor commands for ‘desired’ versus ‘undesired’ outcomes. The possibility of increased plasticity during UD song is also supported by the observations that, relative to FD song, neural activity and immediate early gene (IEG) expression in song control nuclei are greater during UD song, features that have been associated with heightened plasticity (Hessler and Doupe 1999; Jarvis et al. 1998). Our results extend the finding of variability regulation from syllable structure to syllable sequencing and suggest that plasticity of syllable sequencing could also be heightened during UD song. Furthermore, our results are consistent with the possibility that species differences in the intrinsic variability of syllable sequencing are correlated with differences in plasticity of syllable sequencing in adult song; syllable sequencing in adult Bengalese finch song is more variable than in zebra finch song, and adult Bengalese finches demonstrate more rapid plasticity of sequencing following manipulations of auditory feedback than adult zebra finches (Brainard and Doupe 2001; Nordeen and Nordeen 1992; Okanoya and Yamaguchi 1997; Sakata and Brainard 2006; Scott et al. 2000; Woolley and Rubel 1997).

Converging evidence suggests that a component of song variability is actively introduced into the vocal motor pathway by neurons in a forebrain-basal ganglia circuit specialized for song, the anterior forebrain pathway (AFP; Bottjer et al. 1984; Hessler and Doupe 1999; Kao and Brainard 2006; Kao et al. 2005; Ölveczky et al. 2005; Scharff and Nottebohm 1991). In particular, the lateral nucleus of the nidopallium (LMAN), which projects to the robust nucleus of the arcopallium (RA) in the vocal motor pathway, has been found to influence vocal motor variability. Lesions of LMAN in adult zebra finches reduce the variability of syllable structure (Kao and Brainard 2006; Kao et al. 2005), and lesions and inactivations of LMAN in juvenile zebra finches decrease the variability of both syllable structure and sequencing (Bottjer et al. 1984; Kao et al. 2005; Ölveczky et al. 2005; Scharff and Nottebohm 1991). Furthermore, the variability of spiking activity in

LMAN is reduced during renditions of FD song, which could serve to reduce the variability of firing in RA and, consequently, syllable structure (Hessler and Doupe 1999; Kao et al. 2005; Leonardo and Fee 2005; Ölveczky et al. 2005). This causal relationship is supported by the finding that introducing variable amounts of activity into LMAN increases the variability of syllable structure (Kao et al. 2005).

The observation that the variability of both syllable structure and sequence was reduced when Bengalese finches produced FD song suggests that the regulation of both types of variability could be controlled by shared neural substrates. A recent report demonstrating that inactivation of LMAN reduces syllable and sequence variability in juvenile zebra finches supports this notion (Ölveczky et al. 2005). Alternatively, independent mechanisms could drive context-dependent changes in syllable structure versus syllable sequencing in adult songbirds. For example, lesions of LMAN in adult zebra finches affect the variability of syllable structure but not of syllable sequencing, though this could be because adult zebra finches already produce songs with very stereotyped sequences (e.g., Kao and Brainard 2006). Separate medial and lateral basal ganglia circuits project to different areas of the vocal motor pathway, and it has been proposed that the lateral pathway, which includes LMAN and projects to RA, regulates syllable variability whereas the medial pathway, which includes the medial nucleus of the nidopallium (MMAN) and projects to HVC (proper name), regulates sequence variability (Foster et al. 1997; Jarvis et al. 1998; Kubikova et al. 2007; Reiner et al. 2004). Mounting evidence indicates that HVC and its afferents are critically involved in sequence generation for song (reviewed in Fee et al. 2004; Hahnloser et al. 2002; Hosino and Okanoya 2000; Vu et al. 1994; Yu and Margoliash 1996) and that RA is important for the acoustic structure of syllables (Leonardo and Fee 2005; Vu et al. 1994; Yu and Margoliash 1996); therefore, the distinct connectivity of the medial and lateral basal ganglia circuits to the vocal motor pathway is consistent with such differential

contributions to syllable sequencing versus structure. Further support for this notion comes from the observation that IEG expression in the lateral but not the medial pathway is modulated by social context in the zebra finch, a species in which there is substantial evidence for context-dependent changes in adult syllable structure but not in syllable sequencing (Jarvis et al. 1998; Kao and Brainard 2006; Kao et al. 2005; but see Sossinka and Böhner 1980). In this regard, because changes to both syllable structure and sequencing were observed in Bengalese finches from UD to FD song, it would be interesting to assess whether both medial and lateral basal ganglia circuits display context-dependent changes in IEG expression.

Context-dependent changes in syllable variability and neural activity are hypothesized to arise from alterations in neuromodulatory inputs to the AFP, such as catecholaminergic input from midbrain areas like the ventral tegmental area (VTA) or locus coeruleus (reviewed in Ball et al. 2003; Bharati and Goodson 2006; Castelino and Ball 2005; Castelino et al. 2007; Ding and Perkel 2002; Hara et al. 2007; Jarvis et al. 1998; Kao et al. 2005; Maney and Ball 2003; Perkel 2004; Sasaki et al. 2006; Yanagihara and Hessler 2006). The role of neuromodulatory systems in the social modulation of song structure, as opposed to classical effects of steroid hormones or other slower influences on gene transcription, is supported by the rapidity of changes to song variability (Fig. 6). Experimental data from songbirds suggests that dopaminergic and/or noradrenergic inputs to song system nuclei may be regulated by social context and could contribute to social modulation of neural activity and IEG expression within the AFP (Castelino and Ball 2005; Ding and Perkel 2002; Hessler and Doupe 1999; Sasaki et al. 2006; Yanagihara and Hessler 2006). We propose that such changes in catecholamine concentrations in vocal motor circuits, including the AFP, underlie not only the decrease in the variability of syllable structure but also the decrease in the variability of syllable sequencing. Consistent with this perspective are reports that

elevated dopamine levels, particularly in nigrostriatal circuits, can increase the stereotypy and repetitiveness of motor expression in rodents and primates (Berridge and Aldridge 2000; Berridge et al. 2005; Cromwell et al. 1998; reviewed in Ridley 1994; Saka et al. 2004).

In addition to changes to the variability of syllable structure and sequence, we observed that song tempo and FF were elevated during FD song relative to UD song in the Bengalese finch and suggest that these alterations could be mediated by changes in AFP activity. In zebra finches, songs produced to females are faster than songs produced in isolation (Cooper and Goller 2006; Kao and Brainard 2006; Sossinka and Böhner 1980), and lesions of IMAN lead to a gradual acceleration of song and an attenuation of context effects on song tempo (Brainard and Doupe 2001; Kao and Brainard 2006; Williams and Mehta 1999). Alterations in vocal quality are often reported in patients with Parkinson's disease (Goberman et al. 2005; reviewed in Pinto 2004), and interference with dopamine function alters the bandwidth of ultrasonic vocalizations in rats (Ciucci et al. 2007). Therefore, context-dependent changes in activity within the AFP may be responsible for a suite of vocal motor modifications of adult song.

Given the importance of song for mating success in songbirds, social modulation of song features is likely to be relevant for courtship. It has been hypothesized that males emphasize more difficult, effortful or attractive song features when singing to females to enhance their chances of successful reproduction (reviewed in Catchpole and Slater 1995; Gil and Gahr 2002; Lambrechts 1996; Nowicki and Searcy 2004; Searcy and Yasukawa 1996). Consequently, the observed differences between FD and UD song could reflect greater difficulty or energetic investment and highlight features that females might attend to during social interactions. The observed increases in song tempo (Fig. 5) and repeat number (Figs. 4 and 6) are consistent with increased difficulty or energetic investment. Faster songs can require shorter inspirations between syllables

(Calder 1970; Cooper and Goller 2006; Glaze and Troyer 2006; Hartley and Suthers 1989; Suthers and Zollinger 2004; Suthers et al. 1999; Wild 1998), making them potentially more difficult to produce. Repeated syllables are produced with the shortest inter-syllable intervals in the Bengalese finch and are generally the loudest syllable in the repertoire (K. Bouchard, J. T. Sakata, and M. S. Brainard, unpublished observations), thereby making an increase in repeat number potentially more effortful. This perspective also suggests that reduced variability in syllable structure and sequencing could require greater motor control or effort and, therefore, that stereotypy itself could be a feature preferred by female Bengalese finches. The degree to which these features influence female choice remains to be addressed in this species, but in other songbird species, features that are influenced by social context have been found to affect female preferences (e.g., Kreutzer et al. 1999; Vallet and Kreutzer 1995; Vallet et al. 1998). One interesting example that highlights the potential function of changes to song variability is the chestnut-sided warbler: in this species, males produce more stereotyped songs during the time of day when interactions with females are more prevalent, and males with increased syllable stereotypy have been found to enjoy higher reproductive success (Byers 1995, 2007).

CHAPTER 2.

The contribution of LMAN to song variability in the adult Bengalese finch.

An avian basal ganglia-forebrain circuit contributes to syllable but not sequence variability of adult Bengalese finch song.

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ABSTRACT

Behavioral variability is important for motor skill learning but continues to be present and actively regulated even in well-learned adult behaviors. In adult songbirds, two types of song variability can persist and are modulated by social context: variability in syllable structure and variability in syllable sequencing. The degree to which the control of both types of adult variability is shared or distinct remains unknown. The output of a basal ganglia forebrain-circuit, LMAN (the lateral magnocellular nucleus of the anterior nidopallium), has been implicated in song variability. For example, in adult zebra finches, neurons in LMAN actively control the variability of syllable structure. It is unclear, however, whether LMAN contributes to variability in syllable sequencing because sequence variability in adult zebra finch song is minimal. In contrast, Bengalese finches retain variability in both syllable structure and syllable sequencing into adulthood. We analyzed the effects of LMAN lesions on the variability of syllable structure and sequencing and on the social modulation of these forms of variability in adult Bengalese finches. We found that lesions of LMAN reduced the level and social modulation of variability for syllable structure but not for syllable sequencing. These results demonstrate a dissociation in the contribution of LMAN to syllable versus

sequence variability, and suggest that different forms of adult song variability are regulated by distinct neural pathways.

INTRODUCTION

Birdsong is a vocal-motor behavior that is stable once learned, yet maintains some aspects of variability in adulthood. In songbird species such as the zebra finch, a young male initially produces highly variable, unstructured vocalizations. By 3-4 months of age, a juvenile male has listened to, memorized, and closely imitated song from an adult tutor, thereby developing his own learned and highly practiced song (Immelman 1969; Clayton 1987; Tchernichovski et al., 2001; Brainard and Doupe 2002). It has been suggested that this motor skill development is aided by song variability that reflects ‘motor exploration’ important for discovering how to match the tutor song (Sutton and Barto 1998; Doya and Sejnowski 2000; Brainard 2004; Kao et al. 2005; Ölveckzy et al. 2005). Once the song has been learned, it becomes relatively stable or ‘crystallized’, yet retains variability. Mounting evidence in adult songbirds indicates that such song variability is actively driven by the brain, suggesting that it continues to serve a function even following song crystallization (Hessler and Doupe 1999; Jarvis et al. 1998; Bottjer 2004; Kao et al. 2005; Kao and Brainard 2006; Teramitsu and White 2006; Tumer and Brainard 2007; Sakata et al. 2008).

Birdsong consists of spectrally complex sounds (syllables) that are organized into learned sequences. Across renditions of song, there can be variability in the spectral structure of syllables as well as in the sequencing of syllables. Both types of variability are actively modulated by social context. Specifically, the variability of syllable structure and of syllable sequencing is reduced when male songbirds sing courtship songs to females (‘female-directed song’) versus when they sing in isolation (‘undirected song’; Sossinka and Bohner 1980; Kao and Brainard 2006; Sakata et al. 2008).

The output of a basal ganglia-forebrain circuit, LMAN (the lateral magnocellular nucleus of the anterior nidopallium) has been identified as one source of variability in song. In juvenile zebra finches, lesions or inactivations of LMAN can lead to dramatic changes in the structure of developing song, largely manifest as abrupt reductions in the variability of song structure (Bottjer et al. 1984; Scharff and Nottebohm, 1991; Ölveczky et al. 2005). Such lesions also prevent the normal progression of song learning (Bottjer et al. 1984; Scharff and Nottebohm, 1991). In adult zebra finches, lesions of LMAN have relatively little influence on the gross structure of song (Bottjer et al. 1984; Scharff and Nottebohm, 1991; Nordeen and Nordeen, 1993). However, LMAN lesions do cause an abrupt reduction in the rendition-to-rendition variability of syllable structure (Kao et al. 2005; Kao and Brainard 2006) and prevent various forms of adult song plasticity (Morrison and Nottebohm 1993; Williams and Mehta 1999; Brainard and Doupe 2000, Brainard and Doupe 2001; Thompson and Johnson 2006; Thompson et al. 2007; Scott et al. 2007). LMAN lesions additionally eliminate the social modulation of variability in syllable structure (Kao et al. 2005; Kao and Brainard 2006), and variability in the activity of LMAN neurons correlates with variability of syllable structure (Jarvis et al. 1998; Hessler and Doupe 1999; Kao et al. 2005). Finally, artificial introduction of variable activity into LMAN of singing birds drives increased variability of song (Kao et al. 2005). These data strongly implicate LMAN as an active source of variability in syllable structure in both developing and adult zebra finches, and suggest the possible importance of such variability for song learning and adult song plasticity.

Whether shared or distinct neural circuits control the active regulation of different forms of song variability remains unknown. While lesions of LMAN reduce variability in syllable structure, LMAN's contribution to variability in syllable sequencing is unclear. For juvenile zebra finches, variability in syllable sequencing is reduced following lesions or inactivations of LMAN (Bottjer et al. 1984; Scharff and Nottebohm 1991; Ölveczky et

al. 2005). In contrast, for adult zebra finches, variability in syllable sequencing has not been reported to decrease following LMAN lesions (Bottjer et al. 1984; Scharff and Nottebohm 1991; Kao and Brainard 2006). However, syllable sequencing in the adult zebra finch is highly stereotyped (e.g. Zevin et al. 2004; Kao and Brainard 2006) such that it would be difficult to detect any reduction in variability caused by lesions (only increases in variability would be readily detectable). Hence, the zebra finch is a problematic model for investigating contributions of LMAN to sequence variability in adult song.

In contrast, adult Bengalese finch song exhibits variability in both syllable structure and syllable sequencing, making it well suited to address the contribution of LMAN to both of these features (reviewed in Okanoya 2004). These forms of variability are also modulated by social context, providing another opportunity to assess LMAN's role in the control of song variability (Sakata et al. 2008). Moreover, one previous study reported that disruption of input to LMAN, by partial lesions of the basal ganglia homologue Area X, could alter normal syllable sequencing (Kobayashi et al. 2001). This demonstrates the sensitivity of Bengalese finch song to manipulations of basal ganglia circuitry and supports the possibility that LMAN contributes to sequence control. To test LMAN's contributions to syllable structure and syllable sequencing, we measured how lesions of LMAN affected variability of adult Bengalese finch song and the regulation of variability by social context. We found that LMAN lesions significantly reduced the variability of syllable structure but had no systematic effects on the variability of syllable sequencing. Similarly, LMAN lesions eliminated the social modulation of variability for syllable structure but not for syllable sequencing. These data indicate a specific role of LMAN in regulating variability of syllable structure and suggest that distinct neural pathways contribute to regulating variability of syllable sequencing and of other aspects of adult song.

METHODS

Animals: Adult Bengalese finch males (n=16, age: 5-34 months) were raised in our colony. Birds were kept with their parents until 60 days of age, whereupon they were housed in same-sex cages. Prior to experiments, males were housed individually in sound-attenuating chambers (Acoustic Systems, Austin, Texas) maintained on a 14 hour light: 10 hour dark photoperiod. All procedures were performed in accordance with established animal care protocols approved by the University of California, San Francisco Institutional Animal Care and Use Committee (IACUC).

Data collection: Song was recorded and collected as described previously (Sakata et al. 2008). Briefly, sound was recorded using an omnidirectional microphone and threshold-based song detection software (Observer, A. Leonardo, Caltech; C. Roddey, UCSF; Sound Analysis Pro v. 1.04 (http://ofer.sci.ccny.cuny.edu/html/sound_analysis.html); Evtaf, E. Tumer, UCSF). We collected songs produced when males were alone (undirected, or UD song) as well as songs produced to females (female-directed, or FD song). Female-directed song was collected by presenting one of a series of females in a separate cage for 1-2 minutes at intervals of at least 4 minutes. This procedure has been shown to elicit robust differences in both syllable and sequence variability between UD and FD song (Sakata et al. 2008). Most birds (15 of the 16 birds) had a minimum of 10 FD songs within a single collection day; for one bird we collected songs across two consecutive days. UD song was interleaved with FD song and collected up to 30 minutes prior to the first exposure to a female and up to 30 minutes following the last exposure to a female.

Song analysis: Song is defined as a series of complex sounds separated by short silent intervals. For our purposes, a syllable is defined as a spectrally discrete sound element within song at least 10 ms in duration, separated by a minimum of 5 ms of silence (Okanoya and Yamaguchi 1997). Songs were visualized by plotting spectrograms in MATLAB (MathWorks, Natick, MA). Syllables were segmented based upon amplitude thresholds and manually labeled with unique letters (Figure 1a).

To analyze changes to the variability of syllable structure, we calculated the fundamental frequency (FF) of syllables with distinct and stable harmonic structure. For each syllable we calculated the autocorrelation of a segment of the sound waveform. The FF was defined as the distance, in Hz, between the zero-offset peak and the highest peak in the autocorrelation function. Each example of a syllable was visually screened to ensure that only examples devoid of sound artifacts that could affect FF calculation (e.g., sound of movement, female calls in background) were used in the analysis. To improve the resolution of the frequency estimates, we performed a parabolic interpolation of the peak of the autocorrelation function (de Cheveigné and Kawahara 2002). We found that the FF of syllables varied from rendition to rendition and characterized this variation using the coefficient of variation.

$$CV = (\sigma / \mu) * 100.$$

In addition to variability in syllable structure, adult Bengalese finch song exhibits variability in the sequencing of particular syllables. ‘Branch points’ are nodes in song in which transitions vary across renditions. We characterized the variability at branch points using transition entropy:

$$transition\ entropy = \sum -p_i * \log_2(p_i)$$

where the sum is over all possible transitions, and p_i is the probability of the i th transition across all songs (Gil and Slater 2000; Sakata and Brainard 2006, Sakata et al. 2008). Low entropy indicates low variability; completely stereotyped sequences have an

entropy value of zero. Branch points in which the dominant transition occurred >95% of the time were considered stereotyped sequences and not included in the analysis of branch points.

For another measure of variability in syllable sequencing, we analyzed ‘repeats’, syllables that are consecutively repeated a variable number of times across renditions. First, we calculated the transition entropy of repeats. Repeats can be considered a different class of branch point transition in which a syllable can transition to itself or to another syllable. Higher numbers of repeats lead to lower values of repeat entropy as the probability of a repeated syllable increases (i.e., increased predictability in transitions). Second, we calculated the CV of repeat number. This measure directly reflects the variability in the number of times a syllable is consecutively repeated across renditions.

Additionally, we analyzed changes to song tempo and the number of introductory notes. For song tempo, we measured the duration of matched sequences of syllables that were produced often in a bird’s song. We measured the interval from the onset of the first syllable to the onset of the last syllable in the sequence. Onsets were selected as boundaries because the change in amplitude is sharper and less variable for onsets than for offsets, allowing for a more accurate estimate of duration. Introductory notes are low amplitude syllables that precede song and are repeated a variable number of times. We counted the number of introductory notes preceding each song by starting at the first introductory note prior to the first (non-introductory) syllable of the song and then counting backward in time until there was >500 ms of silence (Kao and Brainard 2006). If there were more than one type of introductory note, all were combined in the analysis.

LMAN Lesions: Neurons in LMAN project to the robust nucleus of the arcopallium (RA) in the vocal motor pathway (Figure 1c). Birds were anaesthetized using equithesin or a

combination of ketamine and midazolam supplemented with gaseous isoflurane. Stereotaxic coordinates from the posterior branch of the mid-sagittal sinus were used to locate LMAN (rostral: 5.1-5.5 mm; lateral: 1.2-1.8 mm; depth: 1.7-2.1 mm). For LMAN lesions (n=8 birds), we made 6-8 electrode (30-100k Ω) penetrations passing 50-100 μ A for 60-120 seconds. Silica gel was used to protect the brain during and after surgery. The craniotomy was sealed using bone wax, and skin was sealed together over the skull with veterinary glue (Nexaband, Abbott Laboratories, North Chicago, IL). Sham lesions (n=2) were performed in a similar manner, but lesions were made outside of the boundaries of LMAN and its projections to RA. Control birds (n=6) underwent no surgery, but had song collected at two time points separated by 2-6 weeks. No differences were observed between sham and control groups. Consequently, these are combined in our analysis and collectively referred to as 'control'.

Histology and estimation of lesion size: Upon completion of behavioral experiments, birds were anaesthetized with a lethal dose of isoflurane and perfused with paraformaldehyde or formalin. 30-40 μ m sections were cut on a microtome, and every third section was stained for either Nissl or calcitonin gene-related peptide (CGRP). CGRP staining labels LMAN cell bodies and projections to RA, along with other regions (Bottjer et al. 1997). Lesion size was estimated by at least 2 experienced observers based on residual CGRP staining in LMAN and RA. We compared the amount of residual CGRP staining in lesioned birds to that in control sections from intact birds. All lesion birds in this study had 75% or greater bilateral damage to LMAN. We also confirmed that the medial magnocellular nucleus of the anterior nidopallium (MMAN), a CGRP-positive region medial to LMAN (Foster et al. 1997), remained intact in all birds analyzed.

Table 1: Summary of birds. Experimental group, size and age are listed for each bird in this study.

Bird Name	Group	Lesion (%)	Age (months)
bird1	lesion	75	5
bird2	lesion	75	15
bird3	lesion	75	6
bird4	lesion	80	5
bird5	lesion	80	6
bird6	lesion	90	7
bird7	lesion	90	18
bird8	lesion	100	5
bird9	control	0-sham	7
bird10	control	0-sham	6
bird11	control	0	5
bird12	control	0	5
bird13	control	0	6
bird14	control	0	34
bird15	control	0	6
bird16	control	0	6

Statistical analysis:

We first analyzed how lesions of LMAN affected the organization of undirected song. For this set of analyses, we compared the measurements of UD songs prior to LMAN lesion to those two weeks following lesion using paired t-tests.

We next asked whether lesions of LMAN significantly affected the degree to which social context modulated song organization. For each song parameter we analyzed the percent change caused by social context and used a repeated measures MANOVA to assess how LMAN lesions influenced the degree of social modulation. Percent change was calculated using the following formula:

$$\text{percent change} = 100 * (\mu_2 - \mu_1) / \mu_1$$

where μ_1 and μ_2 refer to the sample means for the feature being examined. In these analyses, Group (lesion vs. control) was the main independent variable and percent change at each time (pre vs. post) were the dependent variables. An effect of LMAN lesions on the social modulation of song would be manifest as a significant Group

x Time interaction; this interaction would mean that the degree to which context-dependent song modulation changed over time was different between lesion and control groups. If a significant interaction was observed, we conducted separate post hoc paired t-tests within lesion and control groups.

The songs of adult Bengalese finches can contain multiple distinct examples of a measured song parameter. For instance, songs from a single Bengalese finch could contain multiple syllables with flat acoustic structure for FF measurement or multiple branch points for entropy measurement. For the analyses presented here, we analyzed each example of a song parameter individually. However, to avoid pseudoreplication in population analyses, we also analyzed a weighted average of percent changes for a given parameter for each bird to get a single, per male percent change value. The weighted average was computed using the following equation:

$$\text{weighted percent change (per male)} = \sum \Delta_i * (n_i / \sum n)$$

where Δ_i represents the percent change of the *i*th example of the parameter (e.g. FF syllable 1, FF syllable 2, or FF syllable 3), n_i represents the sample size for the *i*th example, and $\sum n$ represents the sum of *n*'s across all examples of that parameter. In all instances, the analyses on the per bird level corroborated the results from the per example level. In order to depict all the data, we present only the results from the per example analyses. Because we conducted comparisons of multiple song features, we used a threshold for significance of $\alpha=0.01$ to reduce Type I errors. Analyses were done using JMP 5.0.1 (SAS Institute, Cary, NC) for the MacIntosh and MATLAB.

RESULTS

In this study we analyzed the effects of LMAN lesions (Figure 1b,c) on the organization of song as well as on the social modulation of song. Bengalese finch song

consists of acoustically distinct syllables that are produced in learned sequences (Figure 1a). In the adult Bengalese finch, both syllable production and syllable sequencing are variable. In order to assess the degree to which LMAN contributes to different aspects of song variability, we first consider the effect of LMAN lesions on undirected song where variability is highest. We then consider the effect of LMAN lesions on the social modulation of song variability.

Contributions of LMAN to adult undirected song:

Adult Bengalese finch song continues to be produced without conspicuous alteration following LMAN lesion. An example of Bengalese finch song before and after LMAN lesion is presented in Figure 1a.

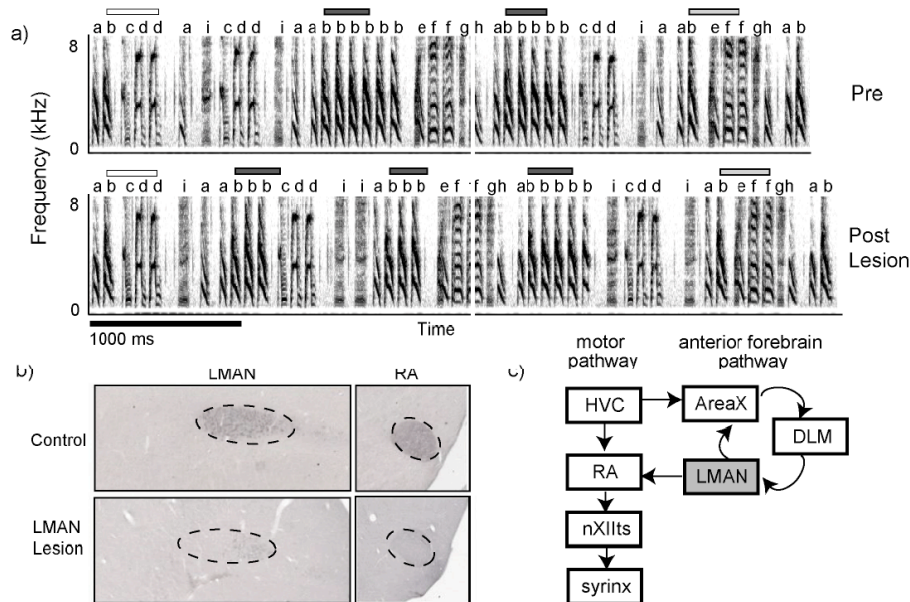


Figure 1: Bengalese finch song before and after LMAN lesion. (a) Spectrograms of undirected songs from a Bengalese finch before (top) and after (bottom) LMAN lesion. Above each spectrogram are labels for each distinct syllable. Bars above labeled syllables highlight examples of sequence variability for syllable 'b', a branch point which could be followed by the syllables 'c' (white bar), 'b' (dark grey bar), or 'e' (light grey bar). (b) Representative images of CGRP staining in LMAN and RA in a control bird (top) and LMAN lesioned bird (bottom). CGRP is expressed in LMAN neurons and their terminals in RA. Lesions of CGRP-expressing cells in LMAN lead to a loss of CGRP-immunoreactivity in RA (Bottjer, et al., 1997). (c) A diagram of two major pathways in the song system, the motor pathway [HVC (proper name), RA (robust nucleus of the arcopallium), nXIIts (tracheosyringeal portion of the hypoglossal nucleus), and syrinx], and the anterior forebrain pathway [AFP: Area X, DLM (medial nucleus of the dorsal lateral thalamus), and LMAN].

LMAN (lateral nucleus of the anterior nidopallium)]. LMAN is the output nucleus of the AFP and is positioned to modulate activity in the motor system.

The song of this bird contained 9 unique syllables, which are labeled above the spectrogram with the letters a-i. As is typical for adult Bengalese finches, these syllables were produced with some variability in their sequencing. For example, there were several 'branch points' at which a given syllable could be followed by variable transitions to other syllables; syllable 'b' is a branch point that could be followed by syllables 'b' (dark grey bar), 'c' (white bar) or 'e' (light grey bar). Similarly, there were several syllable 'repeats' where a given syllable could be repeated variable numbers of times before transition to the next syllable of song; syllables 'f' and 'b' are syllable repeats for this bird.

It is apparent from the songs illustrated in Figure 1a that LMAN lesions did not dramatically alter the structure of individual syllables or the sequences in which they were produced. This lack of gross effect of lesions was typical for all birds studied and indicates that LMAN is not an obligatory part of the pre-motor circuitry for song production. Previous studies in the zebra finch have similarly found that the gross structure of adult song is unaltered by LMAN lesions, but have revealed that such lesions can alter the variability with which song is produced (Bottjer et al. 1984; Scharff and Nottebohm 1991; Kao and Brainard 2006). Hence, we next quantified more precisely the degree of variability present in the structure and sequencing of syllables within songs from individual birds and how these types of variability were influenced by LMAN lesions.

Contributions of LMAN to adult undirected song: syllable structure

Because variability in syllable structure is strongly reduced by LMAN lesions in the adult zebra finch (Kao and Brainard 2006), we first investigated the contributions of

LMAN to syllable structure in the adult Bengalese finch. We measured the fundamental frequency (FF) of syllables with flat harmonic structure, and quantified the variation in the FF of individual syllables across renditions using the coefficient of variation (CV; see Methods).

We found that lesions of LMAN significantly reduced the variability of syllable structure in adult Bengalese finch song. Figure 2a shows an example syllable along with

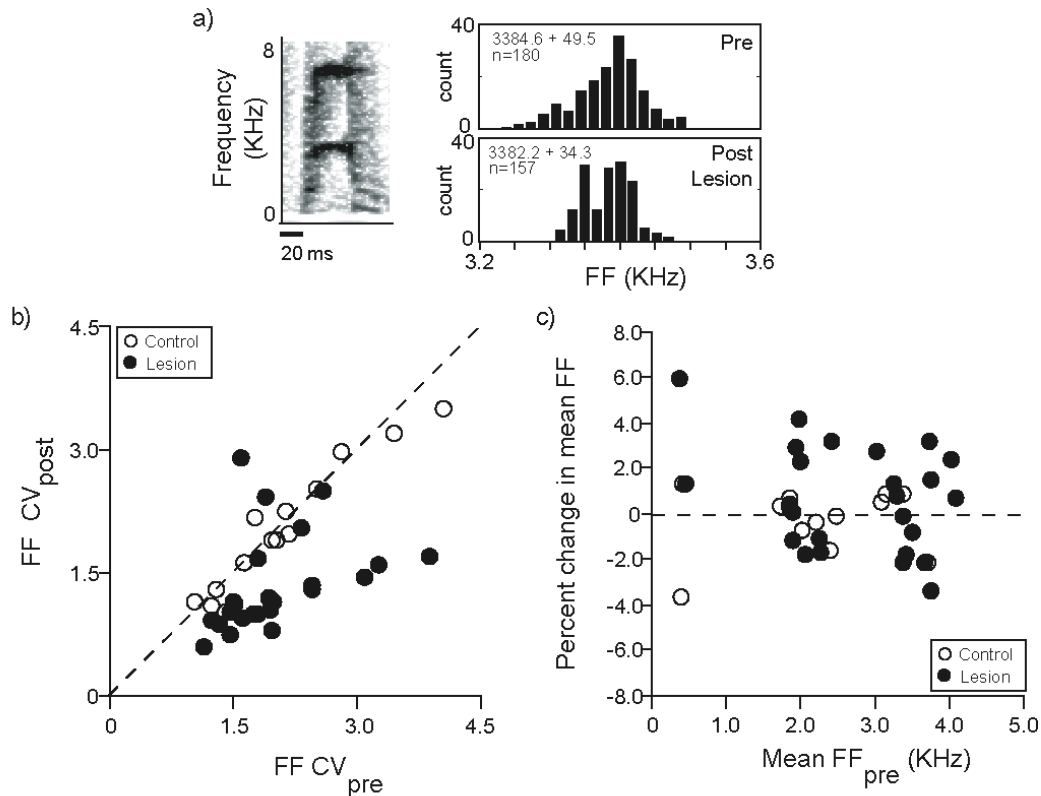


Figure 2: Effect of LMAN lesion on syllable variability for undirected song. (a) Spectrogram of the type of syllable, with flat harmonic structure, used in the analysis of fundamental frequency (FF). To the right is the distribution of FF values for UD song before (pre) and after (post) LMAN lesion. In this example, LMAN lesions led to a decrease in the coefficient of variation (CV) of FF (t-test: $p < 0.001$) but no change in mean FF (t-test: $p = 0.6083$). (b) CVs of 25 syllables from 8 birds before and after LMAN lesion (●) and of 14 syllables from 8 control birds at two time points (○). Overall, CV did not change over time for control birds ($p = 0.3784$) but was significantly reduced after LMAN lesion ($p = 0.0005$). (c) Percent change in mean FF of 25 syllables from 8 lesion birds due to LMAN lesion (●) and of 14 syllables in 8 control birds over time (○), versus pre-values on the x-axis. Mean FF did not change significantly over time for either control or lesion birds.

the distributions of FF for multiple renditions of that syllable before ('pre') and after ('post') lesion of LMAN. For this syllable, the CV decreased significantly from 1.46

before the lesion to 1.01 two weeks following the lesion (31% decrease; two-sample F-test for equal variances: $p < 0.001$). Similar decreases in CV were consistently observed following lesions. Across 25 syllables analyzed in 8 birds with LMAN lesions, the CV decreased on average by $31.1 \pm 6.3\%$ (filled symbols, Figure 2b; paired t-test; $p = 0.0005$). In contrast, there was no significant change in CV across 14 syllables in 8 control birds ($2.2 \pm 3.2\%$; open symbols, Figure 2b). These data indicate that, as in the zebra finch, LMAN contributes strongly to the variability of syllable production in the adult Bengalese finch.

The variability of FF for individual syllables was reduced without any systematic change in the mean of FF. For both lesion and control groups the mean of FF often changed by up to a few percent between measurement time points. However, these small changes in FF were equally likely to reflect increases or decreases (Fig. 2c). Consequently, across the 25 measured syllables in 8 lesion birds, there was no net change to the mean of FF (filled symbols, Fig. 2c). These results indicate that for the Bengalese finch, as for the zebra finch, LMAN lesions consistently affected the variability of syllable FF without systematically altering the mean of FF. In addition to suggesting a conservation of function of LMAN across species, these similarities provide a positive control for the efficacy of the LMAN lesions in our study.

Contributions of LMAN to adult undirected song: syllable sequencing

Unlike the case for syllable structure, the role of LMAN in controlling adult syllable sequencing is unclear from previous studies in the zebra finch. Here, for the adult Bengalese finch, we found that LMAN lesions did not significantly affect the variability of syllable sequencing, indicating a strong dissociation in the contributions of LMAN to variability of syllable sequencing versus syllable structure.

Inspection of syllable transition diagrams before and after LMAN lesions revealed that LMAN lesions did not lead to dramatic changes in the sequencing of syllables. An example of the lack of effect of LMAN lesion on transition probabilities is provided in Figure 3a (same song depicted in Figure 1). In this example all transitions are retained

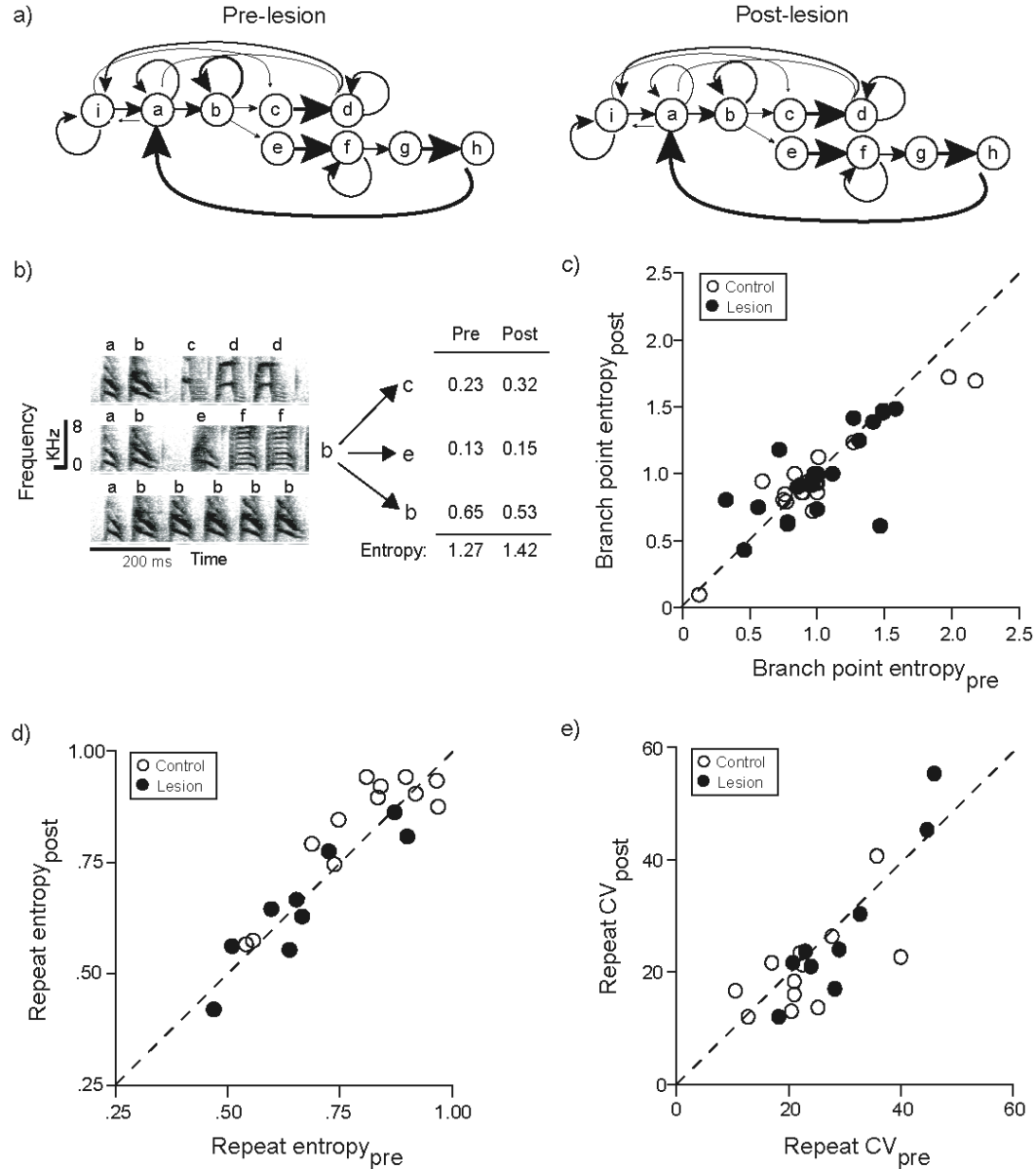


Figure 3: Effect of LMAN lesion on sequence variability for undirected song. (a) Transition diagrams before (left) and after (right) LMAN lesion for the same song shown in Fig. 1. Arrow thicknesses are proportional to the probability of transitions. In this example, all transitions persisted after LMAN lesion, with no gross changes to probabilities. (b) Spectrograms illustrating maintained sequence variation at a branch point for the same bird: syllable 'b' could be followed by 'c', 'e', or 'b'. In this case, the probabilities for each transition and the branch point entropy (right) were only modestly affected by LMAN lesion. (c) Branch point entropy values for 19 branch points in 8 lesion birds before and after LMAN lesion (●) and for 23 branch

points in 8 control birds at two time points (○). Branch point entropy did not change in a consistent manner in lesion or control birds ($p > 0.01$ for both). (d) Repeat entropy values for 9 syllable repeats in 5 lesion birds before and after LMAN lesion (●) and for 12 syllable repeats in 6 control birds at two time points (○). Repeat entropy did not change significantly over time for either lesion or control birds. (e) CVs of repeat numbers for 9 syllable repeats in 5 lesion birds before and after LMAN lesion (●) and for 12 syllable repeats in 6 control birds at two time points (○). The CV of repeat number did not change significantly over time for either lesion or control birds.

following the lesion; stereotyped sequences remained stereotyped and branch points continued to retain sequence variability. This was the case for all lesions birds. For example, in no case did a branch point sequence become completely stereotyped in sequence transitions following lesions. This contrasts with the effect of LMAN manipulations in juvenile zebra finches wherein variable sequences often became stereotyped following LMAN lesions or inactivations (e.g. see Figure 2e in Ölveckzy et al. 2005). To quantitatively assess whether there were any changes to the variability of syllable sequencing, we analyzed how individual branch points and syllable repeats were affected by LMAN lesions. LMAN lesions did not affect the variability of syllable sequencing at branch point transitions. We quantified this variability in transitions using the branch point entropy, with larger entropy values indicating greater variability (see Methods). For the example shown in Figure 3b, syllable 'b' is a branch point that could be followed by the syllables 'c', 'e', or 'b'. All of these transitions were retained following LMAN lesion. The entropy at this branch point increased modestly following lesions of LMAN, from 1.27 to 1.42 (11.0% increase). However, at a second branch point in this bird's song the entropy decreased modestly (6% decrease), so that there was not a consistent direction of entropy change for this bird. Overall, across 19 branch points in 8 lesion birds, LMAN lesions did not cause a net change in branch point entropy (Figure 3c).

LMAN lesions did not affect variability in sequencing for repeats. We quantified this in two ways. First, we found that repeat entropy was not affected by LMAN lesions (see Methods). Across 9 repeats in 5 lesion birds, we found no consistent change

caused by LMAN lesions (Figure 3d). Second, we analyzed how the CV of repeat number across renditions was affected by LMAN lesions. Just as with the analysis of repeat entropy, we found that the CV of repeat number was not significantly affected by LMAN lesions (Figure 3e).

Analysis of both branch points and repeats indicated that the contribution of LMAN to sequence variability is minimal for adult Bengalese finch song. These data suggest that high levels of sequence variability in the adult Bengalese finch do not require an intact LMAN.

Contributions of LMAN to adult undirected song: song tempo and introductory notes

For the zebra finch, LMAN lesions cause a gradual increase in song tempo over a period of weeks (Brainard and Doupe, 2001; Williams and Mehta, 1999; Kao and Brainard 2006). In contrast, we found no systematic change in the tempo of Bengalese finch song over the two weeks that songs were analyzed following lesions. While there

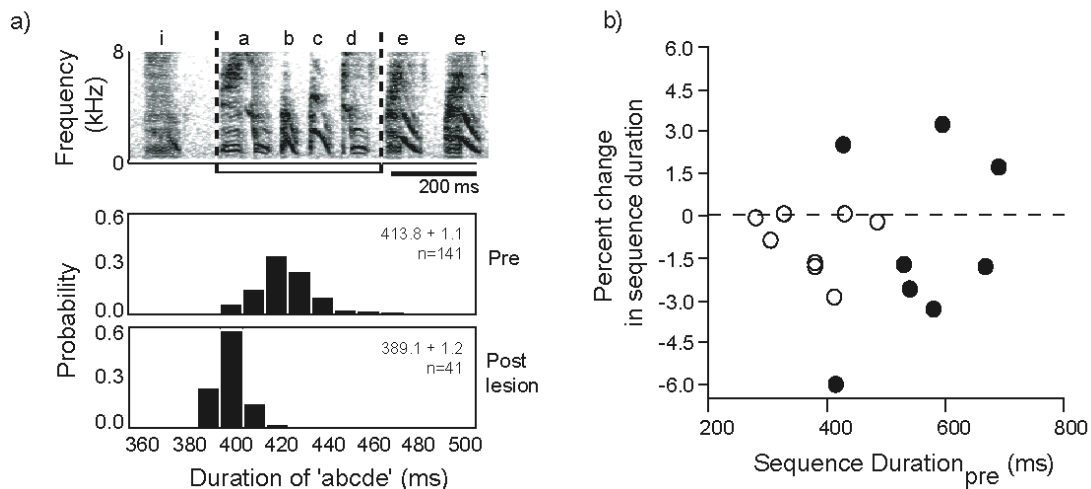


Figure 4: Effect of LMAN lesion on tempo for undirected song. (a) Example of the most extreme change in tempo (6% increase) following LMAN lesion. At top is a spectrogram of the sequence used for measurement of song tempo (sequence duration). The sequence duration was calculated as the time from the onset of 'a' to the onset of 'e', denoted by the dotted lines. Below are histograms of the duration of 'abcde' before and after LMAN lesion. In this case, LMAN lesion led to a significant speeding up of song (t-test: $p < 0.01$). (b) Plotted on the y-axis are values for percent change in sequence duration for lesion (●)

and control birds (○) versus pre-values on the x-axis. Despite individual cases of tempo changes, there was no significant change in song tempo over time for lesion and control birds.

were some individual cases in which the tempo of defined syllable sequences increased (e.g., Figure 4a), the overall effect of LMAN lesions on song tempo was not significant (Figure 4b). These data suggest that the contribution of LMAN to song tempo is reduced in the Bengalese finch relative to the zebra finch.

As observed in the zebra finch, LMAN lesions did not significantly affect the number of introductory notes preceding song (data not shown).

Contributions of LMAN to the social modulation of song

The variability and organization of multiple song features is strongly modulated by social context in Bengalese finches (Sakata et al., 2008). The spectral structure and sequencing of syllables are less variable during female-directed (FD) song than undirected (UD) song. Additionally, FD song is faster and contains more introductory notes than UD song. For the zebra finch, lesions of LMAN eliminate context-dependent modulation of some but not all song features (Kao and Brainard, 2006). Because this affords another opportunity to dissociate the role of LMAN in different aspects of song organization, we investigated how LMAN contributes to the social modulation of song. We compared the magnitude of context-dependent changes before and after LMAN lesions for song features that were previously found to be modulated by social context in the Bengalese finch.

We found that social modulation of syllable variability was strongly affected by LMAN lesions, while social modulation of syllable sequencing and other features were unaffected. As reported previously, we observed strong social modulation of the CV of FF, mean FF, branch point entropy, repeat entropy, introductory notes and song tempo ($p < 0.01$ for all). To assess whether the magnitude of social modulation was affected by

LMAN lesions, we compared the percent change from UD to FD song before and after LMAN lesions (see Methods, Figure 5). For the CV of FF, we found that LMAN lesions

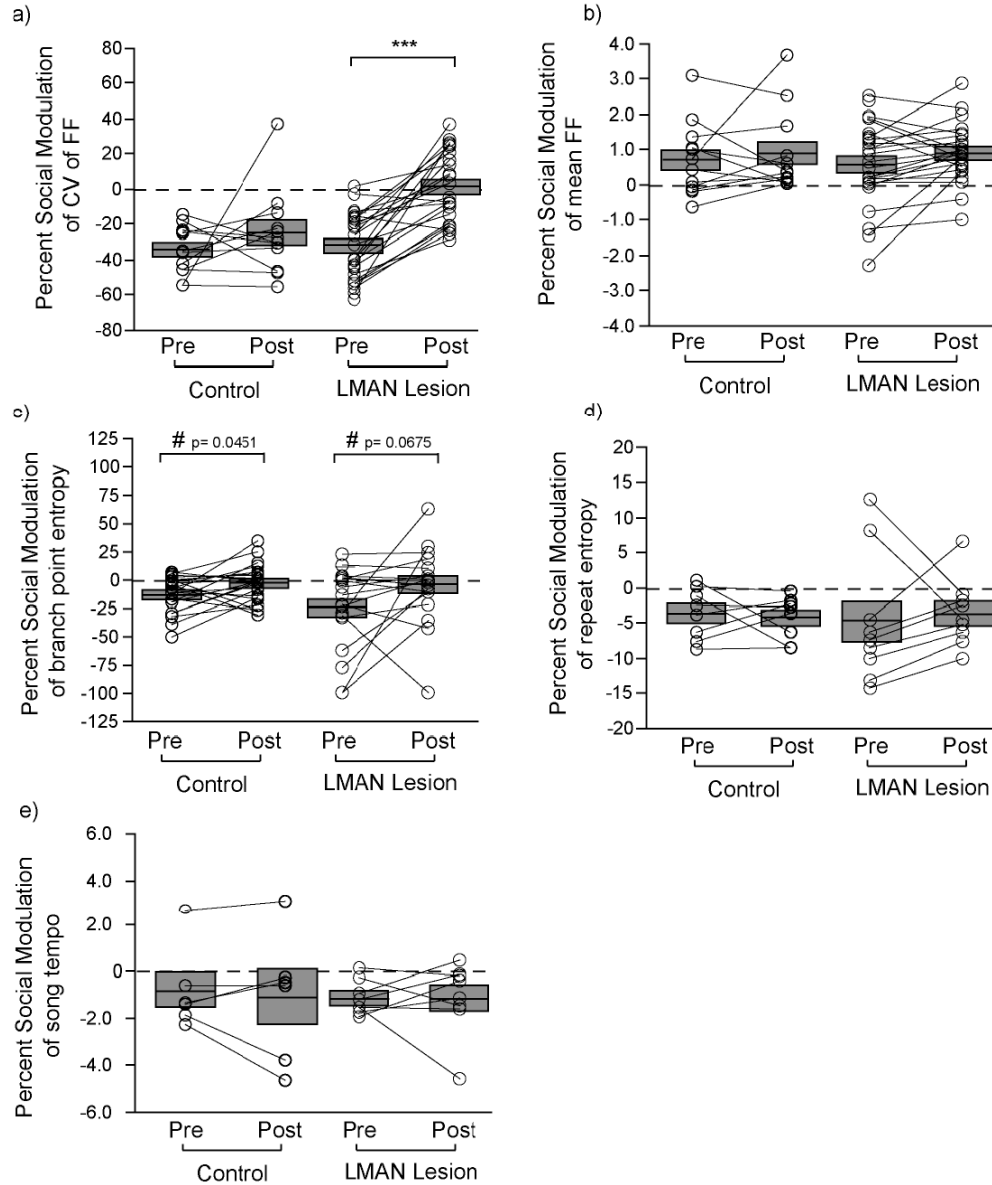


Figure 5: Effect of LMAN lesion on social modulation of song structure. Panels a-d each plot social modulation for one song feature from 'pre' and 'post' time points for control and lesion birds. Gray boxes indicate mean $\pm$ SEM. For all 5 features, there was significant social modulation during the 'pre' period, consistent with prior report (Sakata, et al., 2008). Lesions eliminated social modulation of the CV of FF but did not significantly affect social modulation of other features. (a) Social modulation of the CV of FF for 14 syllables in 8 control birds and 25 syllables in 8 lesion birds. The CV of FF did not change over time for control birds ($p=0.5462$) but decreased significantly from the first to the second session for lesion birds ($p<0.0001$; MANOVA: Group * Time $p=0.0035$). (b) Social modulation of mean FF for 14 syllables in 8 control and 25 syllables in 8 lesion birds. The social modulation of mean FF did not change significantly across testing sessions for control or lesion birds. (c) Social modulation of branch point entropy for 23 sequences in 8 control birds and 19 sequences in 8 lesion birds. There was no significant effect of lesion on

social modulation of branch point entropy (MANOVA: Group * Time; $p=0.2835$), though an attenuation of social modulation of branch point entropy across testing sessions was present across both control ($p=0.0451$) and lesion birds ($p=0.0675$; MANOVA: effect of Time; $p=0.0084$). (d) Social modulation of repeat entropy for 12 sequences in 6 control birds and 9 sequences in 6 lesion birds. There was no significant effect of LMAN lesion on social modulation of repeat entropy. (e) Social modulation of song tempo for 8 control and 8 lesion birds. There was no significant effect of LMAN lesion song tempo.

eliminated the social modulation of the CV of FF (Figure 5a, before lesion; UD>FD by $31.8 \pm 3.6\%$, after lesion: UD>FD by $0.6 \pm 3.9\%$). In contrast, the level of social modulation was not significantly different across time for control birds (Fig. 5a).

These data are consistent with experiments in adult zebra finches (Kao and Brainard, 2006) and indicate that LMAN is required for the social modulation of the variability of syllable structure.

In contrast to the CV of FF, LMAN lesion did not significantly affect the magnitude of social modulation of other song features (MANOVA: Group * Time interaction: $p>0.1$ for all). For example, mean FF continued to increase from UD to FD song following LMAN lesions (Fig. 5b); this provides additional evidence for a dissociation of LMAN contributions to mean FF versus the variability of FF (see also Fig. 2). For branch point entropy, the change in magnitude of social modulation across time was not different between lesion and control groups (Figure 5d). However, we unexpectedly observed an attenuation of social modulation across recording sessions for both control and lesion birds, indicating that the social modulation of branch point entropy changed over time (MANOVA, effect of Time: $p=0.0084$). Branch point entropy was the only song feature that demonstrated an attenuation of context-dependent difference across time. The cause of this attenuation over time could be due to multiple exposures to females across the course of the experiment (Teramitsu and White 2006), though it is unclear why branch point entropy would be differentially affected relative to other song features. For repeat entropy, introductory notes and song tempo the

magnitude of social modulation was unchanged over time in both control and lesion birds (Figure 5c,e,f; data not shown for introductory notes).

DISCUSSION

Our results demonstrate a dissociation in the contribution of LMAN to variability of syllable structure versus syllable sequencing. The songs of adult Bengalese finches exhibit variability at multiple levels of song organization, including variability in syllable structure and sequencing (Clayton 1987; Okanoya and Yamaguchi 1997; Woolley and Rubel 1997; Sakata and Brainard 2006). Both types of variability are actively modulated by social context in adult Bengalese finches (Sakata et al. 2008). Because the variability of syllable structure and sequencing are both reduced when Bengalese finches sing courtship songs to females ('female-directed' or FD song) relative to when they sing in isolation ('undirected' or UD song), it is possible that the variability of syllable structure and sequencing are controlled by shared neural mechanisms. We assessed this possibility by measuring the effect of lesions of the lateral nucleus of the anterior nidopallium (LMAN), a nucleus implicated in some forms of song variability, on the baseline levels and social modulation of syllable structure and sequence variability for adult Bengalese finch song. We found that lesions of LMAN consistently and robustly decreased the variability of syllable structure (fundamental frequency, or FF; Fig. 2) of UD song as well as the social modulation of variability in FF (Fig. 5a). In contrast, LMAN lesions did not affect the variability of syllable sequencing of UD song or its social modulation; lesions did not affect the sequence variability of branch points and syllable repeats or the magnitude of context-dependent changes in syllable sequencing (Figures 3 and 5). These data support a primary role of LMAN in the regulation of the variability of syllable structure and suggest that variability of syllable structure and sequence are regulated by distinct neural pathways.

LMAN is the output of the anterior forebrain pathway (AFP), an avian basal ganglia-forebrain loop that has been hypothesized to inject variability into the song motor pathway. In the adult zebra finch (Kao and Brainard 2006) and Bengalese finch (this study), LMAN lesions reduce the variability of syllable structure. Our results suggest that LMAN's contribution to song variability in adult songbirds is specific to variability of syllable structure and that this function is conserved across songbird species. This specificity contrasts with studies in mammalian species indicating a major role of basal ganglia circuits in sequence control (reviewed in Hikosaka et al. 1999; Saint-Cyr 2003; Rhodes et al. 2004; Seger 2006). Studies in songbirds have focused on the anterior forebrain pathway (AFP), the avian basal ganglia-forebrain loop of which LMAN is the output, as a primary source of song variability (Bottjer et al. 1984; Scharff and Nottebohm 1991; Kobayashi et al. 2001; Kao et al. 2005; Ölveczky et al. 2005; Kao and Brainard 2006). Our results suggest that other regions of the songbird basal ganglia and song system should be investigated as potential sources of sequence variability for adult song (e.g. Foster et al. 2001; Hosino and Okanoya 2000).

The minimal contribution of LMAN to syllable sequencing in adult Bengalese finches is consistent with studies in adult zebra finches, but contrasts with the effects of lesions of LMAN in juvenile zebra finches. Lesions or inactivation of LMAN in juvenile zebra finches leads to significant decreases in the variability of syllable sequencing (Bottjer et al. 1984; Scharff and Nottebohm, 1991; Ölveczky et al. 2005). These studies suggest that neuroanatomical changes across development could lead to a change in the contribution of LMAN to sequence control (e.g. Bottjer et al. 1984; Scharff and Nottebohm, 1991; Aronov et al. 2008). Because the only known connection from the AFP to the vocal motor pathway is the projection from LMAN to RA, it is likely that alterations in LMAN or RA function underlie this difference. For example, the influence of LMAN on RA neurons could change across development, an idea that is supported by

the finding that LMAN lesions lead to greater changes in dendritic morphology and synaptic physiology of RA neurons in juveniles than in adults (Kittelberger and Mooney 1999). It has recently been found that RA is reciprocally connected to HVC (Roberts et al. 2008), a nucleus critically involved in sequence learning and generation (reviewed in Fee et al. 2004), and connections from RA to HVC could be more influential on motor control in juveniles. Neurons in RA may also participate in sequence control (Ashmore et al. 2005; but see Vu et al. 1994), and it is possible that RA's contribution to syllable sequencing is greater in juveniles than in adults. Regardless of the mechanism of developmental change, our results demonstrate that the influence of LMAN on the variability of syllable sequencing is relatively small in adult songbirds.

Mean pitch, the number of introductory notes preceding song, and song tempo are song features that also appear to be controlled independently of LMAN in adult Bengalese finches. With regard to pitch, there was neither a consistent effect of LMAN lesions on the mean FF of syllables nor a significant effect of lesions on the social modulation of mean FF (Figs. 2 and 5). Despite the elimination of social context effects on the variability of FF after LMAN lesions, mean FF continued to increase when males produced FD song following LMAN lesions. These data suggest that control of mean FF is distinct from control of variability in FF and support the hypothesis that changes in social context recruit circuits in addition to the AFP to modulate song. For example, neuromodulatory systems that are differentially active during the production of FD song versus UD song could underlie these LMAN-independent effects (reviewed in Ball et al. 2003; Bharati and Goodson 2006; Castelino and Ball 2005; Hara et al. 2007; Maney and Ball 2003; Sasaki et al. 2006; Yanagihara and Hessler 2006).

In adult zebra finches, lesions of LMAN did not affect the degree to which social context modulated the number of introductory notes preceding song but reduced social modulation of song tempo (Kao and Brainard 2006). Consistent with the previous study,

we found that the number of introductory notes preceding song was greater for FD song than for UD song, and that this social modulation was not significantly affected by LMAN lesions. However, we did not find that LMAN lesions increased song tempo or eliminated the speeding up of song from UD to FD song (Figs. 4 and 5). The lack of changes to UD song tempo following LMAN lesion contrasts with previous studies of LMAN function in the zebra finch (Williams and Mehta 1999; Brainard and Doupe 2000; Kao and Brainard 2006). While it is possible that stronger effects on tempo may have been observed had we followed song for a longer period of time after LMAN lesions, our data suggest that LMAN contributions to song tempo could be reduced in the Bengalese finch.

The experiments described here provide insight into the role of LMAN in the control of adult song, including the variability of syllable structure and sequencing. They demonstrate that different song features are regulated by distinct neural circuits. However, the current study does not address the role of LMAN in the plasticity of these song features. For the adult zebra finch, LMAN lesions do not affect normal syllable sequencing but prevent plasticity in syllable sequencing (Bottjer et al. 1984, Scharff and Nottebohm 1991; Nordeen and Nordeen 1993; Morrison and Nottebohm 1993; Williams and Mehta 1999; Brainard and Doupe 2000, Brainard and Doupe 2001; Thompson and Johnson 2006; Thompson et al. 2007; Scott et al. 2007; reviewed in Brainard 2004). Hence, it remains of interest for future studies to test whether manipulations of LMAN activity in the Bengalese finch alter the capacity for plasticity of even those song features, such as syllable sequencing, that are unaffected by LMAN lesions of normal adult song.

CHAPTER 3.

The contribution of LMAN to adaptive song recovery in the adult Bengalese finch.

ABSTRACT

Birdsong is a vocal motor behavior that retains the ability to change into adulthood. Moment-by-moment as well as long-term changes in song have been studied in adult songbirds. The basal ganglia-forebrain nucleus LMAN has been implicated in many forms of adult song change. It is unclear, however, whether changes to adult song serve any function. It is possible that changes to adult song are simply 'noise' in the system, or a maladaptive response to loss of sensory input. Alternatively, changes in song could be used to actively maintain song. To differentiate these possibilities, we introduced a reversible form of long-term song plasticity, tracheosyringeal nerve crush (tsNC), and investigated the role of LMAN in the recovery from this manipulation. For tsNC, the input to the vocal muscles was temporarily removed by bilaterally crushing the ts nerve. We found that there was an initial acute disruption of song due to loss of ts input, followed by a further, gradual disruption of song structure over a period of several weeks after tsNC. After this degradation we found that song recovered from tsNC. Finally, we lesioned LMAN after tsNC induced song degradation (3 weeks post), and found that recovery from this reversible injury was prevented. Taken together, our results suggest that song changes that are actively driven by LMAN are adaptively used for adult song maintenance and recovery from injury.

INTRODUCTION

The ability to change behavior throughout life in order to adapt to one's environment is necessary for fitness and survival. Birdsong is a precisely learned vocal

motor behavior that retains the ability to change into adulthood. In previous studies, song changes in adult songbirds range from moment-by-moment changes in song structure to long-term plasticity due to sensory feedback manipulations (Nordeen and Nordeen 1992; Williams and McKibben 1992; Leonardo and Konishi 1999; Hough and Volman 2002; Woolley and Rubel 2002; Cynx and Gell 2004; Zevin et al. 2004; Kao and Brainard 2006; Sakata and Brainard 2006; Roy and Mooney 2007; Tumer and Brainard 2007; Sakata et al. 2008). The output of a basal ganglia-forebrain circuit, LMAN (the lateral nucleus of the anterior nidopallium) has been implicated in some forms of adult song change (Williams and Mehta 1999; Doupe and Brainard 2000; Scott et al. 2007; Kao and Brainard 2006; Sakata et al. 2008). Despite evidence of its neural regulation by LMAN, the function of song change in adults is under debate. It is possible that changes to adult song are a result of biological noise or degradation, unimportant to the system. Alternatively, it is possible that the ability to change song may be retained in adult birds for the purpose of adaptive song maintenance.

The hypothesis that adult song change is adaptive is supported by studies that indicate that song change, or song plasticity, is actively regulated by LMAN. Under normal conditions song change is expressed as song variability. Even after song is well-learned in adults, it continues to vary from rendition to rendition, and this variability correlates with variability in LMAN activity (Kao et al. 2005). When LMAN is lesioned in adult songbirds, this moment-by-moment song variability is reduced (Kao and Brainard 2006). During learning in young birds, lesions or inactivations of LMAN also reduce moment-by-moment changes or variations in song, and prematurely stereotype song (Bottjer et al 1984; Scharff and Nottebohm 1991; Olveckzy et al. 2005). This suggests that LMAN actively contributes to song change, and that these changes are regulated by the brain rather than being uncontrolled biological noise.

Further support of the adaptive hypothesis is from evidence that song plasticity is also actively regulated following sensory feedback manipulations. For example, deafening in adult songbirds causes slow, progressive degradation of song (Nordeen and Nordeen 1992). When LMAN is lesioned prior to sensory feedback manipulations, song changes are prevented, suggesting that song changes are actively regulated by LMAN (Williams and Mehta 1999; Brainard and Doupe 2000). Similar to what may occur during vocal development, it has been hypothesized that disruptions of sensory feedback are perceived as errors in vocal-motor production. When sensory feedback is altered, then, it is thought that song is changed in order to expand motor exploration and correct those errors (Doya and Sejnowski 2000; Sutton and Barto 1998; reviewed in Brainard and Doupe 2002; Troyer and Doupe 2000). However, it is difficult to infer whether such changes serve any adaptive function because classic sensory manipulations cause permanent degradation of song.

In contrast, it is possible that LMAN activity is regulated, but not adaptive. One study suggests that LMAN is only responsible for song degradation in adults. Disruption of the motor nucleus, HVC by small ‘microlesions’ in adult zebra finches leads to a remarkably fast degradation of song that is reversible within a few days. Interestingly, LMAN lesions after song was degraded due to HVC microlesion caused immediate reversion of song back to pre-microlesion state (Thompson et al. 2007). This result suggests that activity from LMAN can cause maladaptive song degradation that is reversed by LMAN lesions. It is unclear, however, how this can be linked to the sort of long-term motor changes that were seen in sensory feedback manipulations.

In order to investigate the role of LMAN in song plasticity, we introduced a reversible form of long-term song plasticity, tracheosyringeal nerve crush (tsNC). For tsNC, the input to the vocal muscles is temporarily removed by bilaterally crushing the ts

nerve that provides input to the vocal muscles (syrinx). This causes immediate changes to song, but is reversible because the nerve is able to re-grow. We find that, like classical manipulations of auditory feedback, song changes immediately and further degrades in the weeks after tsNC. However, song also recovers from tsNC over a period of several weeks. Finally, we tested the contribution of LMAN to this recovery process by lesioning LMAN after tsNC induced song degradation. We found that LMAN lesions prevented song recovery, halting song in a degraded state. Our results support the hypothesis that adult song changes can be adaptive and that LMAN contributes to adaptive song recovery.

METHODS

Animals: Adult Bengalese finch males (n=10, >5 months old) were raised in our colony or obtained from an outside vendor. Prior to the experiment, males were housed individually in a sound-attenuating chamber (Acoustic Systems, Austin, Texas) on a 14 hour light: 10 hour dark photoperiod. All procedures were performed in accordance with established animal care protocols approved by the University of California, San Francisco Institutional Animal Care and Use Committee (IACUC).

Data collection: Undirected song was recorded at weekly intervals and collected for 2 time points pre-tsNC and 1, 3, 4 and 8 weeks post-tsNC surgery. Sound was recorded in the bird's home cage using an omni-directional microphone and threshold based song detection software SAP (Sound Analysis Pro v. 1.04 http://ofer.sci.ccny.cuny.edu/html/sound_analysis.html). SAP detects sound based upon amplitude threshold crossings, and then saves "songs" based upon user settings.

Settings for SAP recordings were low threshold, and allowed for calls, short songs and disrupted or atypical songs to be saved.

Behavioral training: Pilot studies suggested that recovery from nerve crush was hindered by social isolation. To prevent adverse affects from social isolation, doors were frequently opened to allow birds to call to each other and all birds were given access to perches that, when displaced by hopping or pecking, activated bird's own song (BOS) stimuli. Perches were provided *ad libitum*, but to ensure sufficient amounts of singing, a maximum of 200 BOS playbacks per day was allowed. Perch hop activity was monitored and recorded in SAP (see song recordings). Birds who failed to learn perch hopping for BOS or who did not consistently perch hop for BOS stimuli after surgery were excluded from this study.

ts nerve crush: Birds were anaesthetized using equithesin supplemented with gaseous isoflurane. Lidocaine was applied topically and under the skin lateral to the midline of the neck, where the trachea is visible underneath the skin. The tracheosyringeal nerve runs on either side of the trachea. A small incision was made and lidocaine was applied to the nerves to prevent any damage induced muscle contraction. Each nerve was crushed for 5 seconds with blunt #2 forceps. The area was rinsed with saline and the skin sutured together with veterinary glue. Post-operative care is critical for tsNC birds, as respiratory wheezing due to stress can be fatal up to 2 weeks post-tsNC. After surgery, birds were transferred immediately to their home cage with plenty of food and water, monitored carefully but left minimally disturbed.

LMAN Lesions: LMAN lesions were performed after song degraded due to tsNC. In N=5 birds, LMAN was lesioned 3 weeks post-tsNC. Birds were anaesthetized (see above).

Stereotaxic coordinates from the posterior branch of the mid-sagittal sinus were used to locate LMAN (rostral: 5.1-5.5 mm; lateral: 1.2-1.8 mm; depth: 1.7-2.1 mm). For LMAN lesions, we made 6-8 electrode (30-100k Ω) penetrations passing 50-100 μ A for 60-120 seconds. Silica gel was used to protect the brain during and after surgery. The craniotomy was covered and skin was sealed together over the skull with veterinary glue. N=2 sham lesions were performed in a similar manner at identical time points post nerve crush, but lesions were made outside of the boundaries of LMAN. N=3 control birds underwent no lesion surgery after tsNC, but had song collected at identical time points. No differences between sham and control birds were found, therefore they were combined in our analysis (collectively referred to as 'control').

Histology and estimation of lesion size: Upon completion of behavioral experiments, birds were anaesthetized with a lethal dose of isoflurane and perfused with paraformaldehyde or formalin. Every third section was used for either Nissl stains or calcitonin gene-related peptide (CGRP) immunohistochemistry. CGRP stains LMAN cell bodies and projections to RA. Lesion size was estimated by at least 2 experienced observers based on residual CGRP staining in LMAN and RA. We compared the amount of residual CGRP staining to that of control tissues to estimate the percent of LMAN lesioned. All lesion birds had 75% or greater bilateral damage to LMAN. All sections were also checked for the presence of CGRP staining in brainstem regions to confirm positive antibody reaction within the experimental tissue. CGRP also stains cell bodies in MMAN (the medial magnocellular nucleus of the nidopallium) a region proximal to LMAN that also projects to the vocal motor pathway (Foster et al. 1997). N=2 lesion birds had a small amount (~10-20%) of damage to the left lateral portion of MMAN. These birds had phenotypically comparable results to the other LMAN lesion birds, and thus were not excluded.

Song analysis: Song is defined as a series of complex sounds separated by short silent intervals. For our purposes, a syllable is defined as a spectrally discrete sound element within song approximately 10-100 ms, separated by a minimum of 5 ms of silence (Okanoya and Yamaguchi 1997). Songs were filtered from 200-8000 Hz and visualized by plotting spectrograms in MATLAB. Syllables were segmented based upon amplitude thresholds and manually labeled with unique letters. At some stages, particularly 3-4 weeks post-tsNC, syllable labels could be difficult to assign. In such cases, an unidentified syllable was labeled '0', and excluded from labeled analysis. Because the majority of unlabeled syllables occurred at the most degraded stages, this led to a conservative under-estimation of degradation in labeled analysis. In our results, we present both labeled and unlabelled data analysis.

Syllables are complex sounds that can be described by many acoustic features. We used 8 acoustic features to characterize the structure of individual syllables: duration, spectral entropy, spectro-temporal entropy, amplitude, entropy of loudness vs. time, frequency slope and mean frequency. Any given syllable is unique in its combination of these features, and each bird has a unique set of syllables. Therefore, we used principle components analysis (PCA) as a non-biased way of clustering syllables within each bird and across time. To normalize all features to the same coordinates, a z-score was taken before PCA. PCA reduces the dimensionality of the data by calculating coefficients for each feature that best account for variability within the data. For example, a first principle component may be calculated to be 35% duration, 15% amplitude, 45% spectro-temporal entropy and 5% other features. Features for individual syllables labeled within each bird across time were combined and used to calculate PCA coefficients. Each syllable has its own PCA score. When principle components are plotted, examples of a single syllable form clusters. Euclidean distances

were measured between pre-tsNC syllable clusters and syllable clusters at each time point. Variability of a syllable within each time point was measured as the spread or density of a cluster: average distance from a given syllable to the center of its cluster. Each bird has multiple syllables. To prevent pseudo-replication in population analyses, an average syllable distance and syllable variability was calculated for each bird at each time point. Introductory syllables were excluded from this analysis.

To make sure that the same effect was true for all data, including non-labeled syllables, we performed a separate analysis on all syllables that did not require song labeling. For this, we used a single feature: spectro-temporal entropy (STE). STE reflects syllable noisiness and is defined as the probability of noise in each pixel of the sound spectrum (s_i), normalized to the total number of sound pixels (n_s).

$$STE = -\sum[s_i \cdot \log_2(s_i)] / [\log_2(n_s)]$$

In this measure, a pure tone has low entropy a sweep is intermediate, and maximal entropy is 1.0, equivalent to white noise.

Song timing is a higher order level of song organization reflecting the overall temporal pattern of song. To analyze song timing, we measured the unlabeled distribution of all intervals. Intervals are defined as the offset of one syllable to the onset of the next syllable up to a maximum of 500ms denoting the end of one song and the beginning of a new song. Each bird has a unique distribution of intervals based upon the pattern of the bird's own song. For example, the distribution of intervals is altered when syllable sequencing is altered: syllables deleted, syllables added, or numbers of repeated syllables change. For comparison, we also report the distribution of durations of syllables. Duration is defined as the onset to offset of a single syllable.

The distribution of intervals, durations, and spectro-temporal entropies are non-normal and complex. Therefore, the difference between pre-nerve crush feature distributions, and feature distributions at other time points was described by the

Kullback-Leibler divergence (KL-div): the amount of information, per sample, in bits that it would take to convert one distribution P (example: post) to another distribution Q (example: pre).

$$\text{KL-div} = \sum_i P_i * \log (P_i/Q_i)$$

Finally, sequence changes were calculated as described previously (see Sakata et al. 2008) for branch point entropy, repeat entropy, and number of introductory notes. 'Branch points' are nodes in song in which transitions vary across renditions. We characterized the variability at branch points using transition entropy:

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

where the sum is over all possible sequence transitions, and p_i is the probability of the i th transition across all songs (Gil and Slater 2000; Sakata and Brainard 2006, Sakata et al. 2008). Low entropy indicates low variability; completely stereotyped sequences have an entropy value of zero. Branch points in which the dominant transition occurred >95% of the time were considered stereotyped sequences and not included in the analysis of branch points. We also analyzed 'repeats', syllables that are consecutively repeated a variable number of times across renditions. We calculated the transition entropy of repeats. Repeats can be considered a different class of branch point transition in which a syllable can transition to itself or to another syllable. Higher numbers of repeats lead to lower values of repeat entropy as the probability of a repeated syllable increases (i.e., increased predictability in transitions). Finally, we calculated the mean number of introductory notes. Introductory notes are low amplitude syllables that precede song and are repeated a variable number of times. We counted the number of introductory notes preceding each song by starting at the first introductory note prior to the first (non-introductory) syllable of the song and then counting backward in time until there was >500 ms of silence (Kao and Brainard 2006). If there were more than one type of introductory note, all were combined in the analysis. Finally, in order to describe global

sequence changes, we calculated whole song entropy. Whole song entropy is the entropy of all pair-wise transitions in song (branch points, repeats, intro notes, etc.).

For the effect of nerve crush over time, all non-lesion data from all birds was pooled and means were compared for pre, 1, 3 and 8 weeks post-tsNC using student's t-tests. To validate the use of individual t-tests or ANOVA's, Least Squares Means analysis was performed on all combined data. Tukey HSD was also performed to compare across multiple time points ($\alpha=0.05$).

For the effect of lesion after nerve crush, measures at each time point were compared across groups (control or lesion) using ANOVA's ($\alpha=0.05$). All statistical analyses were done using Jump 5.01 for the Macintosh (SAS Institute, www.jmp.com).

RESULTS

To test whether LMAN is necessary for adaptive song plasticity we developed a reversible song degradation paradigm: tracheosyringeal nerve crush (tsNC). First, we describe the dynamics of song changes and recovery following tsNC. Then we investigate how LMAN is involved in this form of song plasticity.

The song system has two major pathways, the motor pathway and the anterior forebrain pathway, depicted in Figure 1a. The motor pathway is required for the production of song, while the anterior forebrain pathway (AFP) is involved in song learning and song plasticity. The motor pathway has two major outputs, one to respiratory muscles via respiratory regions of the brainstem, PAm and RAm, and one to the vocal muscles of the syrinx (avian vocal organ) via brainstem region nXIIts. Respiratory regions are ultimately responsible for the timing of song, for example, when a syllable or silent period occurs. The vocal muscles of the syrinx control the spectral structure of syllables such as pitch, or fundamental frequency (reviewed in Vicario 2004;

Suthers and Zollinger 2004; Goller and Cooper 2004). The motor pathway connects to and

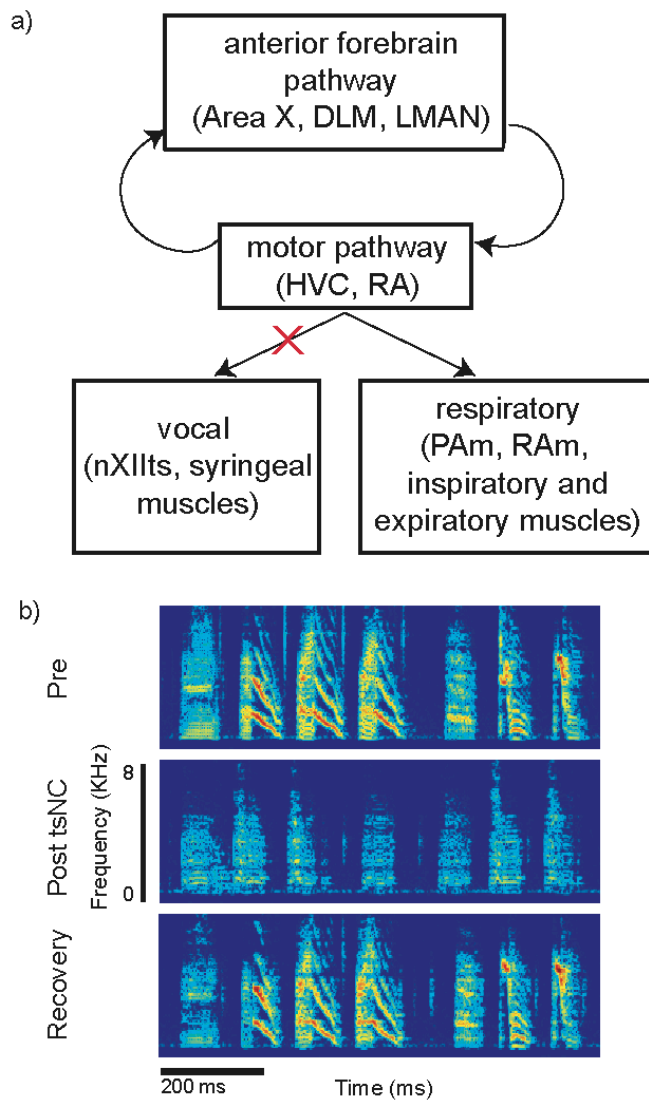


Figure 1: Song recovers after tsNC. a) The two major pathways of the song system are depicted. The motor pathway (HVC, RA) projects to vocal and respiratory centers of the brainstem (nXIIts, Pam, RAm) and is required for song production. The anterior forebrain pathway (Area X, DLM, LMAN) is interconnected with the motor pathway and is involved in song learning and plasticity. TsNC reversibly removes the input to the syrinx via the ts nerve. b) Spectrograms of identified song motifs before, immediately after, and 8 weeks post tsNC. Immediately after tsNC, frequency modulation is lost due to loss of input to the vocal muscles, but song eventually recovers from tsNC, indicating that it is reversible.

receives projections from the AFP. LMAN is the output of the AFP connecting to the RA of the motor pathway such it is in a position to modulate vocal motor activity.

tsNC: song change and recovery

We found that tsNC caused reversible song degradation. The ts nerve provides input to the syrinx, and was reversibly damaged by tsNC (see methods). Figure 1b shows an example of identified song motifs before, 1 and 8 weeks post-tsNC. The loss of ts input acutely removed vocal muscle control, shown by the loss of frequency modulation and the resultant noisy appearance of syllables immediately post tsNC. Despite substantial changes to vocal motor control after tsNC, song recovered indicating that this manipulation was reversible. To quantitatively describe the dynamics of tsNC song changes we measured syllable features and song timing before and after tsNC. Overall, we observed two different types of change following tsNC; acute and delayed. Acute changes in syllable structure occurred immediately after tsNC as measured at 1 week post-tsNC. Delayed changes in syllable variability and song timing were larger at 3 weeks post-tsNC than at 1 week post tsNC.

We found significant changes to syllable structure following tsNC ($p < 0.0001$). Figure 2 is a summary figure for syllable changes using a single feature, spectro-temporal entropy (STE, see methods) where labeling of syllables was not required. This measure showed significant acute changes post-tsNC ($p < 0.0001$). Figure 2a shows an example of STE distributions at 1, 3 and 8 weeks post-tsNC. STE increased immediately after tsNC. This reflects the loss of input to vocal musculature (reviewed in Goller and Cooper 2004), which caused syllable ‘noisiness’ and therefore STE, to increase. 3 weeks post-tsNC, syllables were still significantly degraded, though some structure had returned. Good recovery of STE was observed at 8 weeks post tsNC. Overall, syllable structure degraded immediately after tsNC ($p < 0.0001$), was still degraded 3 weeks ($p < 0.0001$), and returned to pre-levels by 8 weeks post-tsNC ($p = 0.0689$) (Figure 2b: Least Squares Means Tukey HSD, $wk1 \neq wk3 \neq pre2 = wk8$, $\alpha = 0.05$).

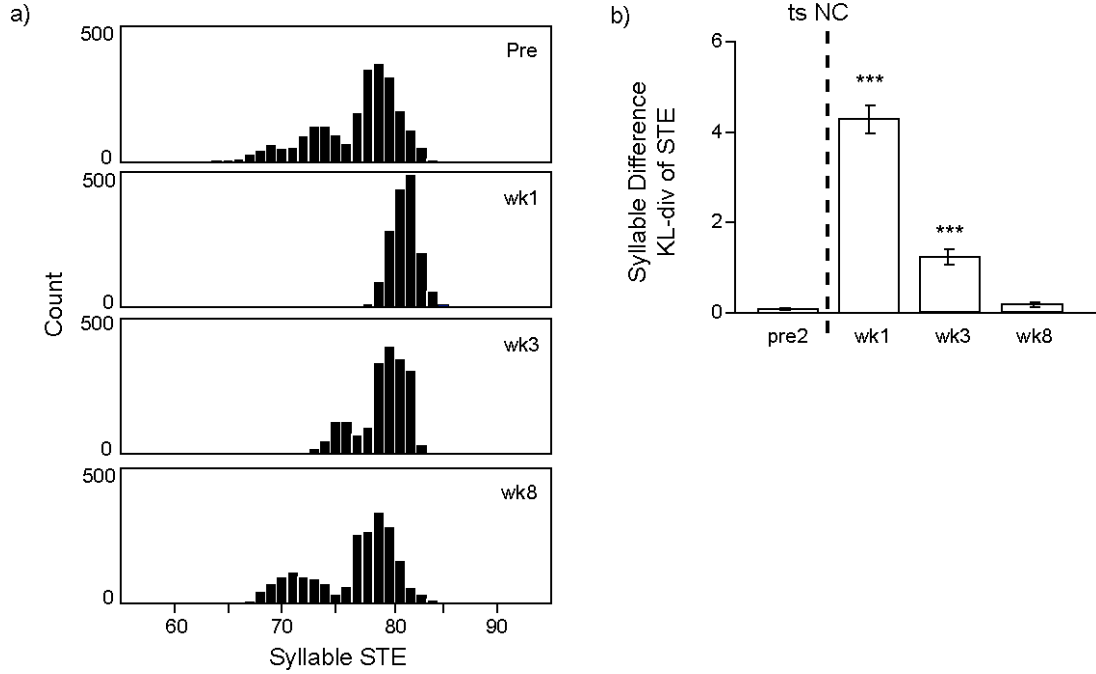


Figure 2: Dynamics of syllable structure changes after tsNC: STE. a) Histograms of spectro-temporal entropy (STE) for all songs in an example bird pre, and week1, 3 and 8 post-tsNC. The distribution shifts toward higher STE at week1 as all syllables become extremely noisy post-tsNC, and gradually recovers. c) Summary of syllable differences measured by the average KL-divergence (vs. pre) of STE per bird for pre2, and 1,3, and 8 weeks post-tsNC. Overall, STE changes occur acutely at week1, and recover by week8 post-tsNC. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$

We also quantified syllable changes using PCA (see methods), where many features of syllables were calculated and combined to describe changes in syllable structure. Figure 3a shows an example syllable and its corresponding clusters at pre-tsNC and 1,3, and 8 weeks post-tsNC. This example is representative of the summary data in Figure 3b, where the largest distance between pre and post syllables occurred immediately after tsNC, 1 week post-tsNC ($p < 0.0001$) indicating, just as in the unlabeled STE analysis, that changes in syllable structure occurred acutely. At 3 weeks post-tsNC, the distance to pre syllable clusters was reduced, but syllables were still significantly different from pre-tsNC ($p < 0.0001$). We found that syllables recovered after tsNC by 8 weeks post-tsNC, when syllable distances were closer to pre-tsNC levels, though modest differences persisted ($p = 0.0156$) likely due to the long time elapsed during the manipulation (LS Means Tukey HSD: syllable difference $wk1 \neq wk3 \neq pre2 = wk8$, $\alpha = 0.05$).

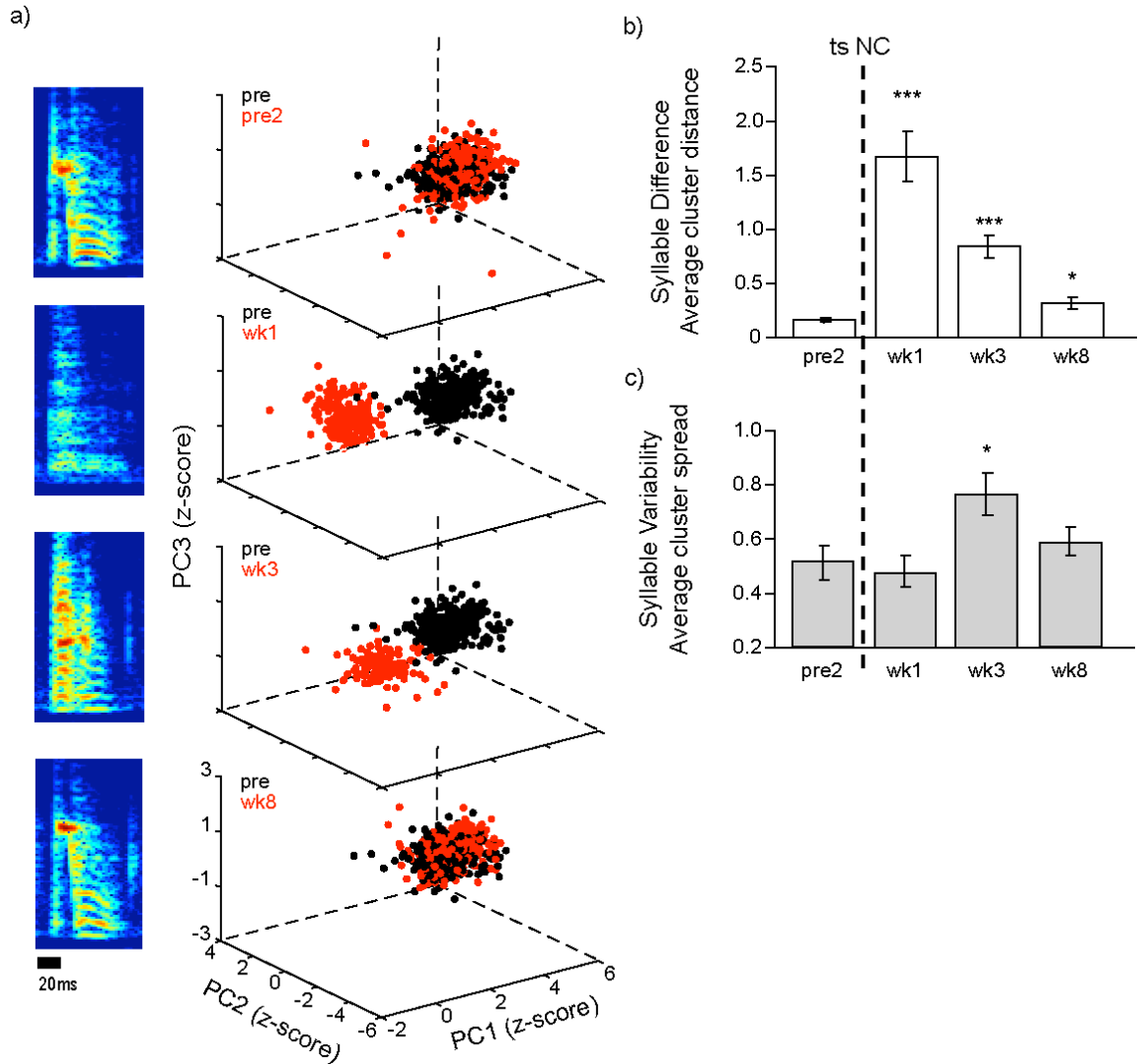


Figure 3: Dynamics of syllable structure changes after tsNC: PCA. a) Spectrograms of an example syllable before, and week1, 3, and 8 post-tsNC, with corresponding PCA clusters of all renditions of this syllable at pre (●), and pre2, weeks 1, 3, and 8 post-tsNC (●). b) Summary data for syllable differences measured by the average syllable cluster distance (vs. pre) per bird for pre2, and weeks 1, 3, and 8 post-tsNC. Overall, syllable differences were greatest immediately after tsNC at week1, still significantly altered at week3, and recovered by week8. d) Summary data for syllable variability measured by the average spread of syllable clusters at each time point. Overall, syllable variability was unchanged immediately after tsNC, but increased at week3 post-tsNC. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$

In addition to changes in syllable structure, we found changes in the variability of syllables ($p = 0.0003$). Unlike syllable structure, however, we found that syllable variability changes were delayed, peaking long after the initial injury, at 3 weeks post-tsNC. As measured by the average spread of PCA clusters (see methods) syllable variability

changes were minimal at 1 week post-tsNC ($p=0.7001$), increased at 3 weeks post-tsNC ($p=0.0250$), and returned to pre-levels by 8 weeks post-tsNC ($p=0.3801$) (Figure 3c: Least Squares Means Tukey HSD, $wk3 \neq pre2 = wk1 = wk8$, $\alpha=0.05$). Changes in song variability have been shown to be actively driven by neural substrates (Kao et al 2005; Kao and Brainard 2006; Olveckzy et al 2005), therefore these changes suggest that, after the initial changes to peripheral syringeal input, central plasticity may have occurred.

Finally, we found significant changes to song timing due to tsNC ($p<0.0001$). Like syllable variability, we found that changes in song timing due to tsNC were delayed. We measured these changes first using the unlabeled distribution of intervals, (Figure 4b).

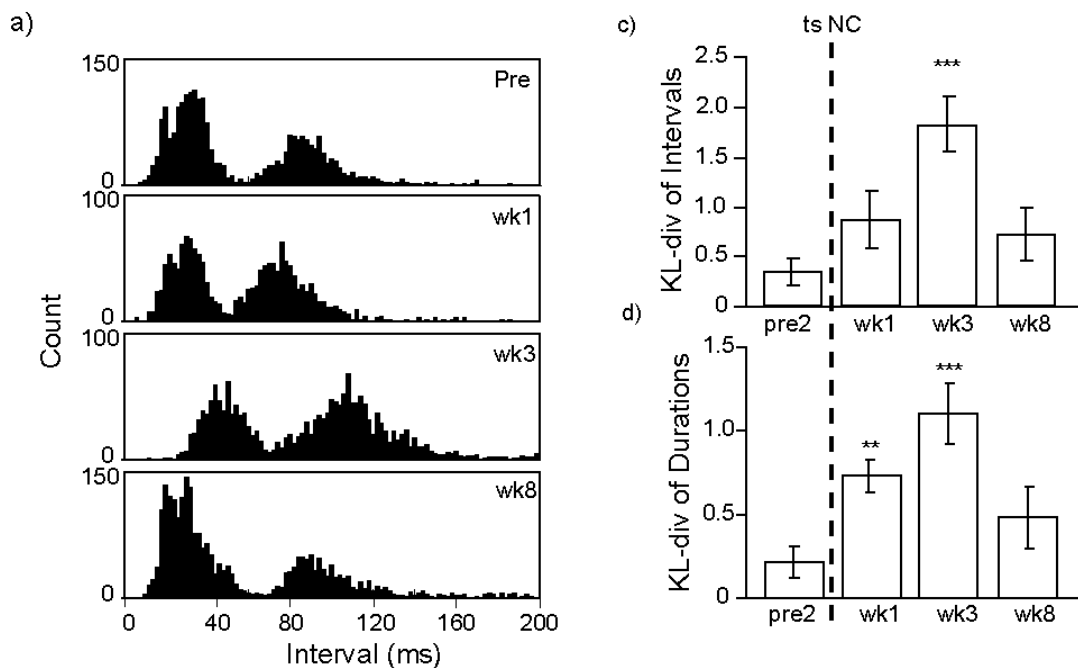


Figure 4: Dynamics of song timing changes after tsNC. a) Histograms of intervals for all songs in this bird pre, and weeks 1,3 and 8 post-tsNC. Weeks 1 and 8 are similar to pre song, but week3 intervals are shifted rightward indicating a delayed degradation of song timing. c) Summary of song timing differences measured by the average KL-divergence of intervals (vs. pre) per bird from pre, weeks 1,3, and 8 post tsNC. The greatest difference from pre occurred at week3 post-tsNC. d) Summary of average KL-divergence of durations (vs. pre) per bird for comparison. Changes in duration reflect a combination of acute (wk1) syllable changes and delayed (wk3) song timing changes. * $p<0.05$, ** $p<0.01$, *** $p<0.0001$

The distribution of intervals was relatively unchanged at 1 week post-tsNC, but highly increased at 3 weeks post-tsNC, and returned to normal at 8 weeks post-tsNC. This example is reflective of the trend in all birds (Figure 4c: week1 vs pre: $p=0.1139$, week3 vs pre: $p=0.0002$, week8 vs pre: $p=0.2519$, LS Means Tukey HSD: $wk3 \neq pre2 = wk8 = wk1$, $\alpha=0.05$). For comparison we also analyzed changes in the distribution of durations. Duration is a feature that is important to song timing, but also reflects changes in syllable structure as each individual syllable has an average duration that is controlled by opening and closing of the vocal musculature. As expected from this combined role, durations have both acute (week1: $p=0.0016$) and delayed (week3: $p=0.0008$) changes that generally recover by week8 post tsNC ($p=0.2749$) (Figure 4d: LS Means Tukey HSD: $wk1 \neq wk3 \neq pre2 = wk8$, $\alpha=0.05$).

Changes in song timing can be due to number of related sequence changes. For example, introductory notes have long intervals, so an increase in intervals like that seen in Figure 4 could be due to an overall increase in introductory notes. Similarly, repeat transitions typically contain short intervals, such that a decrease in the number of repeats would lead to an overall increase in the distribution of intervals. We analyzed branch point transitions, repeats, and introductory notes (see methods, Sakata et al. 2008) in order to describe sequence changes following tsNC.

Consistent with the delayed peak in song timing changes, we observed sequence changes at 3 weeks post-tsNC. An example of 3 week post-tsNC song is compared to pre song in Figure 5a. In this example, song timing at 3 weeks post-tsNC was severely degraded: pauses between syllables were longer, syllable durations shorter, and defined motifs (red, green and blue boxes) are more rare. Across birds, we found a multitude of changes at 3 weeks post-tsNC. For 10/17 branch points analyzed, branch point entropy was decreased relative to pre-tsNC, including 3/17 cases in which

branch points were lost. For 7/8 introductory notes analyzed the mean number of introductory notes increased and the percentage of intro notes out of total song notes was increased. Finally, for 10/13 repeats analyzed, the mean numbers of repeats decreased compared to pre song. Despite these observations, changes in introductory notes, repeats and branch points were varied across individual birds and were minimally or not at all significant 3 weeks post-tsNC (Figure 5: introductory notes: $p=0.0401$, repeat entropy: $p=0.1524$, branch point entropy: $p=0.7381$). We hypothesized, then, that a

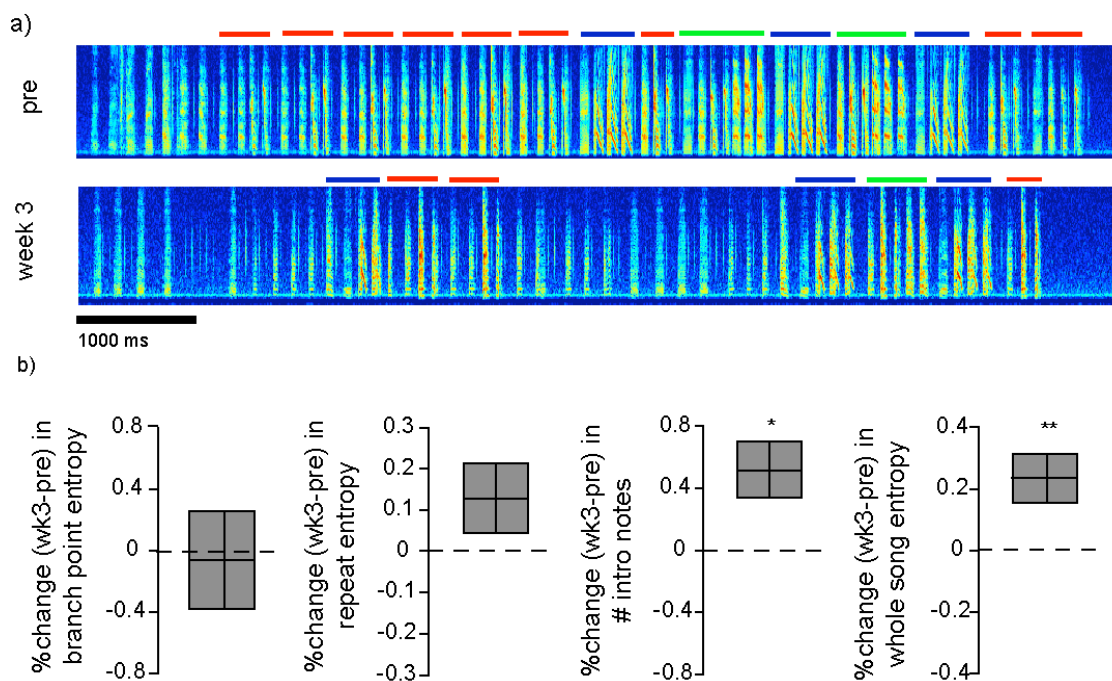


Figure 5: Delayed sequence changes after tsNC. a) Example spectrograms of songs from pre, and week 3 post-tsNC. At 3 weeks post-tsNC intervals appear longer, introductory notes more prevalent, and identified motifs (red, green, blue bars above spectrogram) are more rare. b) Percent change from pre at week3 post-tsNC for branch point entropy, repeat entropy, number of introductory notes, and whole song entropy. Branch point and repeat entropy changes vary across birds and are overall non-significant, while there was consistent increase in the number of introductory notes. The significant increase in whole song entropy at week3 reflects a combination of multiple sequence changes per bird. * $p<0.05$, ** $p<0.01$, *** $p<0.0001$

combination of sequence changes in each individual bird could have contributed to song timing effects overall. To test this, we employed a global sequence measure: whole song entropy (see methods). Whole song entropy was calculated for every pair-wise transition, which includes branch point, repeat, and introductory note transitions. Changes in whole song entropy were robust across birds at 3 weeks post-tsNC

($p=0.0037$). This suggests that changes in song timing at 3 weeks post-tsNC were due to a combination of sequence changes. These changes provide further support for central plasticity due to tsNC.

LMAN lesion and tsNC song recovery

LMAN has been implicated in song plasticity in previous studies. It is unknown, however, whether LMAN has an adaptive role in song recovery. To test this hypothesis, we lesioned LMAN 3 weeks post-tsNC, when song was degraded, and where evidence for central plasticity was the strongest (Figure 6a). We analyzed syllable and song timing

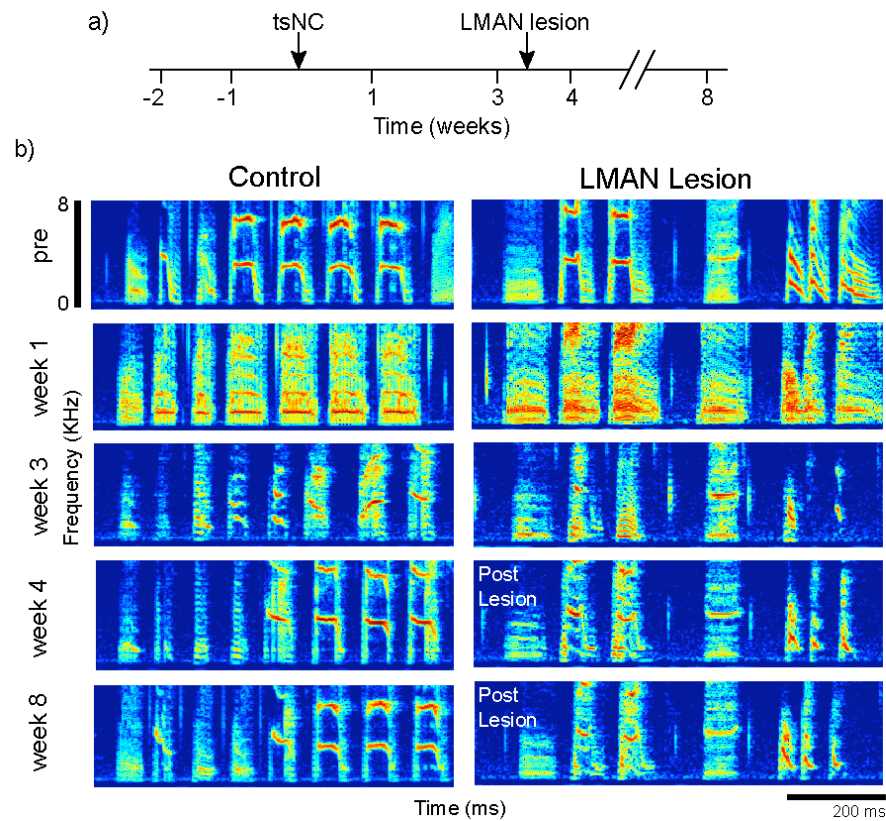


Figure 6: LMAN lesions prevent recovery from tsNC. a) Shown here is the timeline of experimental procedures: LMAN lesions were performed at 3 weeks post-tsNC. b) Example spectrograms of identified song motifs pre, and week 1, 3, 4 and 8 post-tsNC for a control (left) and a lesion bird (right) where week 4-8 is post-LMAN lesion. The control bird's song shows degradation and recovery, while the LMAN lesion bird's song remains degraded post-lesion.

recovery in lesion birds compared to control birds. We found a significant lack of recovery following LMAN lesions. Figure 6b depicts an example song motif over time from a control bird and a lesion bird. Both the control and the lesion bird exhibit similar time-courses of degradation up to 3 weeks post-tsNC. Following LMAN lesion, it is evident from this example that song remained degraded up to 8 weeks post-tsNC in the lesion bird, while the control bird recovered normally. This indicates that recovery from tsNC was halted by LMAN lesion.

Syllable structure recovery was consistently impaired after LMAN lesion. Figure 7a shows example syllables in control and lesion birds across time. While syllable 'm' from the control bird recovered well, syllable 'd' from the LMAN lesion bird remained heavily degraded post-lesion. Syllable spectrograms across time showed little change in syllable structure from 3 to 8 weeks post-tsNC in the lesion bird, while the control syllable recovered well. The difference in recovery at 8 weeks post-tsNC is shown in the lack of match between pre and wk8 syllable clusters for the lesion bird compared to the control bird (Figure 7b). This was true across the population of birds (Figure 7c) where degradation of syllables was equivalent across groups 3 weeks post-tsNC ('pre-lesion' ANOVA $p=0.2544$), but the lesion birds remained degraded at 8 weeks post-tsNC (LS Means Tukey HSD: $wk3=w8 \neq pre2$, $\alpha=0.05$). This led to significantly lower recovery of syllables; a greater distance between pre and 8 week post-tsNC syllable clusters for the lesion group compared to the control group (ANOVA: $p=0.0274$). The same trend holds for unlabeled analysis of STE (ANOVA $p=0.0393$). Because loss of LMAN halted syllable recovery in a degraded state, these data indicate that LMAN is necessary for adaptive recovery of syllable structure following tsNC.

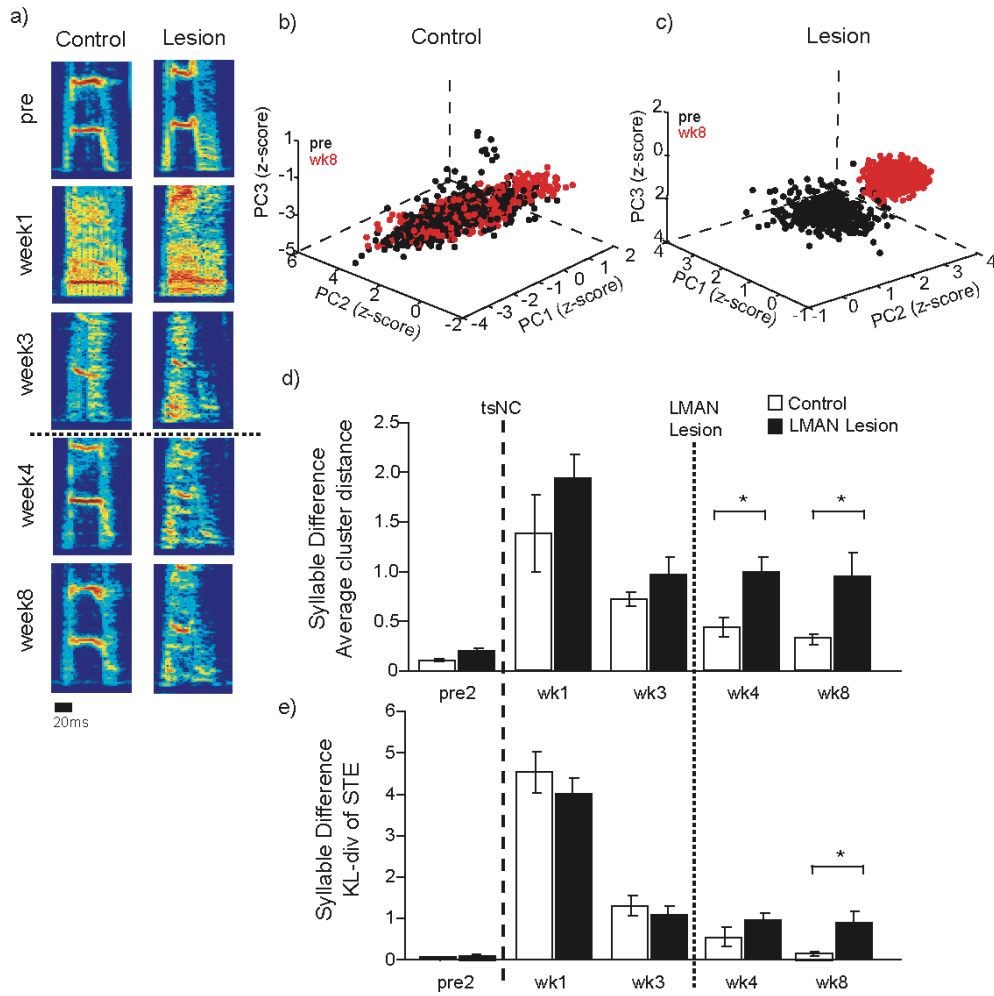


Figure 7: Syllable recovery is halted after LMAN lesion. a) Spectrograms of example syllables at pre, week 1,3, 4 and 8 post-tsNC for a control (left) and a lesion (right) bird where week4-8 is post-LMAN lesion. b-c) PCA clusters of all renditions of the example syllable pre (●) and week8 (●) post tsNC. Syllable clusters are recovered in the control bird (b), but remain degraded in the lesion bird (c). d) Summary data for syllable differences measured by average syllable cluster distances at each time point for control (□) and lesion (■) groups. Average syllable cluster distances are unchanged after week3 LMAN lesion in the lesion group, while they gradually recover in the control group, leading to overall significant differences between groups. d) Summary data for syllable differences measured by STE corroborate with syllable cluster data indicating that birds in the lesion group remained degraded at week8, when control birds were recovered. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$

In contrast, we found no evidence for a lack of song timing or sequence recovery following LMAN lesion in tsNC birds. Figure 8 shows the summary data of song timing for lesion and control birds. Song timing was not significantly different between lesion and controls at 8 weeks post-tsNC. Other measures for sequence including branch points, repeats, and introductory notes were also not significantly different between lesion and control birds at 8 weeks post-tsNC (ANOVA $p > 0.05$ for all, data not shown).

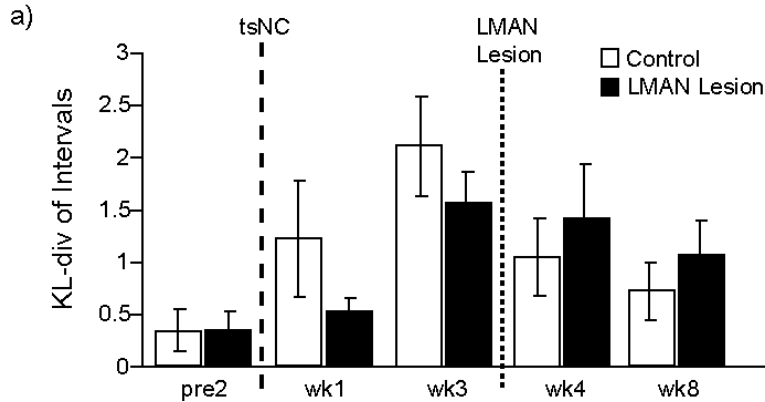


Figure 8: Song timing recovery after LMAN lesion. Song timing measured as the average KL-divergence of intervals is plotted for control (□) and lesion (■) groups across time. Song timing was not significantly different between lesion and control groups at any time point. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$

tsNC and song variability

LMAN lesions have previously been found to reduce the variability in syllable structure in adult songbirds. We therefore asked how variability in tsNC song was affected by LMAN lesions. As described in figure 2, syllable variability was highest at 3 weeks post-tsNC. Figure 9 shows example syllable clusters at 3 (9a, pre-lesion, red) and 4 weeks post-tsNC (9b, post-lesion, red) compared to a pre-tsNC syllable cluster (black) in an LMAN lesion bird. The variability of this syllable was reduced by 26.31% following LMAN lesion. Consistent with previous studies on the effects of LMAN lesion (Kao and Brainard 2006; Chapter 2), the loss of variability from week3 (pre-lesion) to week4 (post-lesion) was greater in the lesion group (Figure 9c $23 \pm 17\%$ decrease, paired t-test, $p = 0.05$) than in the control group ($5 \pm 12\%$ decrease, paired t-test $p = 0.6548$), though there was no overall difference between groups in this smaller dataset (ANOVA $p = 0.4426$).

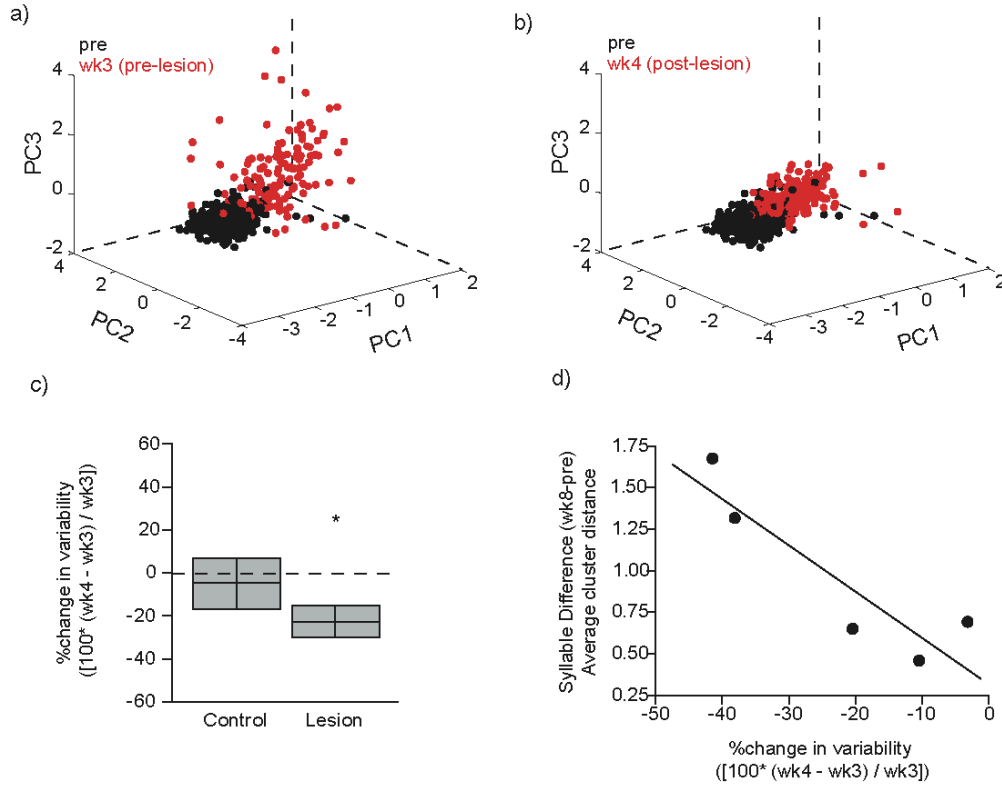


Figure 9: LMAN lesions reduce tsNC syllable variability. a) (left) PCA clusters are plotted for all renditions of an example syllable pre (●) and week3 post tsNC (●) when variability was the highest. b) A few days post-lesion, at week4 post tsNC (●) the cluster distribution is less dense indicating that syllable variability was reduced. Comparison with the pre cluster (●) shows that despite the reduction in variability, this syllable was not recovered after lesion. b) Overall, the reduction in variability at week4 was significant for lesion birds, but not for control birds. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$

We hypothesized that a lack of recovery in the tsNC lesion group could be attributed to a loss of syllable variability after LMAN lesion. Consistent with this hypothesis, loss of syllable variability, on average, in lesion birds correlated with worsened recovery ($R^2=0.81$, $p=0.0385$). Figure 9d shows that the birds with the greatest reduction in variability had the worst recovery, i.e. the greatest distance of week8 to pre syllables. This affect cannot be attributed to lesion size alone as there was no significant correlation with lesion size and syllable recovery. This suggests that the syllable variability present at week3 post-tsNC was not just degradation but possibly an important component of motor exploration needed for song plasticity and recovery.

DISCUSSION

Changes to adult song behavior have been hypothesized to be due to residual biological noise, unimportant to behavior. Alternatively, studies have shown there is active regulation of adult song change suggesting that it may serve a function. We found that tsNC induced changes to adult song that subsequently recovered, indicating that adult birds retain the capacity to recover from song degradation. In support of an adaptive function of LMAN, we found that LMAN lesions, at a time when song was degraded due to tsNC, prevented song recovery.

Even though tsNC is a peripheral nerve injury, our data suggest that central nervous system was involved in the process of degradation and recovery. The ts nerve innervates the vocal organ (syrinx) that is responsible for the spectral structure of syllables (reviewed in Vicario 2004; Suthers and Zollinger 2004; Goller and Cooper 2004). As in previous studies involving manipulations or deinnervation of the syrinx, we found that loss of ts nerve input to the syrinx led to immediate change in the spectral structure of syllables as well as delayed degradation of song timing (Figure 2-4; Simpson and Vicario 1990; Williams and McKibben 1992; Hough and Volman 2002; Roy and Mooney 2007). Song timing is controlled by central nuclei in the song system upstream of the ts nerve (reviewed in Margoliash 1997; Fee et al. 2004; see also Vu et al. 1994; Ashmore et al. 2005). We found that song timing changes peaked 3 weeks after the initial nerve injury, suggesting that central plasticity occurred. In addition, syllable variability also increased at 3 weeks after tsNC. Unlike song timing, syllable variability could have increased due to peripheral (e.g. excessive reinnervation of the syringeal muscles) or central changes. LMAN lesions, however, reduced syllable variability, suggesting that at least some component of tsNC syllable variability was central. Our data are consistent with studies in other systems, including mammals and reptiles, suggesting that central plasticity occurs during recovery from nerve injury (reviewed in

Navarro et al 2007; Beazley et al. 2006; Chen et al 2002). Such central plasticity could be manifested by a range of central changes, from remapping of existing motor connections to integration of new neurons (reviewed in Buonomano and Merzenich 1998; Nottebohm 2004).

Many other studies have implicated LMAN in adult song change. LMAN lesions reduce the variability of syllable structure (Kao and Brainard 2006) a moment-by-moment change in baseline song behavior. LMAN lesions also prevent long-term song degradation due to sensory feedback manipulations (Williams and Mehta 1999; Brainard and Doupe 2000; Scott et al. 2007). Here, we suggest that LMAN lesions after song degradation prevent song recovery. To our knowledge, this is the first evidence to suggest that LMAN is necessary for any adaptive function beyond its role in song learning. Some studies have addressed the consequences of LMAN lesions at the cellular level (Kittelberger and Mooney 1999) and in relation to addition of new neurons (Wilbrecht et al. 2004; Scott et al. 2007). Similar approaches could be used to address the mechanism of LMAN's contribution to song recovery.

Our LMAN lesion data contrast with the results of LMAN lesions found in one other study of a reversible manipulation, HVC microlesions. HVC is a nucleus in the motor pathway required for song production. When small lesions are made in HVC, song degrades and recovers in a matter of days (Thompson and Johnson 2007). LMAN lesions after HVC microlesion degradation cause abrupt recovery of song (Thompson et al. 2007), while LMAN lesions after tsNC degradation prevent recovery of song. It is possible that LMAN was not required in the case of HVC microlesions because 'relearning' may not have been required. The discrepancy between our results and Thompson et al. may also be due to fundamental differences in plasticity states of the motor pathway. TsNC degradation takes several weeks, and may thus reflect stronger changes to the motor pathway and central rearrangement. HVC microlesions are acute,

and may have caused changes in activity but not full degradation of motor structures. HVC and LMAN project to the same cells in RA (Mooney 1992). It is possible that removal of LMAN may have, in effect ‘uncovered’ the state of the motor pathway, by releasing the influence of LMAN at HVC-RA synaptic sites. In the case of HVC microlesions, the motor pathway was sufficiently intact to produce a good copy of song when LMAN was removed. The lack of immediate recovery after LMAN lesion in this study could be due to stronger changes induced in the motor pathway by tsNC. We suggest that these results, then, are not at odds, but rather show that LMAN is contributing to motor changes, both degradation and recovery, at different stages and for different forms of plasticity. In the case of tsNC, motor changes from LMAN were necessary for long-term song recovery.

Our data also suggest that the involvement of LMAN in song recovery is greatest for syllable structure. This is supported by previous studies of LMAN lesions in adult zebra finches (Kao et al. 2006) and adult Bengalese finches (see Chapter 2) on baseline song. In both studies, LMAN lesions significantly reduced moment-by-moment changes in syllable structure. In neither case was evidence found for the involvement of LMAN in syllable sequencing in adult songbirds. This is consistent with one study in which syllable recovery after vocal distortion appeared to be separate from sequence recovery (Hough and Volman 2002). It is at odds, however, with other plasticity studies that suggest that LMAN lesions affect all changes to song during plasticity states in the zebra finch (Williams and Mehta 1999; Brainard and Doupe 2000; Thompson et al. 2007). We found that recovery of both song timing and syllable sequencing were not significantly different between lesion and control groups. We note that sequence recovery after tsNC was not as strong as syllable recovery, thus possibly masking an effect of LMAN lesions on sequence recovery. On the other hand, this raises the interesting possibility that a separate neural circuit exists for variability and plasticity of syllable sequencing.

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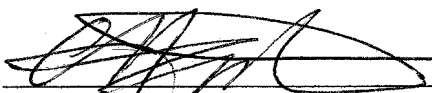
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