An Avian Basal Ganglia-Forebrain Circuit Contributes Differentially to Syllable Versus Sequence Variability of Adult Bengalese Finch Song

Cara M. Hampton, Jon T. Sakata, and Michael S. Brainard
Keck Center for Integrative Neuroscience, University of California, San Francisco, California

Submitted 29 September 2008; accepted in final form 1 April 2009

Hampton CM, Sakata JT, Brainard MS. An avian basal ganglia-forebrain circuit contributes differentially to syllable versus sequence variability of adult Bengalese finch song. J Neurophysiol 101: 3235–3245, 2009. First published April 8, 2009; doi:10.1152/jn.91089.2008. Behavioral variability is important for motor skill learning but continues to be present and actively regulated even in well-learned 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 adult 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 significantly reduced the variability of syllable structure but not of syllable sequencing. We also found that LMAN lesions eliminated the social modulation of the variability of syllable structure but did not detect significant effects on the modulation of sequence variability. These results show that LMAN contributes differentially to syllable versus sequence variability of adult song and suggest that these forms of 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 mo 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 (Brainard and Doupe 2002; Clayton 1987; Immelmann 1969; Tchernichovski et al. 2001). 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 (Brainard and Doupe 2004; Doya and Sejnowski 2000; Kao et al. 2005; Olˇveczky et al. 2005; Sutton and Barto 1998). 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 (Bottjer 2004; Hessler and Doupe 1999; Jarvis et al. 1998; Kao and Brainard 2006; Kao et al. 2005; Sakata et al. 2008; Sober et al. 2008; Teramitsu and White 2006; Tumer and Brainard 2007).

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 (FD) song] relative to when they sing in isolation [undirected (UD) song]; Kao and Brainard 2006; Sakata et al. 2008; Sossinka and Böhner 1980].

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; Olveczky et al. 2005; Scharff and Nottebohm 1991). 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; Nordeen and Nordeen 1993; Scharff and Nottebohm 1991). However, LMAN lesions do cause an abrupt reduction in the rendition-to-rendition variability of syllable structure (Kao and Brainard 2006; Kao et al. 2005) and prevent various forms of adult song plasticity (Brainard and Doupe 2000, 2001; Morrison and Nottebohm 1993; Scott et al. 2007; Thompson and Johnson 2006; Williams and Mehta 1999). LMAN lesions additionally eliminate the social modulation of variability in syllable structure (Kao and Brainard 2006; Kao et al. 2005), and variability in the activity of LMAN neurons correlates with variability of syllable structure (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. Although 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 inactiva-
tions of LMAN (Ölveczky et al. 2005; Scharff and Nottebohm 1991). In contrast, for adult zebra finches, variability in syllable sequencing has not been reported to decrease following LMAN lesions (Bottjer et al. 1984; Kao and Brainard 2006; Scharff and Nottebohm 1991). However, syllable sequencing in the adult zebra finch is highly stereotyped (Kao and Brainard 2006; Zevin et al. 2004), 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 studying 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 the role of LMAN in the control of song variability (Sakata et al. 2008). Moreover, one previous study reported that disruption of another part of this avian basal ganglia-forebrain circuit, by partial lesions of the basal ganglia homolog Area X, could transiently alter normal syllable sequencing (Kobayashi et al. 2001). This shows 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 the variability of adult Bengalese finch song and the regulation of variability by social context.

METHODOLOGIES

Animals

Adult Bengalese finch males (n = 21, age: 5–34 mo) were raised in our colony. Birds were kept with their parents until 60 days of age, after which they were housed in same-sex cages. Before experiments, males were housed individually in sound-attenuating chambers (Acoustic Systems, Austin, TX) maintained on a 14-h light: 10-h 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://oberlin.edu/observer/sound_analysis.html); Evtuf, E. Tumer, UCSF]. We collected songs produced when males were alone (UD song) and songs produced to females (FD song). UD song was collected by presenting one of a series of females in a separate cage for 1–2 min at intervals of 4–6 min. UD song was usually produced within 15 s of the introduction of the female. This procedure has been shown to elicit robust differences in both syllable and sequence variability between UD and FD song for Bengalese finches (Sakata et al. 2008). Most birds (20 of 21 birds) sang ≥10 FD songs within a single collection day; for one bird, we collected songs across 2 consecutive days. Within a recording session, UD song was interleaved with FD song and collected ≤30 min before the first exposure to a female and ≤30 min 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 ≥10 ms in duration, separated from other elements 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 on amplitude thresholds and manually labeled with unique letters (Fig. 1A).

To analyze changes to the variability of syllable structure, we first calculated the fundamental frequency (FF) of syllables that had distinct and stable harmonic structure (Fig. 2A). For each syllable, we calculated the autocorrelation of a segment of the sound waveform. The FF was defined as the distance, in hertz, 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 Cheveigneé and Kawahara 2002). We characterized the rendition-to-rendition variation in FF using the CV

$$CV = \left( \frac{\sigma}{\mu} \right) \times 100$$

where the sum is over all possible transitions, and p, is the probability of the ith transition across all songs (Gil and Slater 2000; Sakata and Brainard 2006; Tchernichovski et al. 2000). Completely stereotyped sequences have an entropy value of zero, and sequences with greater variability have higher entropy values. Branch points at 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. Such repeated syllables were present in the songs of eight of the lesion birds. First, we calculated the transition entropy of repeats. Repeats can be considered a different class of branch point transition at 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.

We also 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 sequence 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 before the first (nonin-
troductory) 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.

Thirteen birds received bilateral LMAN lesions. Of these 13, the effect of social context on song organization was assessed before and after LMAN lesions for eight birds. Social context effects were assessed only after the lesion for five birds (see RESULTS). For two of the lesion birds, songs before lesions were not available for analysis; consequently, the effect of LMAN lesions on the organization of UD song was analyzed for 11 birds.

LMAN lesions

Birds were anesthetized using equithesin or a combination of ketamine and midazolam supplemented with gaseous isoflurane. Stereotaxic coordinates from the posterior branch of the midsagittal sinus were used to locate LMAN (rostral: 5.1–5.5 mm; lateral: 1.2–1.8 mm; depth: 1.7–2.1 mm). As in previous studies (Bottjer et al. 1984; Brainard and Doupe 2000, 2001; Kao and Brainard 2006; Kittelberger and Mooney 1999; Scharff and Nottebohm 1991; Thompson and Johnson 2006; Thompson et al. 2007), we removed LMAN by performing electrolytic lesions. Electrolytic lesions not only destroy the cell bodies of neurons in focal brain areas but can also damage fibers that pass through the area. Consequently, it is possible that some of the effects observed following electrolytic lesions could be attributed to damage to the fibers of passage. However, no major fiber tracts are known to pass through LMAN, and pharmacological inactivations of LMAN cause song changes that are comparable to those observed with electrolytic lesions (Ölveczky et al. 2005), suggesting that observed effects are likely to derive from damage to LMAN rather than fibers of passage.

For LMAN lesions (n = 13 birds), we made six to eight electrode (30–100 kΩ) penetrations passing 50–100 μA current for 60–120 s. Silica gel was used to protect the brain during and after surgery. The craniotomy was sealed using bone wax, and skin was sealed 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 the robust nucleus of the arcopallium (RA). Intact birds (n = 6) underwent no surgery but had song collected at comparable time intervals. No differences were observed between sham and intact groups. Consequently, these eight birds were combined in our analysis and collectively referred to as control.

Histology and estimation of lesion size

On completion of behavioral experiments, birds were anesthetized with a lethal dose of isoflurane and perfused with paraformaldehyde...
or formalin. Sections were cut at 30–40 μm using a microtome, and every third section was stained for either Nissl or calcitonin gene-related peptide (CGRP). Neurons in LMAN project to RA in the vocal motor pathway (Fig. 1C), and CGRP is expressed in LMAN cell bodies and projections to RA, along with other regions (Fig. 1B) (Bottjer et al. 1997). Lesion size was estimated by at least two 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 bodies and projections to RA, along with other regions (Fig. 1C), and CGRP is expressed in LMAN cell bodies and projections to RA, along with other regions (Fig. 1B) (Bottjer et al. 1997). Lesion size was estimated by at least two 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 (Table 1). We found no correlation between the size of lesions and any of the reported effects (see Supplementary Table S1).1 For all birds included in this study, the medial magnocellular nucleus of the anterior nidopallium (MMAN), a CGRP-positive region medial to LMAN (Foster et al. 1997), remained intact.

### Statistical analysis

We first analyzed how lesions of LMAN affected the organization of UD song. For this set of analyses, we compared the measurements of UD songs before LMAN lesion with those 2 wk following lesion using paired t-test within each group. We also used t-tests to compare the percent change over time in UD songs for lesion versus control groups to determine whether changes with lesion were different from that expected across control conditions. Percent change was calculated using the following formula

\[
\text{percent change} = 100 \times \frac{\mu_2 - \mu_1}{\mu_1}
\]

where \(\mu_1\) and \(\mu_2\) refer to the sample means before lesion and after lesion, respectively, for the feature being examined.

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 (see above where \(\mu_1\) and \(\mu_2\) refer to the sample for UD and FD song, respectively, for the feature being examined) and used a repeated measures multivariate ANOVA (MANOVA) to assess how LMAN lesions influenced the degree of social modulation. In these analyses, group (lesion vs. control) was the main independent variable and percent change values at each time (pre vs. post) were the dependent variables. An effect of LMAN lesion on the social modulation of song would be manifest as a significant group \(\times\) 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.

---

**Table 1. Summary of birds**

<table>
<thead>
<tr>
<th>Bird Name</th>
<th>Group</th>
<th>Lesion (%)</th>
<th>Age, mos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bird1</td>
<td>Lesion</td>
<td>75</td>
<td>5</td>
</tr>
<tr>
<td>Bird2</td>
<td>Lesion</td>
<td>75</td>
<td>15</td>
</tr>
<tr>
<td>Bird3</td>
<td>Lesion</td>
<td>75</td>
<td>6</td>
</tr>
<tr>
<td>Bird4</td>
<td>Lesion</td>
<td>80</td>
<td>5</td>
</tr>
<tr>
<td>Bird5</td>
<td>Lesion</td>
<td>80</td>
<td>6</td>
</tr>
<tr>
<td>Bird6</td>
<td>Lesion</td>
<td>90</td>
<td>7</td>
</tr>
<tr>
<td>Bird7</td>
<td>Lesion</td>
<td>90</td>
<td>18</td>
</tr>
<tr>
<td>Bird8</td>
<td>Lesion</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>Bird9</td>
<td>Lesion</td>
<td>85</td>
<td>4</td>
</tr>
<tr>
<td>Bird10</td>
<td>Lesion</td>
<td>100</td>
<td>15</td>
</tr>
<tr>
<td>Bird11</td>
<td>Lesion</td>
<td>100</td>
<td>6</td>
</tr>
<tr>
<td>Bird12</td>
<td>Lesion</td>
<td>90</td>
<td>6</td>
</tr>
<tr>
<td>Bird13</td>
<td>Lesion</td>
<td>75</td>
<td>6</td>
</tr>
<tr>
<td>Bird14</td>
<td>Control</td>
<td>0—sham</td>
<td>7</td>
</tr>
<tr>
<td>Bird15</td>
<td>Control</td>
<td>0—sham</td>
<td>6</td>
</tr>
<tr>
<td>Bird16</td>
<td>Control</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Bird17</td>
<td>Control</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Bird18</td>
<td>Control</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Bird19</td>
<td>Control</td>
<td>0</td>
<td>34</td>
</tr>
<tr>
<td>Bird20</td>
<td>Control</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Bird21</td>
<td>Control</td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

Experimental group, size, and age are listed for each bird in this study.

---

1 The online version of this article contains supplementary data.
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

\[ \text{weighted percent change (per male)} = \frac{\sum_n \Delta_i \times (n/\Sigma n)}{n} \]

where \( \Delta_i \) 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 \( \Sigma n \) 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 (Supplementary Table S1). To depict all the data, we present only the results from the per example analyses.

For univariate tests (i.e., unpaired or paired t-test), we also conducted nonparametric tests (e.g., Wilcoxon rank sum or signed-rank tests) to assess the possible influences of assumption violations for parametric tests. Results were identical for parametric and nonparametric tests (see also Supplementary Table S1). 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 on the organization of song and on the social modulation of song. Bengalese Finch song consists of acoustically distinct syllables that are produced in learned sequences. In the adult Bengalese finch, both syllable production and syllable sequencing are variable. To assess the degree to which LMAN contributes to different aspects of song variability, we first consider the effect of LMAN lesions on UD 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: syllable structure

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 Fig. 1A. The song of this bird contained nine 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 gray bar), “c” (white bar), or “e” (light gray bar). Similarly, there were several syllable repeats where a given syllable could be repeated variable numbers of times before transitioning to the next syllable of song; syllable “b” is a syllable repeat for this bird.

It is apparent from the songs shown in Fig. 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 (\( n = 11 \)) and indicates that LMAN is not an obligatory part of the premotor 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 shown that such lesions can alter the variability with which song is produced (Bottjer et al. 1984; Kao and Brainard 2006; Scharff and Nottebohm 1991). 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: parameter

Because variability in syllable structure is strongly reduced by LMAN lesions in the adult zebra finch (Kao and Brainard 2006), we first investigated the contribution of LMAN to syllable structure in the adult Bengalese finch. We measured the FF of syllables with flat harmonic structure (Fig. 2A) and quantified the variation in the FF of individual syllables across renditions using the 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 the distributions of FF for multiple renditions of that syllable before (Pre) and after (Post Lesion) lesion of LMAN. For this syllable, the CV decreased significantly from 1.46 before the lesion to 1.01 2 wk following the lesion (31% decrease; 2-sample \( t \)-test for equal variances: \( P < 0.001 \)). Similar decreases in CV were consistently observed following lesions. Across 31 syllables analyzed in 11 birds with LMAN lesions, the CV decreased on average by 34.1 \( \pm 4.5\% \) (Fig 2B, filled symbols; paired \( t \)-test, \( P < 0.0001 \)). In contrast, there was no significant change in CV across 14 syllables in eight control birds (2.2 \( \pm 3.2\% \); Fig. 2B, open symbols). 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 FF. For both lesion and control groups, the mean 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 31 measured syllables in 11 lesion birds, there was no net change to the mean FF (Fig. 2C, filled symbols). 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 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.

In addition to the mean and variability of FF, we measured the effects of LMAN lesions on the mean and CV of other syllable features including the entropy of the spectral density, spectro-temporal entropy, entropy of loudness versus time, duration, and amplitude (Sakata and Brainard 2006). LMAN lesions did not significantly affect the mean or CV of any of these other features; (Supplementary Table S2). The lack of an effect of lesions on the amplitude of adult song is particularly noteworthy, because previous studies in juvenile zebra finches...
have noted a decrease in amplitude following lesions (Bottjer et al. 1984; Scharff and Nottebohm 1991; Sohrabji et al. 1990). This contrast is consistent with the idea that LMAN contributes a significant component of the premotor drive for song in juvenile birds but contributes only minimally to premotor drive in adults, at least under normal circumstances (Aronov et al. 2008).

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 contribution of LMAN to variability of syllable sequencing versus syllable structure.

Inspection of syllable transition diagrams before and after LMAN lesions showed 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 shown in Fig. 3A (same song as depicted in Fig. 1). In this example, all transitions are retained following the lesion; stereotyped sequences remained stereotyped and branch points continued to exhibit sequence variability. This was the case for all lesion birds. For example, in no case was a branch point eliminated following lesion.

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. First, 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 Fig. 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 28 branch points in 11 lesion birds, LMAN lesions did not cause a net change in branch point entropy (Fig. 3C).

![Figure 3](http://jn.physiology.org/)

**Fig. 3.** Effect of LMAN lesion on sequence variability for undirected song. **A:** transition diagrams before (Pre-lesion; left) and after (Post-lesion; 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 showing 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 28 branch points in 11 lesion birds before and after LMAN lesion (filled symbols) and for 23 branch points in 8 control birds at 2 time points (open symbols). Branch point entropy did not change in a consistent manner in lesion or control birds. **D:** repeat entropy values for 12 repeated syllables (repeats) in 8 lesion birds before and after LMAN lesion (filled symbols) and for 12 repeats in 6 control birds at 2 time points (open symbols). Repeat entropy did not change significantly over time for either lesion or control birds. **E:** CVs of repeat numbers for 12 repeats in 8 lesion birds before and after LMAN lesion (filled symbols) and for 12 repeats in 6 control birds at 2 time points (open symbols). The CV of repeat number did not change significantly over time for either lesion or control birds. Power analyses corroborated these findings (see Supplementary Table S3).
LMAN lesions also 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 12 repeats in eight lesion birds, we found no consistent change caused by LMAN lesions (Fig. 3D; see also Supplementary Table S3). Second, we analyzed the CV of repeat number across renditions and also found that it was not significantly affected by LMAN lesions (Fig. 3E).

These analyses showed that high levels of sequence variability in the adult Bengalese finch do not require an intact LMAN and indicated that the contribution of LMAN to sequence variability is minimal for adult Bengalese finch song.

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; Kao and Brainard 2006; Williams and Mehta 1999). Here, we found no systematic change in the tempo of Bengalese finch song over the 2 wk that songs were analyzed following lesions. While there were some individual cases in which the tempo of defined syllable sequences increased (e.g., Fig. 4A), the overall effect of LMAN lesions on song tempo was not significant (Fig. 4B).

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 FD song than 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 examine the role of LMAN in different aspects of song organization, we studied 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 LMAN lesions eliminated the social modulation of syllable variability but had comparatively little effect on the social modulation of syllable sequencing. As reported previously (Sakata et al. 2008), 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 this social modulation was affected by LMAN lesions, we compared the percent change from UD to FD song for each of these features before and after lesions (see Methods; Fig. 5). We found that LMAN lesions eliminated the social modulation of the CV of FF (Fig. 5A; before lesion: UD > FD by 31.8 ± 3.6%; after lesion: UD > FD by 0.6 ± 3.9%). The level of social modulation of the CV of FF 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 lesions did not significantly affect the magnitude of social modulation of other song features (MANOVA: group × time interaction: $P > 0.1$ for all; Fig. 5, B, D, and E). 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 social modulation across time was not different between control and lesion groups (Fig. 5C). However, we unexpectedly observed an attenuation of social modulation across recording sessions for both control and lesion birds (MANOVA, effect of time: $P = 0.0084$). Branch point entropy was the only song feature that showed such an attenuation of context-dependent modulation over time ($P > 0.1$ for all others: MANOVA, effect of time). This attenuation of social context effects, even in birds without LMAN lesions, might reflect habituation because of repeated exposures to females over the course of the experiment (Teramitsu and White 2006). Because there was not a significant modulation of branch point entropy for either the control or lesion group at the post time point, we could not determine from these data whether lesions altered the magnitude of social modulation of branch point entropy.

To further study the contribution of LMAN to the social modulation of branch point entropy, we compared the songs of...
birds with intact and lesioned LMAN during a first session with females (so that there would not be opportunity for any exposure-dependent habituation of social modulation). For this purpose, we lesioned LMAN in five additional birds and tested them for social modulation of branch point entropy for the first time 2 wks following lesions (12 branch point sequences in 5 birds). We compared the effects of social context in LMAN lesion birds with the effects observed in control birds during their first session with females (37 branch point sequences in 13 birds). The magnitude of the social modulation of the CV of FF did not change over time for control birds (P = 0.5462) but decreased significantly from the 1st to the 2nd timepoint 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 time 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), although an attenuation of social modulation of branch point entropy across testing sessions (MANOVA: effect of time; P = 0.0084) was present for both control (P = 0.0451) and lesion birds (P = 0.0675). 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 on the social modulation of song tempo.

FIG. 5. Effect of LMAN lesion on social modulation of song. A–E: social modulation is plotted for each song feature from pre- and post-time points for control and LMAN lesion birds. Gray boxes indicate means ± SE. For all 5 features, there was significant social modulation during the pre period, consistent with a 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 magnitude of the social modulation of the CV of FF did not change over time for control birds (P = 0.5462) but decreased significantly from the 1st to the 2nd timepoint 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 time 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), although an attenuation of social modulation of branch point entropy across testing sessions (MANOVA: effect of time; P = 0.0084) was present for both control (P = 0.0451) and lesion birds (P = 0.0675). 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 on the social modulation of song tempo.

As a second measure of sequence variability, we examined repeat entropy (see METHODS). For control birds, there was significant social modulation of repeat entropy, and this effect did not attenuate over time (Fig. 5D). Therefore we could effectively ask whether LMAN lesions caused a change in social modulation of repeat entropy between pre- and post-lesion time points. We found that LMAN lesions did not significantly affect the magnitude of social modulation of repeat entropy (MANOVA: group × time interaction; P > 0.1; Fig. 5D). Together, the data from analysis of branch point entropy and repeat entropy suggest that there is comparatively little effect of LMAN lesion on the social modulation of sequence variability relative to the social modulation of syllable variability.

DISCUSSION

Our results demonstrate a dissociation in the contribution of LMAN to the 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; Sakata and Brainard 2006; Woolley and Rubel 1997). 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 (FD song) relative to when they sing in isolation (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 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 (FF; Fig. 2) of UD song. In contrast, LMAN lesions did not affect the variability of syllable sequencing of UD song; lesions did not affect the sequence variability of branch points or repeats (Fig. 3). Finally, LMAN lesions eliminated the social modulation of variability in FF (Fig. 5A) but had no detectable effects on the social modulation of variability in syllable sequencing (Figs. 5, C and D, and 6). 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; Rhodes et al. 2004; Saint-Cyr 2003; Seger 2006). Studies in songbirds have focused on the AFP, the avian basal ganglia-forebrain loop of which LMAN is the output, as a primary source of song variability (Bottjer et al. 1984; Kao and Brainard 2006; Kao et al. 2005; Kobayashi et al. 2001; Ölveczky et al. 2005; Scharff and Nottebohm 1991). Our results suggest that other regions of the songbird basal ganglia circuitry and song system should be studied as potential sources of sequence variability for adult song (Foster and Bottjer 2001; Hosino and Okanoya 2000; Kobayashi et al. 2001).

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 lead to significant decreases in the variability of syllable sequencing (Bottjer et al. 1984; Ölveczky et al. 2005; Scharff and Nottebohm 1991). These studies suggest that neuroanatomical changes across development could lead to a change in the contribution of LMAN to sequence control (Aronov et al. 2008; Bottjer et al. 1984; Scharff and Nottebohm 1991). 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 showed 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 relative to UD 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 eliminated the speeding up of song from UD to FD song (Fig. 5). Nor did we find that LMAN lesions increased song tempo of UD song (Fig. 4). The lack of changes to UD song tempo following LMAN lesion contrasts with previous studies of LMAN function in the zebra finch (Brainard and Doupe 2000; Kao and Brainard 2006; Williams and Mehta 1999). Although 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 show that different song features are regulated by distinct neural circuits. However, this 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 (Bot-tjer et al. 1984; Scharff and Nottebohm 1991; Nordeen and Nordeen 1993). Such lesions prevent plasticity in some aspects of syllable sequencing within song motifs (Brainard and Doupe 2000, 2001; Morrison and Nottebohm 1993; Scott et al. 2007; Thompson and Johnson 2006; Thompson et al. 2007; Watanabe et al. 2006; Williams and Mehta 1999) but do not prevent plasticity in other aspects of sequencing, especially between song motifs (Horita et al. 2008). Hence, it remains of interest for future studies to test whether manipulations of LMAN activity in the adult Bengalese finch alter the capacity for plasticity of even those song features that are unaffected by LMAN lesions.

ACKNOWLEDGMENTS

We thank J. Wong and R. Mazumder for technical support, S. Woolley and E. Turner for critical reading of the manuscript, and E. Turner for assistance in data collection.

GRANTS

This work was supported by T32 MH20006 and an ARCS scholar award to C. M. Hampton, F32 MH068055-01 to J. T. Sakata, and the McKnight Foundation and NIH to M. S. Brainard.

REFERENCES


