Lesions of an avian basal ganglia circuit prevent context-dependent changes to song variability

Abbreviated title: LMAN lesions prevent changes to song variability

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Abstract

Trial-by-trial variability is important in feedback-based motor learning. Variation in motor output enables evaluation mechanisms to differentially reinforce patterns of motor activity that produce desired behaviors. Here, we investigate neural substrates of variability in the performance of adult birdsong, a complex, learned motor skill used for courtship. Song performance is more variable when male birds sing alone (‘undirected’) than when they sing to females (‘directed’). We test the role of the anterior forebrain pathway (AFP), an avian basal ganglia–forebrain circuit, in this socially driven modulation of song variability. We show that lesions of the lateral magnocellular nucleus of the anterior nidopallium (LMAN), the output nucleus of the AFP, cause a reduction in the moment-by-moment variability in syllable structure during undirected song to the level present during directed song. This elimination of song modulation is immediate and long lasting. We further show that the degree of syllable variability and its modulation are both attenuated in older birds, in concert with decreased variability of LMAN activity in these birds. In contrast to the requirement of LMAN for social modulation of syllable structure, we find that LMAN is not required for modulation of other features of song, such as the number of introductory elements and motif repetitions and the ordering of syllables, or for other motor and motivational aspects of courtship. Our findings suggest that a key function of avian basal ganglia circuitry is to regulate vocal performance and plasticity by specifically modulating moment-by-moment variability in the structure of individual song elements.

Keywords: birdsong, motor learning, language, motor exploration
Introduction

A hallmark of well-learned skills is that they exhibit a high degree of trial-by-trial reproducibility. Such stereotyped motor output is often considered an endpoint of motor learning, and residual variability in motor performance may be construed as biological 'noise' that either the nervous system is unable to eliminate or that is below threshold for behavioral importance. In the context of learning and ongoing calibration of motor performance, however, trial-by-trial motor variability plays a potentially critical role; variation in motor output enables 'motor exploration' such that evaluation mechanisms can differentially reinforce patterns of motor activity that produce more desired outcomes and weaken or 'punish' patterns of activity that produce worse outcomes. While such an adaptive function of variability is well established in models of reinforcement-based motor learning (Doya and Sejnowski 2000; Sutton and Barto 1998), whether and how the nervous system actively regulates variability in the context of motor practice and ongoing motor performance is little understood. Here, we investigate neural substrates of variability in the motor control of adult birdsong.

Song learning shares many features with other forms of vertebrate motor skill learning (Doupe and Kuhl 1999). Initially, during a sensory learning period, birds memorize the sound of an adult 'tutor' song. Subsequently, during a sensorimotor learning period, young birds begin to vocalize and use auditory feedback to gradually refine their vocalizations until they closely approximate the tutor song. Initial vocalizations are highly variable from one rendition to the next. However, as the bird's
song approaches the end state (a good match to the tutor song), it becomes increasingly stereotyped. In the zebra finch, this process is completed by approximately 90 days post-hatch (dph), at which point the song is said to be 'crystallized' (Immelmann 1969). Despite the apparent stability of adult zebra finch song, disruptions of feedback, such as deafening, lead to a gradual deterioration of performance, indicating that adult experience continues to contribute to maintenance of song (Leonardo and Konishi 1999; Nordeen and Nordeen 1992). Several studies have suggested that learning and maintenance of song may rely in part on the regulation of song variability for purposes of motor exploration (Doya and Sejnowski 2000; Jarvis et al. 1998; Kao et al. 2005; Ölveczky et al. 2005; Scharff and Nottebohm 1991).

Here, we investigate the role of the anterior forebrain pathway (AFP), an avian basal ganglia–forebrain circuit (Perkel 2004), in the regulation of song variability. The AFP is an accessory loop that is interconnected with the motor structures for song production (Fig. 1A) and, hence, is well positioned to influence song. Previous lesion studies have demonstrated that the AFP is critical for song learning and feedback-based vocal plasticity and have suggested a possible role for this pathway in modulating song variability (Bottjer et al. 1984; Brainard and Doupe 2000, 2001; Kao et al. 2005; Ölveczky et al. 2005; Scharff and Nottebohm 1991; Williams and Mehta 1999). In juvenile zebra finches, lesions or inactivation of LMAN (lateral magnocellular nucleus of the anterior nidopallium), the output nucleus of the AFP, disrupt song development and induce premature stereotypy, resulting in highly repetitive, simplified songs (Bottjer et al. 1984; Ölveczky et al. 2005; Scharff and Nottebohm 1991). In adult zebra finches, lesions of LMAN have little overt effect on previously learned song, but they prevent song
plasticity induced by experimental manipulation of auditory and/or proprioceptive feedback (Bottjer et al. 1984; Brainard and Doupe 2000, 2001; Nordeen and Nordeen 1993; Scharff and Nottebohm 1991; Williams and Mehta 1999). Consistent with a role in regulating song plasticity, LMAN neurons exhibit robust singing-related activity that is correlated with song structure (Hessler and Doupe 1999a, b). Moreover, manipulation of neural activity in LMAN during singing can alter the spectral structure of individual song elements, or ‘syllables’, on a moment-by-moment basis (Kao et al. 2005). These findings suggest that a key function of the AFP in regulating vocal plasticity may be to modulate ongoing patterns of activity and synaptic connections in the motor pathway (Jarvis et al. 1998; Kao et al. 2005; Kittelberger and Mooney 1999; Scharff and Nottebohm 1991). Such a modulatory influence could serve to introduce variability in the motor pathway and subsequent song, which is critical in reinforcement learning (Brainard 2004; Doya and Sejnowski 2000; Kao et al. 2005; Ölveczky et al. 2005; Sutton and Barto 1998).

To examine the hypothesis that LMAN actively introduces variability into patterns of activity in the motor pathway, we focused on naturally occurring, context-dependent regulation of variability in adult song structure. Song serves as a courtship signal in zebra finches, and previous studies have shown that a variety of aspects of song structure, including its variability, are sensitive to social context (Kao et al. 2005; Sossinka and Böhner 1980). When a male zebra finch sings alone (‘undirected’ song), variability in syllable structure is consistently greater than when he sings to a female (‘directed’ song; Kao et al. 2005; Ölveczky et al. 2005). Moreover, these context-dependent changes in song structure are associated with context-dependent changes in neural activity in LMAN. Singing-related activity in LMAN is greater in magnitude and
more variable in pattern across repeated renditions during undirected song than during
directed song (Hessler and Doupe 1999b; Jarvis et al. 1998; Kao et al. 2005). These
findings suggest that greater variability in LMAN activity during undirected singing may
actively generate variability in ongoing activity in the song motor pathway and
subsequent song output (Fig. 1B). Alternatively, or in addition, stereotyped patterns of
LMAN activity during directed song may facilitate reproducibility in motor pathway
activity and song.

To examine how LMAN modulates song variability, we compared songs
produced in the two social contexts before and after lesions. We previously reported that
lesions of LMAN eliminate context-dependent differences in the variability of syllable
structure (Kao et al. 2005). Here, we further investigate the nature of the effects of
LMAN lesions. If LMAN actively generates variability in undirected song, lesions
should reduce or eliminate variability in undirected song (Figs. 1B–C). Alternatively, if
LMAN actively reduces variability during directed song, then lesions should cause an
increase in variability in directed song towards the level present in undirected song. In
addition, to test the specificity and nature of the role of LMAN in modulating variability,
we measured the influence of LMAN lesions on other features of song and behavior that
are known to differ with social context. In particular, we asked whether lesions
specifically eliminate the social modulation of individual syllables or whether they more
generally eliminate modulation of the entire suite of behaviors that differentiate directed
and undirected song (Sossinka and Böhner 1980). Finally, to further test the relationship
between LMAN activity and song variability, we measured these variables in older adult
birds whose songs are less plastic than those of young adults (Brainard and Doupe 2001;
Lombardino and Nottebohm 2000). Our findings support a role for LMAN in actively introducing variability to specific aspects of song structure during undirected song.

**Materials and Methods**

**Subjects**

Thirty male zebra finches (*Taeniopygia guttata*) were used for experiments. All birds were raised in individual breeding cages with their parents and siblings until at least 60 dph. Birds were selected on the basis of their size, singing frequency, and song complexity, and were then housed individually in sound-attenuating chambers (Acoustic Systems, Austin, TX). All procedures were performed in accordance with protocols approved by the UCSF Institutional Animal Care and Use Committee.

**Surgical procedures**

Before all surgical procedures, birds were deprived of food and water for 1 hr and then anesthetized with an intramuscular injection of Equithesin (0.85 g chloral hydrate, 0.21 g pentobarbital, 0.42 g MgSO₄, 2.2 ml 100% ethanol, and 8.6 ml propylene glycol to a total volume of 20 ml with water). After surgery, all skin incisions were sealed with a cyanoacrylate adhesive.

**Lesions.** Bilateral electrolytic lesions were stereotaxically targeted at the lateral magnocellular nucleus of the anterior nidopallium (LMAN), with 5 penetrations per side and one or two current injections per penetration (50 or 100 µA for 60 s). The amount of LMAN that was removed bilaterally ranged from 40% to 100% (median lesion size:
80%; see Table 1; \( n = 5 \) birds), as confirmed by histology at the end of experiments (see below). “Sham” lesions were entirely dorsal to LMAN (Table 1; \( n = 2 \) birds). In all birds, the medial magnocellular nucleus of the anterior nidopallium (MMAN) remained intact. Lesions were made in birds between 101 and 123 dph (\( n = 7 \) birds). We did not find any correlation between the extent of the lesions and their effects on song output using a variety of measures, including the average degree of modulation of fundamental frequency by social context (see below) following lesions of LMAN (\( p = 0.69 \)) and the average degree of modulation of song tempo by social context (see below) following lesions of LMAN (\( p = 0.49 \)). Moreover, the reported effects of LMAN lesions on the song of the bird with the smallest lesion ranked third overall (median) for changes in the variability of fundamental frequency (70% lesion on the left; 10% lesion on the right as well as a visible reduction in CGRP labeling in the lateral RA on the left and dorsal RA on the right). Hence, we pooled all lesion birds in the subsequent analyses. Restriction of the data set to the four birds with the largest lesions (mean lesion size of 88%) did not change the significance of any of the reported results.

**Chronic implants.** Electrodes were implanted chronically as described previously (Hessler and Doupe 1999a). Briefly, a lightweight microdrive (UCSF and Caltech Machine Shops) carrying two tungsten electrodes (2-5 MOhm) insulated with epoxylite (FHC, Bowdoinham, ME) or glass (A. Ainsworth, Northhamptonshire, UK; Merrill and Ainsworth 1972) was positioned stereotaxically such that the electrode tips were \( \sim 700 \) \( \mu \)m above LMAN. A reference ground electrode ( uninsulated tungsten electrode; A-M Systems, Carlsborg, WA) was implanted such that its tip was located within \( \sim 2 \) mm of the targeted LMAN. The microdrive and connector socket (FHC) were secured to the
skull with epoxy (Devcon, Wood Dale, IL) and dental cement (Dentsply, Milford, DE),
and a protective cap was fixed around the microdrive. All electrodes were implanted in
the right hemisphere.

Anatomy

After the final recordings, birds were deeply anesthetized with Metofane
(Schering-Plough, Union, NJ) and transcardially perfused with 0.9% saline, followed by
3.7% formaldehyde in 0.025M phosphate buffer. Brains were post-fixed for 4 hours,
cryoprotected, and cut coronally in 40 µm sections with a freezing microtome. Every
third section was stained with cresyl violet acetate, and a second set of intervening
sections was reacted with an antibody to calcitonin gene-related peptide (Fig. 2B; CGRP;
Sigma, St. Louis, MO; Bottjer et al. 1997; Brainard and Doupe, 2001).

Sound recording

Acoustic signals were recorded by a microphone located above the birdcage and
filtered between 200 and 9000 Hz (Krohn-Hite, Avon, MA). Custom-written acquisition
software (C. Malek and A. Leonardo, Caltech, and C. Roddey, UCSF) recorded the
acoustic signals before and after the sound amplitude crossed a threshold level. The
threshold level was determined experimentally to ensure detection of all vocalizations
(calls and songs) and to minimize detection of other sounds, such as the bird flapping its
wings. Each bird’s behavior was monitored and recorded by a video camera.

For all experiments, undirected song was recorded when the male was isolated in
a sound-attenuating chamber. The amount of undirected song was often greater when the
experimental bird could hear the calls of other birds outside the recording chamber. To elicit directed song, one or more female zebra finches was presented in a separate cage to the male zebra finch being recorded. The recorded bird usually moved to the edge of its cage and sang while facing the female(s). Each female presentation lasted for \( \leq 2 \) minutes, regardless of whether or not the male sang, and songs were classified as ‘directed’ only when the male bird faced the female(s). Bouts of directed song were interleaved with undirected song during each recording. Recordings lasted between 30 minutes and several hours depending on the amount of singing.

For experiments examining the effects of lesions of LMAN on context-dependent differences in song, interleaved bouts of directed and undirected song were recorded as illustrated in Fig. 2A. Recordings were made in each bird once a week for 2–3 weeks preceding surgery, and then again on the day before bilateral lesions, and after lesions as follows: Birds were recorded every day following lesions until enough songs were collected in both social conditions to characterize initial effects of lesions (1–3 d) and then once a week for four weeks following the lesions. A final recording was made during the eighth week following lesions (\( n = 5 \) birds with LMAN lesions; \( n = 2 \) birds with sham lesions). Three additional age-matched control birds were also recorded weekly. The range of ages of the birds at the first recording was 88–102 dph. Data from a subset of these recordings have been presented in a brief report (Kao et al. 2005).

To investigate the effect of age on context-dependent differences in song structure, we recorded songs from an additional 6 birds that were greater than 4 years old and 11 birds less than 6 months old.
Physiological recording

In a separate group of birds (n = 7), we recorded neural activity at multiple sites in LMAN (1–6 sites per bird) in directed and undirected conditions. During each recording, a flexible lead terminating in a small operational amplifier circuit was connected to the socket on the bird’s head, and the other end was connected to a commutator (Caltech Machine Shop). Electrodes were positioned at sites in LMAN where action potentials of single and multiple neurons could clearly be differentiated from the background neural activity (spike amplitudes ranged from 300 µV to > 1 mV, peak to peak; Hessler and Doupe 1999a). The neural activity signal passed through the commutator to a differential amplifier (A-M Systems) and was filtered between 300Hz and 10kHz. The acoustic signal was recorded as described above. Custom-written software (A. Leonardo, Caltech, and C. Roddey, UCSF) recorded the acoustic and neural signals, and the bird’s behavior was monitored and recorded by a video camera.

Simultaneous song and neural recordings were made at intervals of 1d to several weeks, over a period of weeks to months. Neural activity was recorded during non-singing and singing periods in both undirected and directed conditions. After completion of recordings in each bird, small electrolytic lesions (30 µA for 10 s) were made at previously recorded sites. Locations of recording sites were confirmed in 40 µm Nissl-stained brain sections by their positions relative to the depth of the marker lesions.

Behavioral analysis.

Song structure. Zebra finch song can be classified into three levels of organization: ‘syllables’, which are individual song elements separated by silent intervals
at least 5 ms in duration; ‘motifs’, which are stereotyped sequences of syllables; and ‘bouts’, which are defined as periods of singing separated by silent intervals at least 2 s in duration (Sossinka and Böhner, 1980). Song bouts usually consist of a series of introductory elements followed by a variable number of repetitions of the same motif. Song bouts may either be aimed at another bird (‘directed’) or sung when the male is alone or not orienting towards any other bird in particular (‘undirected’, Dunn and Zann 1996; Sossinka and Böhner 1980).

**Analysis of syllable structure.** To characterize differences in the structure of individual syllables, we measured the fundamental frequency (FF) of syllables that have constant frequency components (Kao et al. 2005). For a particular syllable, we calculated the autocorrelation of a segment of the sound waveform that had clear harmonic structure (median segment used to calculate FF: ~50% of the total syllable duration; range = 30–85%). The FF was defined as the distance, in frequency, between the zero-offset peak and the highest peak in the autocorrelation function (ACF) within a range of time lags corresponding to fundamental frequencies typical of zebra finch syllables. To improve the resolution of the frequency estimates, we performed a parabolic interpolation of the peak of the ACF (de Cheveigné and Kawahara 2002). This algorithm was applied to syllables that had clear harmonic structure with a well-defined FF. To examine differences between directed and undirected songs, the FF was calculated for a minimum of 16 renditions (range: 16–90 renditions) in each behavioral context.

**Analysis of other song features.** We analyzed four other features of song that previously have been reported to differ across social context (Sossinka and Böhner 1980):
number of introductory elements, number of motifs per bout, the stereotypy of syllable ordering, and song tempo.

To count the number of introductory elements per song bout, we started with the introductory element preceding the first syllable in the song motif and counted backwards until there was at least 500 ms of silence or a different type of vocalization, such as a loud distance call. Using this method, we found consistent differences in the number of introductory elements between directed and undirected song bouts (see Results). For the number of motifs per bout, we counted all motifs in which at least the first half of the motif was sung because zebra finches often truncate the last motif in a song bout (e.g., if the canonical motif was ‘abcdef’, all motifs that included at least ‘abc’ were counted).

The stereotypy of syllable ordering was quantified by using measures of ‘sequence linearity’ and ‘sequence consistency’, similar to those originally described by Scharff and Nottebohm (1991). We followed the practice of recent studies that have examined stereotypy of syllable sequencing and excluded introductory elements from these measures (Foster and Bottjer 2001; Ölveczky et al. 2005; Zevin et al. 2004).

‘Sequence linearity’ quantifies the different possible transitions that can be observed following each unique syllable of song. In this study, we used the ‘internal linearity’ measure of Foster and Bottjer (2001), which excludes variability in the syllable that precedes song terminations. Internal linearity is defined as:

\[
\text{Linearity} = \frac{\text{(number of different syllables)} - 1}{\text{number of syllable-to-syllable transitions}}
\]

For example, if a bird always sings the motif ‘abcde’, there are five unique syllables and four unique transitions, and the internal linearity yields a value of 1.00. Any variation in the sequencing of syllables within the motif will yield a value less than 1.00. However,
songs in which motifs are terminated following more than one syllable still receive a score of 1.00 (i.e., occurrences of truncated motifs ‘abc’ and ‘abcd’ would not reduce the internal linearity score). Because internal linearity excludes variability in song terminations, it always yields a value that is equal to or higher than the sequence linearity score calculated by Scharff and Nottebohm (1991).

To explicitly characterize any context-dependent differences in premature song terminations, we calculated the percentage of syllable types within a motif that were followed by song terminations. For example, if a bird’s typical motif was ‘abcde’, but the truncated versions ‘abc’ and ‘abcd’ were also observed, then the percent of syllables that terminate song would be 60 (3/5).

To measure the frequency with which the main motif sequence occurred, we used the ‘sequence consistency’ score of Scharff and Nottebohm (1991), excluding variability in introductory elements. Sequence consistency measures the proportion of syllable transitions that conform with the most frequent, or dominant, transition for a given syllable and is defined as:

\[
\text{Consistency} = \frac{\sum \text{dominant transitions/song}}{\sum \text{all transitions/song}}
\]

Complete consistency yields a score of 1.00. Because transitions to the end of a song bout are included in this measure, it is influenced by variability at the end of songs.

Since these measures of sequence stereotypy are sensitive to the number of songs that are analyzed, we calculated linearity, consistency, and percent syllables terminating song from randomly selected subsets of recorded songs that were matched to have equal numbers of motif initiations in the directed and undirected conditions (mean and range of motifs per bird: 67; 36–125).
To characterize any context-dependent differences in song tempo, or the rate at which song is delivered, we measured for each bird the durations of complete motifs during directed and undirected songs. For each recording, we computed the mean motif duration for both behavioral conditions.

**Analysis of courtship behavior.** Directed song serves as a courtship signal and is often accompanied by a rhythmic, pivoting dance that includes orienting towards and approaching the female, hopping to and fro, head-tail twists and bows, beak wipes, and a characteristic posture (reviewed in Zann 1996). To characterize these motor aspects of courtship, we scored video clips of directed and undirected singing. Video clips of directed and undirected song bouts (~30–60 s) recorded 1 d pre-lesions and 7 d post-lesions were interleaved, and the clips were organized in blocks by bird because the exact form of the courtship dance varies across males. Two observers highly familiar with zebra finch behavior and blind to the experimental manipulation of each bird (lesions of LMAN or sham lesions) judged the degree of arousal and the vigor of the courtship display of each male using a scale of 0 (relatively inactive and no dance movements) to 3 (highly aroused courtship display). The observers were instructed to note the following features of the courtship dance: the male’s posture, orienting towards the female, approaching the female, hopping to and fro, and beak wiping. During undirected singing, when no female was present, scores could be > 0 if the male sang in the same position or oriented in the same direction as it did during directed singing. Scores were averaged across song bouts in a particular social context on each day to derive a mean behavioral score. Scores were then averaged across the observers.
Analysis of neural signals

Analysis of singing-related activity in LMAN was performed as described previously (Hessler and Doupe 1999a, b). Briefly, rectified, smoothed neural activity waveforms were aligned using a template for the amplitude envelope of each bird’s motif. Both the mean activity level and the coefficient of variation of activity across motif renditions were calculated.

Results

Previous studies have characterized several differences in song depending upon the social context in which it is produced (Kao et al. 2005; Ölveczky et al. 2005; Sossinka and Böhner 1980). Sossinka and Böhner (1980) reported that when a male sings to a female (‘directed’ song), song is preceded by more introductory elements per bout, includes more repetitions of a stereotyped sequence of syllables, or ‘motif’, per bout, is more stereotyped in the sequencing of syllables, and is delivered at a faster tempo than when the male sings alone or to no particular audience (‘undirected’ song; Figs. 3A, 8A). In addition, stereotypy in the structure of individual song elements is greater during directed song than during undirected song (Fig. 3B; Kao et al. 2005). Finally, directed song is often accompanied by changes in the orientation, posture, and position of the male, which collectively constitute a courtship dance (Williams 2001; Zann 1996). In the following sections, we describe the contribution of LMAN to each of these context-dependent changes to song and behavior.
Context-dependent differences in variability of syllable structure require LMAN

To assess the contribution of LMAN to ongoing motor performance and song plasticity, we first characterized the spectral structure of individual syllables during directed and undirected song. We focused our analysis on the fundamental frequency of syllables that contain constant frequency components with clear harmonic structure. Fundamental frequency (FF) is a learned parameter of song that is precisely controlled (Tchernichovski et al. 2001) and robust to differences across recording conditions. In addition, microstimulation in LMAN during singing can induce changes in FF on a moment-by-moment basis, supporting the idea that neural activity in LMAN modulates ongoing performance of this vocal feature (Kao et al. 2005).

As previously reported (Kao et al. 2005; Ölveczky et al. 2005), we found that in normal adult zebra finches, there was a striking modulation in the variability of FF by social context. Figure 3B illustrates the variability of FF for two syllables (‘a' and 'c’) produced by an adult male in interleaved bouts of directed and undirected song. As shown here, the distribution of FF for an individual syllable during undirected song was typically broader than the distribution of FF for the same syllable produced during directed song.

In control and sham lesioned birds, context-dependent differences in the variability of syllable structure were extremely robust. Each point in Figure 4A illustrates the variability in FF expressed as the coefficient of variation (c.v.) for a single syllable produced in interleaved recordings of directed and undirected song. Data are
shown for all measured syllables in all recording sessions from two control and two sham lesioned birds. The diagonal line indicates equal variability under the two social conditions. For all groups of birds, the data points are distributed significantly above the diagonal, indicating greater variability during the undirected condition (control: 2–6 syllables per bird; 9 recording sessions; \( n = 72 \); pre-sham lesion: 2–4 syllables per bird; 3 recording sessions; \( n = 18 \); and post-sham lesion: 2–4 syllables per bird; 6 recording sessions; \( n = 34 \); \( p < 0.0001 \) for all cases, paired sign test).

Lesions of LMAN completely eliminated context-dependent differences in the variability of FF that were present in normal adults. Figure 4B shows variability in FF before and after bilateral lesions of LMAN for all measured syllables and recording sessions from five birds. Before lesions (open symbols) there was a robust modulation of syllable variability by social context; data points lie above the diagonal line (2–6 syllables per bird; 3 recording sessions; \( n = 57 \); \( p < 0.0001 \), paired sign test), indicating greater variability during undirected song. Following lesions (filled symbols, 2–6 syllables per bird; 6 recording sessions; \( n = 111 \)), the data points were distributed along the diagonal, and there was no longer a significant modulation of variability by social context.

The elimination of context-dependent modulation of syllable variability by lesions of LMAN was rapid and long lasting. Figure 5 illustrates the variability of FF at each experimental time point for both undirected (open bars) and directed conditions (shaded bars). For control and sham lesioned birds, there were significant context-dependent differences in song variability for all experimental time points (Fig. 5A). In contrast, context-dependent differences in the variability of syllable structure were absent in the
first recordings post-lesions (1–3 d post) and remained absent for as long as birds were followed (up to 8 weeks; Fig. 5B).

The loss of context-dependent differences in variability could be attributed primarily to a reduction in the variability of syllables produced during undirected song to the level present during directed song. This is illustrated in Fig. 4B by the predominantly downward shift in the population of syllables to the diagonal line (open and filled 'X' indicates population means for pre- and post-lesion recordings, respectively). Indeed, lesions of LMAN caused a significant decrease in the variability of undirected songs (Fig. 5B, \( p < .005 \), paired sign test for 1 d pre-lesions versus first recording post-lesions) but had no effect on the variability of directed songs (Fig. 5B; \( p > 0.05 \), paired sign test). Moreover, the residual variability present in the undirected songs in the first recording post-lesions was not significantly different from the variability present in directed songs 1 d pre-lesions (Fig. 5B, \( p > 0.05 \), paired sign test). These data are thus consistent with a model in which greater variability in syllable structure during undirected song is actively introduced by LMAN (Figs. 1B–C).

Lesions of LMAN, however, did not abolish all variability in syllable structure. We did not observe a decrease in the minimum level of variability in FF (present during directed song) following lesions (Figs. 4B and 5B). This suggests that variability in FF during directed singing, when the patterns of activity in LMAN are stereotyped across repeated renditions of the same motif, represents a residual level of variability in syllable structure in adult birds. This residual variability may reflect motor constraints in the periphery, such as limits in the efficacy of the vocal musculature. Alternatively, other
sources of variability may exist elsewhere in the brain, such as variability intrinsic to the motor structures for song production.

**Courtship behavior is not affected by lesions of LMAN**

The effect of lesions of LMAN on song variability raises the question of whether or not other behaviors of males are grossly affected by lesions. Are the context-dependent differences in syllable variability eliminated by lesions of LMAN simply because the males no longer court females? To examine this issue directly, we scored video clips of directed and undirected bouts of song recorded 1 d pre-lesions and ~7 d post-lesions. In contrast to undirected song, directed song is often accompanied by a rhythmic dance that includes approaching the female, pivoting the body from side to side, and changing the head position (e.g., bowing and beak wiping), partly to display to the female the physical traits that are specifically male (reviewed in Williams 2001; Zann 1996).

To assess the courtship behavior of males, human observers who were familiar with zebra finch behavior but blind to the experimental manipulation were asked to judge the degree of arousal and the vigor of the courtship display of each male using a scale of 0 (relatively inactive and no dance movements) to 3 (highly aroused courtship display). The scores were averaged across song bouts in each social context before and after lesions to assess the degree to which each male’s courtship behavior had changed. Fig. 6 illustrates the difference in the behavior of male zebra finches between social contexts. One day prior to lesions of LMAN, all birds exhibited robust courtship displays when a
female was present but were less active when they were alone (Fig. 6 left; n = 5 birds pre-lesions of LMAN and 2 birds pre-sham lesions). One week after lesions of LMAN, the males continued to vigorously court females (Fig. 6, right), and their behavior was similar to that of birds with sham lesions. Thus, the elimination of context-dependent differences in song variability by lesions of LMAN cannot be attributed simply to a reduction in the motivation of males to court females. Moreover, these results suggest that the modulation of the courtship display by social context is mediated by structures other than the AFP.

**Other song features modulated by social context and their dependence on LMAN**

This study (Figs. 4–5) and other recent work (Kao et al. 2005; Ölveczky et al. 2005) have demonstrated a robust modulation of the variability of syllable structure by social context. In addition, a previous study by Sossinka and Böhner (1980) examined whether other features of song structure are modulated by social context. They reported context-dependent differences in four parameters of song: 1) the number of introductory elements per bout was consistently greater in directed versus undirected song (7/7 birds); 2) the number of motifs per bout was consistently greater in directed versus undirected songs (6/7 birds); 3) the regularity of the sequence of syllables within a motif appeared to be greater during directed songs than during undirected songs (2/3 birds); and 4) directed songs were usually produced at a faster tempo than undirected songs (6/8 birds). It has been suggested that such differences in song may derive from the differential activation
of neurons in the AFP across social context (Jarvis et al. 1998). We therefore examined each of these song features to determine the degree to which it is modulated by social context and whether this modulation is affected by lesions of LMAN.

(Number of introductory elements. Consistent with Sossinka and Böhner (1980), we found a robust modulation of introductory elements by social context. Figure 7B illustrates the social modulation of the number of introductory elements in interleaved bouts of directed and undirected song produced by one adult male prior to lesions of LMAN. In this case, the average number of introductory elements preceding song was approximately twice as large during directed versus undirected song. In all birds, prior to lesions of LMAN, the number of introductory elements was significantly greater during directed song than during undirected song (Fig. 7D, left; \( p < 0.0005 \), Mann–Whitney \( U \) test for comparisons within one recording session for each bird).

Lesions of LMAN did not affect the modulation of introductory elements by social context. For each of the birds whose song was characterized prior to lesions, we counted introductory elements in songs produced one week following lesions of LMAN (Fig. 7D, right). We found that the number of introductory elements continued to be significantly greater during directed songs than during undirected songs for all birds (\( p < 0.0005 \); \( M–W U \) test), indicating that signals from LMAN are not required for social modulation of this song feature.

(Number of motifs. Figure 7C illustrates the social modulation of the number of motifs in interleaved bouts of directed and undirected song produced by one adult male prior to lesions of LMAN, and Figure 7E summarizes data from each of the birds. Our results were again consistent with those of Sossinka and Böhner (1980). We found a
consistent increase in the number of motifs per bout in directed song versus undirected song. This difference was present in all birds and achieved significance for 4/5 birds (Fig. 7E, left; \( p < 0.005 \), M–W U test for comparisons within one recording session for each bird).

Lesions of LMAN did not affect the social modulation of numbers of motifs per bout. One week after lesions, directed songs continued to have more motifs per bout than undirected songs in 4/5 birds (Fig. 7E, right), indicating that signals from LMAN also are not required for social modulation of this higher-level song feature.

**Stereotypy of syllable sequencing.** Sossinka and Böhner (1980) also reported a tendency for the sequence of syllables within a motif to be more stereotyped during directed song than during undirected song. This phenomenon was less robust than the other socially driven changes to song (observed in 2/3 birds), and the study did not provide any quantitative analysis of the variability of syllable sequence. From the example shown in that study (Fig. 5, Sossinka and Böhner, 1980) and the corresponding description, it is apparent that the principle difference observed in the stereotypy of syllable sequencing was greater variability in the transitions that could occur following a given syllable during undirected song than for the same syllable during directed song.

In order to quantify the stereotypy of syllable sequencing, we used measures of ‘sequence linearity’ and ‘sequence consistency’ (see Materials and Methods), which have been employed successfully for this purpose in previous studies of zebra finch song (Foster and Bottjer 2001; Ölveczky et al. 2005; Scharff and Nottebohm 1991; Zevin et al. 2004). Sequence linearity quantifies the number of types of transitions that can follow a given syllable but is insensitive to how frequently these different possible transitions
occur. For this measure, we followed recent studies and included only transitions between two syllables of the bird's motif and did not include transitions from syllables to song terminations (see Materials and Methods and see Foster and Bottjer 2001; Ölveczky et al. 2005; Scharff and Nottebohm 1991; Zevin et al. 2004). Sequence consistency quantifies how often the observed transitions from a given syllable conform to the most common transition for that syllable. Both of these measures should be sensitive to the types of changes in syllable sequencing reported by Sossinka and Böhner (1980). Indeed, for the example bird illustrated in that study, this measure of sequence linearity increases from 0.64 during undirected song to 0.82 during directed song.

We calculated these two measures for interleaved directed and undirected songs from control birds and from experimental birds prior to lesions (Table 2, n = 10). In contrast to the report of Sossinka and Böhner (1980), we found that for both measures, there was no difference in the stereotypy of syllable sequencing between directed and undirected conditions. Mean linearity scores were 1.00 ± 0.00 (s.d.) for directed songs and 1.00 ± 0.00 (s.d.) for undirected songs from the same birds (Table 2), and mean consistency scores were 0.91 and 0.92, respectively (Table 2; p = 0.73; paired sign test). The difference between our findings and those of Sossinka and Böhner is likely to reflect a difference in the degree of stereotypy in the undirected songs of birds in the two studies. In our study, the undirected songs of adult zebra finches were very stereotyped, with high linearity and consistency scores similar to those of other recent studies. For example, Zevin, et. al. (2004) reported a mean linearity score of 0.98 and a mean consistency score of 0.96 for the undirected songs of 27 adult zebra finches. This high level of sequence stereotypy presents little opportunity for songs to become even more
stereotyped in the directed condition. In contrast, for the one bird for which data is available in the study by Sossinka and Böhner (1980), the linearity score of undirected song can be calculated as 0.64 (insufficient data is available to calculate sequence consistency). This value is lower than that for any of the adult zebra finches to which this measure was applied in our study ($n = 10$) or in the study by Zevin et al. (2004, $n = 27$). Because the birds from the Sossinka and Böhner study were a combination of wild caught and domesticated birds of unknown ages, we speculate that the greater variability in the sequence of undirected songs in their study reflects either age or strain differences. With respect to the former possibility, it will likely be informative to test the social modulation of song stereotypy in a population of juvenile birds prior to song crystallization.

Because we found no evidence for the social modulation of sequence stereotypy, we did not have a strong expectation that lesions of LMAN would affect this parameter of song. However, in juvenile birds, lesions and inactivation of LMAN have been reported to increase the stereotypy of syllable sequencing (Bottjer et al. 1984; Ölveczky et al. 2005; Scharff and Nottebohm 1991). Hence, we measured sequence linearity and consistency for directed and undirected songs from birds one week post-lesion and compared these values to those from the same birds pre-lesions (Table 2). We found no influence of LMAN lesions on these measures for either directed or undirected songs (linearity: $p > 0.99$, one sample sign test; consistency: $p > 0.99$, paired sign test). Our finding that LMAN lesions do not increase sequence stereotypy in adult birds may again result from a 'ceiling effect': the pre-lesion songs in our study were already sufficiently stereotyped as to make it difficult to detect any further increases.
Although song linearity and consistency did not differ significantly across social context, we did notice a difference between these conditions in the stereotypy with which birds terminated their songs. In particular, there were often more types of song terminations in directed songs than in undirected songs. We quantified this by calculating the percentage of syllables within a motif that were observed to precede song terminations. Across the population of normal adult birds, there were significantly more types of song terminations in directed versus undirected songs (Table 2; 9/10 birds, $p = 0.02$, paired sign test). This difference, which reflects greater variability in directed versus undirected songs, may result from the less predictable, and potentially disruptive, auditory and visual stimulation from the female bird that is present in the directed context. Indeed, it has been reported previously that unexpected auditory and visual stimuli can elicit premature song terminations Cynx 1990; Cynx and Von Rad 2001). Regardless of the source, we found that this difference between directed and undirected song persisted in all birds following lesions of LMAN (Table 2).

**Song tempo.** To characterize context-dependent changes to song tempo, we measured for each bird the durations of complete motifs produced in directed and undirected songs. Figure 8A illustrates data from one bird for which there was a robust modulation of song tempo by social context. In this case, song was 2.6% slower in the undirected condition than in the directed condition ($p < 0.0001$; M–W $U$ test). The degree of tempo modulation by social context varied across birds and across recording sessions for individuals. Across all control recordings of interleaved directed and undirected songs (3 recording sessions each for 5 birds pre-lesions, 2 birds pre-sham lesions, and 3 control birds), song was faster in the directed context for 24/30 recording
sessions. This difference was significant for 20 of these sessions \((p < 0.05; \text{M–W } U\text{ test})\) for comparisons within one recording session for each bird). For 1 out of 30 sessions, song was significantly slower in the directed context \((p < 0.02; \text{M–W } U\text{ test})\). Overall, there was a highly significant effect of social context on song tempo, with an average slowing of song by 1% in the undirected context \((p < 0.0001; \text{paired sign test, } n = 30\) control recordings; \(p = 0.02, \text{one sample sign test for average effect per bird, } n = 10\) birds). This finding is in accord with that of Sossinka and Böhner (1980), who reported an average slowing of undirected song by approximately 2.7% relative to directed song and significant context-dependent tempo differences in 6/8 birds.

To assess whether the modulation of song tempo by social context is dependent on inputs from LMAN, we compared the magnitude of tempo modulation for recordings one day prior to lesions and one week following lesions (Fig. 8B). In all cases, prior to lesions, the tempo was faster in the directed context than the undirected context. This difference was significant for 4/5 birds considered individually \((p < 0.01, \text{M–W } U\text{ test})\). In contrast, one week after lesions of LMAN, context-dependent differences in song tempo were no longer consistent: 4/5 birds did not exhibit a significant difference in tempo between directed and undirected contexts \((p > 0.05, \text{M–W } U\text{ test for recordings in each bird})\), and in the remaining bird, undirected song was significantly faster than directed song \((p < 0.01, \text{M–W } U\text{ test})\). Hence, lesions of LMAN abolished the social modulation of song tempo.

**Stabilization of syllable structure with age**
Previous studies in adult zebra finches have demonstrated that song plasticity in response to manipulation of auditory feedback declines with age (Brainard and Doupe 2001; Lombardino and Nottebohm 2000). Because variability in motor output is an important component of feedback-based motor learning, we asked whether the decline in plasticity is accompanied by an age-dependent decline in song variability. Such a decline is suggested by the gradual reduction in the variability of undirected song for control and sham lesioned birds over the 8–10 week period of recordings (Fig. 5A). To explicitly examine this issue, we recorded directed and undirected songs for 17 additional adult zebra finches. For these birds, we indeed found that socially driven modulation of song variability is attenuated with age. In young adults (< 6 months old), variability in FF was significantly greater during undirected song than during directed song (Fig. 9A; left; 28/29 syllables in 11 birds; \( p < 0.0001 \), paired sign test). These differences were significant for 19/29 syllables (\( F \)-test for equality of variance across conditions for each syllable). In contrast, variability in FF did not differ across social context in birds greater than four years old (Fig. 9A, right; \( n = 13 \) syllables in 6 birds; \( p = 0.5811 \), paired sign test). The lack of context-dependent differences in syllable variability in older birds appeared to be the result of an age-dependent reduction in the absolute level of variability in FF during undirected song to the level present during directed song (Fig. 9A). That is, variability in FF during undirected song was significantly lower in older birds than in young adults (Fig. 9A; \( p = 0.0015 \), M–W \( U \) test), reminiscent of the lesion-induced stabilization of syllable structure (compare Figs. 5B and 9A).

As with LMAN lesioned birds, the age-dependent loss of socially driven modulation of FF did not result from a general disruption of courtship behavior. Older
adult males exhibited robust context-dependent differences in higher-level features of song. In 6/6 birds, the number of introductory elements was significantly greater during directed song than during undirected song (Fig. 7F; \( p < 0.05 \), Mann–Whitney \( U \) test for comparisons within one recording session for each bird), and in 4/6 birds, the number of motifs per bout was significantly greater during directed song (Fig. 7G; \( p < 0.05 \), M–W \( U \) test). Thus, the social modulation of higher-level features of song persisted despite the absence of context-dependent differences in syllable structure, consistent with the hypothesis that different parameters of song are independently controlled.

The stabilization of syllable structure induced by age and by lesions of LMAN raises the question of whether or not the mechanism for song stabilization is the same in the two groups of birds. The observed age-dependent stabilization of syllable structure may reflect a normal developmental decline in factors from the AFP that promote variability. For example, variability in the pattern of AFP activity may decline with age or may not differ across social context in older adult birds. Alternatively, signals from the AFP may not change with age. Rather, as synaptic connections in RA are strengthened, the ability of extrinsic signals to modulate ongoing motor patterns and subsequent song output may decline with age (Lombardino and Nottebohm 2000).

To examine these possibilities, we compared the singing-related activity in LMAN in birds over a wide range of ages. Figure 9B illustrates the singing-related activity during one recording session for a male bird greater than five years old. Across repeated renditions of the bird's motif, the pattern of activity was more reproducible during directed song than during undirected song. Consistent with previous reports, we found that variability in the pattern of multi-unit activity in LMAN was significantly
greater during undirected song than during directed song in young adult birds (< 6 months old; Fig. 9C, left; 10/10 sites in 5 birds; \( p = 0.002 \), paired sign test). Similarly, in older adults (> 4 years old), variability in LMAN activity was also greater during undirected song (Fig. 9C, right, 9/9 sites in 2 birds; \( p = 0.004 \), paired sign test).

Moreover, the degree to which variability in LMAN activity differed across social context was similar between birds of different ages (mean ratio of the undirected neural coefficient of variation (c.v.) to the directed neural c.v. in young and older adults: 1.78 and 1.81, respectively).

However, despite the presence of context-dependent differences in LMAN activity in birds of different ages, we found that the overall level of variability in LMAN activity was significantly lower in older adults than in young adults (Fig. 9C). This age-dependent decline in the absolute level of LMAN variability was apparent in both behavioral contexts (Fig. 9C; undirected c.v. 0.155 versus 0.123; \( p = 0.01 \), M–W \( U \) test; directed c.v.: 0.087 versus 0.068; \( p = 0.004 \), M–W \( U \) test).

Together, these findings suggest that multiple factors contribute to the age-dependent stabilization of syllable structure: 1) the susceptibility of the motor pathway to sources of perturbation may decline with age; and 2) the factors that promote variability in motor activity, such as variability in LMAN activity, may decline with age.
Discussion

Contributions of LMAN to context-dependent regulation of song and behavior

In zebra finches, adult song and behavior are strongly modulated by social context. During 'directed' songs produced in a courtship context, the number of introductory notes, the number of song motifs, and song tempo are increased and the variability of syllable structure is decreased relative to that of 'undirected' songs produced by birds in isolation (Dunn and Zann 1996; Kao et al. 2005; Sossinka and Böhner 1980). Moreover, directed songs are accompanied by a courtship dance that is not produced when the birds are alone (Williams 2001; Zann 1996). This switching of song and behavioral state occurs within the first moments following introduction of a female to the presence of a male and reverses equally rapidly following her removal (Hessler and Doupe 1999b).

Several previous observations have supported the possibility that signals from the AFP contribute to this rapid, context-dependent modulation of song and behavior: 1) neural activity in the AFP is greater and more variable under conditions of undirected song than directed song, indicating that modulation of neural activity in the AFP is correlated with modulation of behavior (Hessler and Doupe 1999b; Jarvis et al. 1998; Kao et al. 2005); and 2) active manipulation of signals from the AFP, by microstimulation of LMAN during singing, can alter the acoustic structure of song elements, indicating that signals from the AFP are sufficient to drive changes in song in real time (Kao et al. 2005). Here, we demonstrate that the nucleus LMAN is necessary for a specific subset of the context-dependent changes in song and behavior. We show
that in normal adult birds, there is a robust decrease in the variability of syllable structure in directed (courtship) song versus undirected song and that this difference in song variability is eliminated by lesions of LMAN. In addition, lesions of LMAN eliminate the social modulation of song tempo. In contrast, lesions do not prevent context-dependent changes in other features of song, including the number of introductory elements that precede song and the number of motifs that are produced during a bout of singing. Similarly, lesions of LMAN do not prevent the postural changes and courtship dance that normally accompany directed song.

The selectivity of the effects of LMAN lesions has two major implications. First, the effects of lesions on song modulation are not the consequence of globally disrupting the male bird's ability to detect and respond to social cues. When females are presented, the courtship vigor of lesioned males is as robust as that of control birds. Moreover, lesions of LMAN did not affect the social modulation of syntactic features of song, such as the number of introductory elements and the number of motifs per bout. These results indicate that LMAN is not an obligatory structure for integrating and representing information about social context. Rather, LMAN must be 'downstream' from brain regions that register such information.

Second, the selectivity of the effects of lesions of LMAN indicates that separate brain regions participate in the modulation of different components of song and courtship behavior. This finding is consistent with the anatomy of the song system: neurons in LMAN project directly to the premotor nucleus RA (Fig. 1A), which is thought to provide motor commands that control the precise structure of individual song elements (Vu et al. 1994; Yu and Margoliash 1996). Moreover, microstimulation in LMAN at low
intensities modulates syllable structure of adult birds without affecting syllable sequencing (Kao et al. 2005; Vu et al. 1994). In contrast, other aspects of song structure, such as patterning of introductory elements and motifs, are thought to be controlled by nuclei earlier in the premotor pathway for song, such as HVC and its inputs (Foster and Bottjer 2001; Vu et al. 1994).

Recent measurements of the social modulation of song tempo have shown that expiratory pulses, which contribute to syllable production, are longer during undirected song than during directed song, while inspiratory pulses are largely unchanged across social context (Cooper and Goller 2006). These observations are consistent with the social modulation of song tempo reported by Sossinka and Böhner (1980) and with the observation by Glaze and Troyer (2006) that durations of syllables can be regulated independently from durations of intervening intervals. These data suggest that increases in song duration may arise from an accumulation of increases in the durations of individual song elements. In this context, it is possible that the observed effect of lesions of LMAN on context-dependent differences in song tempo reflects the direct modulation by LMAN of pattern generation circuitry in RA that contributes to the structure of individual syllables. Alternatively, or in addition, lesions of LMAN might affect song tempo by indirectly influencing activity in other song premotor areas, such as HVC, which are ultimately modulated by activity in LMAN and RA via recurrent connections (Ashmore et al. 2005; Vu et al. 1998).

Regardless of the mechanism, the notion that LMAN can modulate song tempo is consistent with previous reports that lesions of LMAN result in a gradual acceleration of the tempo of undirected songs (Williams and Mehta 1999; Brainard and Doupe 2001).
Although such increases in tempo occur over a period of many days to weeks, in contrast to the rapid modulation of song tempo by social context, they are nonetheless consistent with our observation that LMAN can influence song tempo.

**Possible functions of different song types (practice versus performance?)**

For song learning, variability in motor output may be required in order for evaluation mechanisms to differentially reinforce those patterns of motor activity that produce better songs (i.e., closer to desired targets) and/or to punish the motor commands that result in worse songs. After song is learned, the ability to modify it likely remains important, in order to compensate for age-related perturbations in the motor program and song structure. Such perturbations may arise from changes in hormone levels, changes in the peripheral motor structures for song production (e.g., muscle tone and innervation), and/or the birth, death and incorporation of new neurons in the motor pathway (Alvarez-Buylla 1990; Nordeen and Nordeen 1988; Scharff 2000).

Given the importance of variability in motor learning, it is quite striking that behavioral variability between directed and undirected conditions is robustly modulated. While the reasons for this modulation of song variability remain unclear, one intriguing possibility is that undirected song is a state of motor practice in which male birds try out alternative motor commands, produce a range of vocalizations, and use auditory feedback to optimize and/or maintain the song. In contrast, directed song may reflect a state of motor performance in which individuals exploit what they have already learned to select the patterns of motor activity that produce their 'best' current version of song (Jarvis et al. 1998; Kao et al. 2005; Sutton and Barto 1998).
These considerations render it plausible that the AFP is part of nervous system circuitry that specifically contributes to the generation of motor variability for the purposes of learning (Bottjer 2004; Brainard 2004; Doya and Sejnowski 2000; Kao et al. 2005; Ölveczky et al. 2005). In principle, the reduced variability of directed song could reflect an active process in which the nervous system tightly controls activity in the motor pathway to produce optimal performance, while the greater variability of undirected song could reflect a 'noisier' default motor performance in the absence of an audience. However, our data show that removal of inputs from the AFP to the motor pathway by lesions of LMAN causes a reduction in the variability in syllable structure present during undirected song to the level present during directed song (Figs. 4B and 5B). These results support the view that the increased variability present in undirected song is actively introduced by the AFP. They are therefore consistent with the hypothesis that one of the critical contributions of the AFP to vocal plasticity may rely on its ability to introduce variability to the song motor pathway in order to generate motor exploration during undirected song. A further implication is that the AFP, and perhaps basal ganglia circuits in other species, may contribute to an active switching of motor activity and behavior between states of practice and performance.

Previous work has shown that lesions of LMAN can prevent experience-dependent changes to adult song (Brainard and Doupe 2000; Morrison and Nottebohm 1993; Williams and Mehta 1999), suggesting two possible roles for LMAN: 1) LMAN may provide experience-dependent 'instructive signals' that actively drive changes in the song motor pathway to produce a good match to the tutor song or 2) LMAN may provide 'permissive factors' for song plasticity, without which song cannot change, but which by
themselves need not provide the direction for change (for discussion, see Brainard 2004). In principle, variability introduced into adult song by the AFP could be construed as a permissive factor for plasticity: the songs of adult birds with LMAN lesions may remain stable because motor exploration is effectively prevented. However, the finding that LMAN can direct moment-by-moment changes in song also indicates that signals from the AFP have the potential to actively instruct song plasticity, perhaps by persistently biasing motor output towards desired targets (Kao et al. 2005; Troyer and Doupe 2000).

**Stabilization of song by age and by lesions of the AFP**

If variability of syllable structure contributes to motor exploration and song plasticity, then one might expect to see changes in the degree of variability under natural conditions associated with song stabilization. One such condition is aging, which in adult zebra finches (beyond the normal age of crystallization at ~90d) leads to a progressive stabilization of song. This stabilization has been documented as a decline in the effects of removing auditory feedback as birds age (Brainard and Doupe 2001; Lombardino and Nottebohm 2000). Here, we show that the age-dependent stabilization of song is indeed accompanied by changes in the variability of syllable structure. Although there was no apparent change in the variability of syllables produced by older males during directed singing, the variability of syllables produced during undirected singing dropped to the level present in directed song (Fig. 9A). Hence, aging and LMAN lesions had remarkably similar effects on both the level of syllable variability and its modulation by social context.
This similarity between the effects of aging and LMAN lesions on syllable variability raises the question of whether LMAN is functionally inactive in older birds. We found that there was indeed a decline in variability of LMAN neural activity between younger and older birds (Fig. 9C). However, even in the oldest birds (> 4 years), where social modulation of song variability was no longer present, there continued to be social modulation of variability of neural activity within LMAN (Fig. 9C). This suggests that enhanced stability of adult song derives from multiple processes, including: 1) reduced variability in the patterns of neural activity present within LMAN and 2) reduced sensitivity of the motor pathway to such activity. Such reduced sensitivity could derive from a progressive weakening of inputs from LMAN to RA and/or a progressive strengthening of connections within the motor pathway itself, such that it becomes less influenced by inputs from LMAN.

The finding that the modulation of song variability declines with age despite the persistent modulation of LMAN activity suggests that factors in addition to patterned neural activity from LMAN are likely to contribute to regulation of song variability. These factors may include slower timescale processes, such as neural or trophic influences on synapse formation and the survival and incorporation of new neurons in the motor pathway (Johnson and Bottjer 1994; Kittelberger and Mooney 2005; Kittelberger and Mooney 1999; Scharff and Nottebohm 1991). Indeed, previous studies have demonstrated that LMAN itself provides trophic support to RA during development (Akutagawa and Konishi 1994; Johnson and Bottjer 1994). Hence, LMAN may well influence both rapid (synaptic) and slower (trophic) timescale processes that contribute to the physiology of the motor pathway and the structure and plasticity of song.
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Figure legends

FIG. 1 Schematic model for the contribution of an avian basal ganglia–forebrain circuit to social influences on song production.  

A. Song system nuclei. The ‘motor pathway’ (gray) is required for normal song production throughout life and includes HVC, the robust nucleus of the arcopallium (RA), and the tracheosyringeal portion of the hypoglossal nucleus (nXIIIts). The ‘anterior forebrain pathway’ (AFP, black) is necessary for vocal motor plasticity but is not required for song production. The AFP includes Area X, which is homologous to mammalian basal ganglia, the medial nucleus of the dorsolateral thalamus (DLM), and the lateral magnocellular nucleus of the anterior nidopallium (LMAN).  

B. When a male sings alone (‘undirected’ song), variability in the patterns of LMAN activity may introduce variability into the activity in the premotor nucleus RA, resulting in variable song output. In contrast, when a male sings to a female (‘directed’ song), stereotyped patterns of LMAN activity may facilitate reproducibility in the activity of RA neurons and subsequent song output.  

C. According to this hypothesis, lesions of LMAN should eliminate socially driven differences in song variability.

FIG. 2 Experimental design and song system nuclei in intact and lesioned birds.  

A. Interleaved bouts of directed and undirected song were recorded weekly prior to and following lesions at each time point indicated by tick marks.  

B. Coronal sections through the AFP (top) and arcopallium (bottom) labeled with an antibody against calcitonin gene-related protein (CGRP; Bottjer, et al., 1997). Electrolytic lesions were stereotaxically targeted either at LMAN or at a site dorsal to LMAN (sham). In a sham
control (left), LMAN, its axonal projections to the target nuclei RA and Area X, and medial MAN (MMAN) are all immunolabeled. In a lesioned bird (right), CGRP labeling in LMAN is absent, and there is a corresponding loss of labeled axonal projections to RA and Area X. MMAN remains intact.

**FIG. 3** **Context-dependent differences in song output.** A. Spectrogram (plot of frequency versus time) of one bout of song recorded when a male was alone (‘undirected’ song (US); top) and when it sang to a female (‘directed’ song (DS); bottom). Song bouts consist of a series of introductory elements followed by a variable number of repetitions of a stereotyped sequence of syllables, or ‘motif’ (‘abc’, indicated by bars). The number of introductory elements and motifs per bout are typically lower during US than during DS (Kao et al. 2005; Sossinka and Böhner 1980). B. Variability in syllable structure is greater in the undirected condition. Spectrogram of the same bird’s motif (‘abc’; top). Syllables ‘a’ and ‘c’ contain constant frequency components with a well-defined fundamental frequency (FF). Dotted red lines indicate the portion of the syllable for which FF was measured. Histograms of the FF for the indicated portions of syllables ‘a’ (middle) and ‘c’ (bottom). Variability in the FF was greater during US than during DS (syllable ‘a’: s.d.: 8.8 versus 3.4; p < 0.0001; syllable ‘c’: s.d.: 7.2 versus 2.5; p < 0.0001, F test for equality of variance).

**FIG. 4** Lesions of LMAN reduce the moment-by-moment variability in FF during undirected song to the level present during directed song. Variability in the FF during directed song versus variability in the FF during undirected song. Each point represents
the coefficient of variation (c.v.) of the FF for one syllable in both social contexts during one recording session. A. Context-dependent differences in FF were present in control birds (open black circles; n = 9 syllables in 2 birds) and in birds before (open red triangles) and after sham lesions (filled red triangles; n = 6 syllables in 2 birds). B. Data are plotted for 19 syllables for all recordings before (open symbols) and after (filled symbols) lesions of LMAN. Black X’s denote the means for the populations. Note that following lesions of LMAN, the absolute level of the variability in FF during undirected song was reduced to the level present during directed song (downward shift in the mean following lesions of LMAN).

FIG. 5 Lesions of LMAN eliminate context-dependent differences in the variability in FF. Bars indicate the mean coefficient of variation (c.v.) of the FF (± s.e.m.) for syllables during undirected song (open bars) and directed song (shaded bars). Data are plotted for the same syllables in the same birds across all recording sessions. A. Summary for all syllables in birds with sham lesions and in age-matched control birds that were recorded weekly. Context-dependent differences in song variability persisted in control birds and in birds with sham lesions (n = 15 syllables in 4 birds; p < 0.01 for each recording, paired sign test). B. Summary for all syllables before and after lesions of LMAN. Bilateral lesions of LMAN abolish the socially driven differences in song variability by reducing variability in the undirected condition to the level present in the directed condition (19 syllables in 5 birds; p < 0.0001 for each recording pre-lesions, paired sign test; no significant differences in song variability were found for any recording post-lesions). Variability in FF during US was significantly lower in the first
recording post-lesion compared to that one day pre-lesions ($p < 0.005$, paired sign test).

For this and all subsequent figures, triple asterisks denote $p < 0.0001$, double asterisks denote $p < 0.005$, and a single asterisk denotes $p \leq 0.01$.

**FIG. 6 Lesions of LMAN do not abolish other courtship behaviors.** Summary for all birds of the average level of arousal and/or vigor of the courtship display (see Materials and Methods) in each social context one day before and one week after lesions of LMAN (*open circles*) or sham lesions (*filled squares*). Males continued to vigorously court females following lesions of LMAN and sham lesions. Lines connect data points for each bird across social context.

**FIG. 7 Context-dependent differences in higher-level features of song are not affected by lesions of LMAN.** A. Schematic of undirected (US) and directed song (DS) bouts. B–C. Frequency distributions of the number of introductory elements per motif and the number of motifs per bout during US (*open bars*) and DS (*shaded bars*) for one recording session pre-LMAN lesion (*bird pur24w55*). Both the number of introductory elements and the number of motifs per bout were significantly lower during US than during DS ($p < .0001$, Mann–Whitney $U$ test). D–E. The number of introductory elements and the number of motifs per bout remain higher during DS following lesions of LMAN. Each point represents the average number of introductory elements per song bout or the average number of motifs per bout for one recording session, and lines connect data points for the same bird across behavioral contexts ($n = 5$ birds). Bars indicate group means; error bars indicate s.e.m. The data plotted in Figs. 7B–C are
indicated by closed squares.  

\[ F-G \]  

Context-dependent differences in higher-level features of song are also present in older adult birds (same convention as above; \( n = 6 \) birds).

FIG. 8  Context-dependent differences in song tempo are dependent on input from LMAN.  

\( A. \ Top. \) Directed songs are typically produced at a faster tempo than undirected songs. Spectrograms of a representative motif during US (top) and DS (bottom) of one bird are aligned by the onset of the first syllable. Dotted lines indicate the onset time and offset times of the motifs.  

Bottom. Histogram of the motif durations during DS (open bars, \( n = 37 \)) and US (closed green bars, \( n = 35 \)). Motif duration was significantly shorter during DS (means: 640.6 versus 657.2; \( p < 0.0001 \), M–W U test).  

\( B. \) Lesions of LMAN eliminate context-dependent differences in song tempo. Undirected motifs were significantly slower than directed motifs in 4/5 birds prior to lesions of LMAN (\( p < 0.01 \), M–W U test for comparisons within one recording session for each bird). Following lesions of LMAN, song tempo was not significantly different in 4/5 birds (\( p > 0.14 \), M–W U test). In the remaining bird, undirected song was significantly faster than directed song (\( p < 0.04 \), M–W U test). Lines connect data points from the same bird. Bars indicate group means; error bars indicate s.e.m.

FIG. 9 Age-dependent stabilization of syllable structure despite persistent context-dependent differences in LMAN activity.  

\( A. \) Context-dependent differences in song variability decline with age. The c.v. of the FF is plotted for syllables recorded in both behavioral contexts (1-7 syllables per bird); lines connect data points for the same
syllable. Bars indicate group means. Variability in FF was significantly greater during US than during DS in birds less than 6 months old (left: 28/29 syllables in 11 birds; $p < 0.0001$, paired sign test) but not in older birds (right: > 4 years old; 13 syllables in 6 birds; $p = 0.5811$, paired sign test). Variability in FF during US was significantly lower in older adults compared to young adults ($p = 0.0015$, M–W U test). B. Context-dependent differences in LMAN multi-unit activity in an adult male (> 5 years old). Top: Singing-related activity in LMAN during three renditions of the motif during US (left) and DS (right). Middle: Rectified, smooth waveforms of LMAN multi-unit activity during 10 renditions of the motif are superimposed for each behavioral condition. The waveforms are normalized by the mean spontaneous activity (dotted line). LMAN activity was greater in magnitude and more variable in pattern across repeated renditions during US. Bottom: Coefficient of variation (c.v.) of the activity level was calculated in 1 ms bins across repeated undirected (left) and directed (right) motifs (thick black lines). The c.v. across undirected motifs (gray) is re-plotted for comparison with the c.v. across directed motifs (right). Arrows denote the mean time-averaged c.v. in each condition. C. Context-dependent differences in LMAN activity are present in birds across a range of ages. Time-averaged c.v. during US and DS are plotted for each site. The data in Fig. 9B are indicated by closed squares. Variability in LMAN activity was significantly greater during US than during DS in birds of different ages (left: < 6 months old: 10/10 sites in 5 birds; $p = 0.002$, paired sign test; right: > 4 years old: 9/9 sites in 2 birds; $p = 0.004$, paired sign test). Although context-dependent differences were present in older birds, the absolute level of variability in LMAN activity declined significantly in both
behavioral contexts with age (undirected c.v.: 0.155 versus 0.123; $p = 0.01$, Mann–Whitney $U$ test; directed c.v.: 0.087 versus 0.068; $p = 0.004$, M–W $U$ test).
Figure 1

A

B  Undirected song  Directed song

C  Undirected/Directed song

HVC

RA

LMAN

Area X

nXilla

larynx

expiratory motor neurons

song 1  song 2  song 3

song
Figure 2

A

Lesion

-2 wk -1 wk -1 d <3 d +1 wk +2 wk +3 wk +4 wk +8 wk

B

LMAN MMAN

Area X

MMAN

Area X

RA

RA
Figure 3
Figure 4

A

B
Figure 5

A

Sham

B

Lesion

Variability in FF (t x v)

0.016

0.012

0.008

0.004

0

-2 wk

-1 wk

-1 d

-(1-3) d

+1 wk

+2 wk

+3 wk

+4 wk

+8 wk

US

DS

-2 wk

-1 wk

-1 d

-(1-3) d

+1 wk

+2 wk

+3 wk

+4 wk

+8 wk
Figure 6
Figure 8
Figure 9

A

< 6 months old

> 4 years old

Variability in FF (c.s.)

US

DS

US

DS

B

US

DS

Activity levels (c.s.)

Modulated frequency

Variable across trials (c.s.)

C

< 6 months old

> 4 years old

Variability in LMN activity (c.s.)

US

DS

US

DS