Title
Contributions of an avian basal ganglia-forebrain circuit to song production and plasticity

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Publication Date
2005

Peer reviewed|Thesis/dissertation

Contributions of an avian basal ganglia-forebrain circuit to song production and plasticity

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

ACKNOWLEDGEMENTS

This thesis would not have been possible without the encouragement and support of my advisor, Allison Doupe. Allison's intensity and enthusiasm for science is unmatched, and her wide range of scientific interests, coupled with an eagerness to learn new methods for approaching problems, have made my graduate education truly exciting. Her example and encouragement created an environment that inspired me to undertake challenges that I was not certain I was capable of meeting.

I have been very fortunate to have a second mentor and advisor during my graduate career. Much of the work described in chapters 3 and 4 was performed in collaboration with and under the guidance of Michael Brainard, whose ability to see the heart of a problem and critically dissect the issues is a quality that I will strive to emulate.

In addition, I greatly value the encouragement and advice of my undergraduate advisor, Russell Fernald, and the friendship of Stephanie White, who supervised my first research project in neuroscience. Both Russ and Stephanie helped me at an important time when I was deciding on whether or not to pursue a career in neuroscience.

I am also grateful for the guidance and advice of my thesis committee members–Steve Lisberger, Michael Stryker, and Krishna Shenoy–who asked many good questions that led to substantial improvements of this thesis.

All of the work in this thesis benefited greatly from the critical assessment of the entire Doupe lab, whose members have been a continuous source of feedback and lively discussions. It has been my pleasure to collaborate with and learn from a number of current and former members of the lab. In particular, I am grateful to Neal Hessler, who taught me how to perform chronic recordings in awake, behaving birds. Neal's patience,

thoughtfulness, and support for a new student who had no experience in electrophysiology are truly appreciated. I am also grateful to Brian Wright, who contributed significantly to my thinking about the data presented in chapter 2 and whose programs have been critical for both song analysis and the analysis of neural activity associated with a variable motor behavior. My fellow graduate students in the lab– Michele Solis, Rhea Kimpo, Charlotte Boettiger, Gabriela Carrillo, and Kathy Nagel– have been a great support group, and I value both their insights into the song system as well as their experiences and thoughts about navigating a career in science. I would also like to thank Adria Arteseros, who manages and provides support to the lab in countless ways.

Finally, I thank my parents, James and Lily, for their confidence in me, which has allowed me to do things that I believed myself incapable of doing, as well as my brother Jimmy, who was the first to introduce me to neuroethology.

The text of Chapter 3 is a reprint of the material as it appears in *Nature*. The co-authors listed (Allison Doupe and Michael Brainard) directed and supervised the research that forms the basis for the dissertation.

Contributions of an avian basal ganglia–forebrain circuit to song production and plasticity

Mimi H. F. Kao

ABSTRACT

The anterior forebrain pathway (AFP) of songbirds is a specialized basal ganglia–forebrain circuit that is critical for vocal plasticity during song learning and in adulthood but is not required for the production of learned song. Here, we investigate the functional contribution of this circuit to the control of song, a complex, learned behavior. We test the hypothesis that neural activity in the lateral magnocellular nucleus of the anterior nidopallium (LMAN), the output nucleus of the AFP, contributes to song plasticity by directing moment-by-moment changes in the primary motor areas responsible for generating song.

Singing-related activity of single neurons in LMAN was characterized by extracellular recordings in adult zebra finches. Individual neurons in LMAN exhibited distinct patterns of activity correlated with song, suggesting that LMAN neurons form a population that jointly encode the acoustic features of song. In addition, the firing properties of single neurons differed markedly across behavioral context. Firing rate, burst rate, and trial-by-trial variability in the activity of single LMAN neurons were significantly greater when a male sang alone than when the male sang to a female. Such naturally occurring differences in the variability of LMAN activity correlated with natural variability in song output, suggesting that neural activity in LMAN may modulate ongoing song on a moment-by-moment basis.

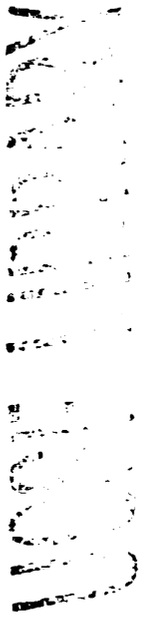
To directly test whether LMAN can direct changes in ongoing song, neural activity in LMAN was manipulated by applying electrical stimulation during singing. Song-triggered microstimulation induced specific changes in learned parameters of song in real-time. In addition, the artificial introduction of variability in LMAN activity caused a significant increase in song variability. Finally, lesions of LMAN eliminated the naturally occurring modulation of song variability.

Together, these findings demonstrate the capacity of the AFP to direct moment-by-moment changes in song. They suggest that neural activity in LMAN may contribute to song plasticity in multiple ways: 1) the specific pattern of activity in LMAN may guide adaptive changes and bias song output towards the desired target; and 2) variability in LMAN activity may introduce variability into motor output, which is a requisite component of feedback-based reinforcement learning.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES



Chapter 1: Background and experimental rationale

Songbirds are a promising model system for investigating the neural mechanisms that underlie learning and memory. Vocal learning in birds is a classic example of sensorimotor learning in which sensory feedback is used for the gradual refinement of motor behavior. Song learning occurs in two stages, both of which depend critically on hearing (Figure 1-1a). In the sensory phase, a young bird listens to and memorizes the song of an adult tutor. The sensorimotor phase begins when the bird starts to sing, producing "subsong", followed by "plastic song". Subsong is soft and variable, has poor spectral structure, and shows little evidence of tutor song copying. Plastic song is also variable but begins to show evidence of the rehearsal of memorized material. The juvenile birds listens to its own vocalizations and gradually modifies its plastic song until it matches the memorized song model, or 'template'. Birds that learn to sing have a specialized set of brain nuclei dedicated to song production and song learning (the 'song system'; Nottebohm et al., 1976) that is not found in closely related species that do not learn to sing. These nuclei are likely locations for neural mechanisms underlying song learning, and studies of such specialized structures can reveal basic principles of nervous system organization and function.

The anterior forebrain is required for song plasticity

The song system consists of a motor pathway and an anterior forebrain pathway (Figure 1-1b). The motor pathway generates and coordinates the patterned breathing and vocal muscle activity necessary for normal song production throughout life. It includes HVC (used as a proper name), the robust nucleus of the arcopallium (RA), and the tracheosyringeal portion of the hypoglossal nucleus (nXIIts), which contains the motor

neurons that control the muscles of the syrinx, the avian vocal organ. RA also projects to brainstem respiratory nuclei that control airflow through the syrinx. Lesions of HVC or RA result in substantial degradation of song structure or muteness (Nottebohm et al., 1976). In contrast to the motor pathway, the anterior forebrain pathway (AFP) is a specialized basal ganglia–thalamo–'cortical' loop (Reiner et al., 1998; Perkel, 2004) that is required for normal song learning and adult vocal plasticity but is not necessary for the production of learned song (Bottjer et al., 1984; Scharff and Nottebohm, 1991; Williams and Mehta, 1999; Brainard and Doupe, 2000). This pathway indirectly connects the motor nuclei HVC and RA and consists of Area X, the medial nucleus of the dorsolateral thalamus (DLM), and the lateral magnocellular nucleus of the anterior nidopallium (LMAN). Anatomically, the AFP is well situated to influence the connectivity and ongoing activity in the motor pathway. The AFP output nucleus LMAN sends a direct excitatory projection to the motor nucleus RA (Mooney and Konishi, 1991), where inputs from the two pathways converge.

Lesion studies have demonstrated that the AFP plays an important role in regulating vocal plasticity. In juvenile zebra finches, lesions of LMAN prevent the normal progression of song learning towards a good match of the memorized song model and induce premature stereotypy, resulting in highly repetitive, simplified songs that consist of a few abnormally structured elements (Bottjer et al., 1984; Scharff and Nottebohm, 1991). Similarly, lesions of LMAN prevent the incorporation of new syllables into the songs of adult birds that have been experimentally manipulated to undergo late learning (Morrison and Nottebohm, 1993). In addition, in adult zebra finches, lesions of LMAN prevent the gradual deterioration of song that is induced either

by deafening or by transecting one of the peripheral nerves to the vocal musculature (Williams and Mehta, 1999; Brainard and Doupe, 2000). The apparent loss of experience-dependent song plasticity after lesions of LMAN in three different experimental conditions implicates the AFP in regulating vocal plasticity in juvenile and adult birds.

Consistent with a role in enabling plasticity, neurons in LMAN increase their firing rate before singing begins and exhibit robust singing-related activity that is correlated with the acoustic features of song (Hessler and Doupe, 1999a, b; Leonardo, 2002, 2005). It is not known, however, whether, and how, such patterned activity from LMAN influences the motor pathway and contributes to song production and plasticity.

Functional contributions of the AFP to song production and plasticity

Two models have been proposed for the functional contribution of LMAN to song plasticity (reviewed in Brainard, 2004). In one model, patterned neural activity in LMAN serves as an instructive signal to guide adaptive changes in the motor pathway so that song progresses towards a better match of the memorized song (Troyer and Doupe, 2000; Brainard and Doupe, 2000). According to this "instructive" model, neural activity in LMAN reflects the deviation between the bird's current song and the memorized song template and biases patterns of motor activity towards a particular target (i.e., the memorized song).

An alternative model for the functional contribution of the AFP to song plasticity postulates that LMAN provides factors that permit plasticity in the motor pathway and are required for changes in song output, but does not provide specific guidance to steer

song towards a better match of the memorized song template. One version of this "permissive" model proposes that a critical function of LMAN activity is to introduce variability into the ongoing patterns of activity in the premotor nucleus RA and subsequent song output (Doya and Sejnowski, 2000). Trial-by-trial variability in motor output is an important component of feedback-based reinforcement learning. In order for song to change, it must vary from rendition to rendition so that evaluation mechanisms can differentially reinforce those patterns of motor activity that produce the desired behavior (i.e., renditions close to the memorized model) and/or punish the motor patterns that result in worse songs.

These two models for the contribution of LMAN activity to vocal plasticity are not mutually exclusive. Rather, the influence of LMAN on the motor pathway and song output will depend on the actual pattern of activity during singing.

Whether neural activity in LMAN can modulate ongoing activity in the motor pathway and subsequent song output was examined first by characterizing the firing properties of single neurons in LMAN. Chapter 2 describes the singing-related activity of single LMAN neurons in two behavioral contexts. Individual neurons in LMAN exhibit unique, time-varying firing patterns correlated with song, consistent with the idea that the pattern of activity carries information about the song. This finding suggests that LMAN neurons form a population that jointly encodes the acoustic features of song. In addition, the firing properties of single neurons were strikingly modulated by the social context in which the bird sang. Firing rate, burst rate, and trial-by-trial variability in the activity of single LMAN neurons were greater when a male sang alone ('undirected song') compared to when the male sang to a female ('directed' song). To examine

whether such naturally occurring differences in the activity of single neurons could modulate ongoing song output on a moment-by-moment basis, songs produced in the two behavioral conditions were analyzed. Chapter 3 describes a correlation between greater variability in LMAN activity and greater variability in syllable structure during undirected song, consistent with a direct modulatory influence of LMAN on song under natural conditions.

To directly test whether LMAN activity can direct moment-by-moment changes in song, neural activity in LMAN was manipulated by applying electrical stimulation during singing. Chapter 3 also characterizes the acute and specific changes in learned parameters of syllable structure that were induced by song-triggered microstimulation in LMAN. Artificially altering the pattern of activity in LMAN induced changes in the frequency and amplitude of individual song elements, or 'syllables', with a latency of 40–45 msec, consistent with a direct modulation of RA by LMAN. Moreover, in a given experiment, stimulation with a fixed pattern of LMAN activation induced systematic changes in the mean value of syllable parameters, not a general degradation of song structure or an enhancement of song variability. These results are consistent with the hypothesis that the pattern of activity in LMAN serves as an instructive signal to RA to systematically bias the motor pathway towards a particular goal.

To test explicitly whether trial-by-trial variability in LMAN activity can give rise to variability in motor output and contribute to song plasticity, LMAN activation was altered across song renditions by varying the current intensity. The artificial introduction of variability in LMAN activity caused a significant increase in the variability of syllable structure, recapitulating a natural difference between directed and undirected song. This

finding provides further evidence that a critical contribution of LMAN to song plasticity may be to introduce into song the variability that is required for reinforcement learning.

If activity in LMAN is responsible for the moment-by-moment differences in song variability under natural conditions, then removing this activity should eliminate the observed context-dependent difference. Chapter 4 characterizes differences in directed and undirected songs before and after bilateral lesions of LMAN. Lesions of LMAN eliminate context-dependent differences in syllable variability by reducing the moment-by-moment variability in undirected song to the level present during directed song.

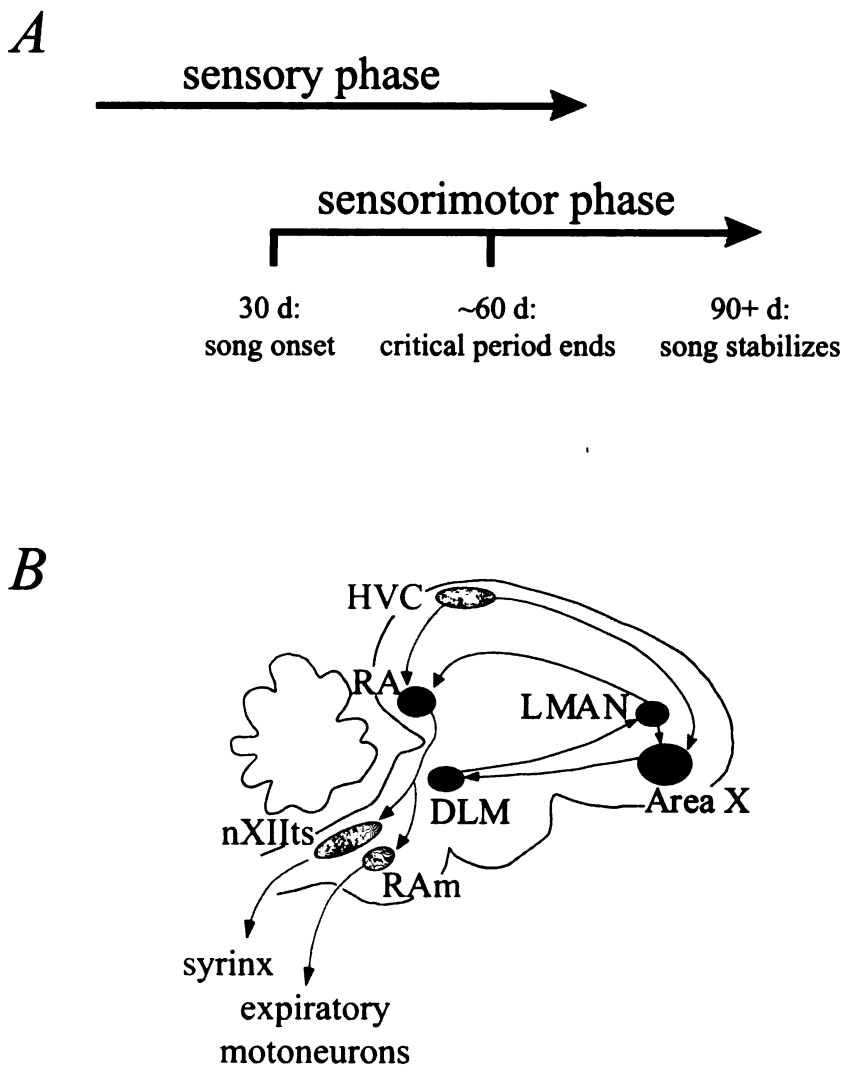
To further investigate the hypothesis that LMAN regulates vocal plasticity by modulating variability in motor output, the contribution of LMAN to the modification of song was examined under conditions of increased plasticity (deaf birds). In the context of reinforcement learning, variability in motor output should depend on the current state of motor performance. When song is far from the desired target, greater variability is appropriate in order to explore the motor space in search of better solutions. Chapter 4 describes the contribution of LMAN to changes in songs in birds that have been deafened. Lesions of LMAN prevent the increase in syllable variability that is induced by deafening. Together with the effects of lesions on context-dependent differences in syllable variability, these results support the hypothesis that a critical contribution of LMAN to vocal plasticity is the modulation of variability in motor output.

In summary, this thesis finds evidence for both instructive and permissive roles of neural activity in an avian basal ganglia–forebrain circuit in ongoing motor performance and plasticity. Activity in LMAN could contribute to song production and plasticity in multiple ways: 1) the specific pattern of activity in LMAN may provide an instructive

signal to the premotor neurons in RA to guide the modification of song towards the desired target; and 2) variability in the activity of LMAN neurons may introduce variability in RA activity and subsequent song output, which is a requisite component of reinforcement learning. Chapter 5 outlines outstanding questions and future experiments suggested by these studies; as in this thesis, future studies will be well served by detailed analysis of song behavior.

Figure 1-1 Vocal learning in songbirds. *A.* Time course of zebra finch song learning. Zebra finches learn to sing in two overlapping phases: the sensory phase ends at ~60 days; the sensorimotor phase begins at ~30 days and continues to adulthood (90+ days). *B.* Schematic diagram of the song system. 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 (nXIIts). 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).

Figure 1-1


A
sensory phase
sensorimotor phase
30 d:
song onset
~60 d:
critical period ends
90+ d:
song stabilizes
B
HVC
RA
LMAN
DLM
Area X
nXIIts
RAm
syrinx
expiratory
motoneurons

Chapter 2: Reproducibility and variability in neural spike trains in an avian forebrain nucleus that is required for song plasticity

ABSTRACT

The anterior forebrain pathway (AFP) of songbirds is a specialized basal ganglia–forebrain circuit critical for vocal plasticity both during song learning and in adulthood. Previous studies have demonstrated a premotor component in the activity of the output nucleus of the AFP, the lateral magnocellular nucleus of the anterior nidopallium (LMAN). In addition, multi-unit activity in LMAN has been shown to be more variable when a male sings alone ('undirected' song) than when it sings to a female ('directed' song) (Hessler and Doupe, 1999a). To investigate how LMAN activity could contribute to song production and plasticity, we examined the activity of single neurons in LMAN, both across sites in the nucleus and across behavioral contexts.

Extracellular single neuron recordings in adult zebra finches revealed that different LMAN neurons in individual birds exhibit distinct time-varying firing patterns during song, suggesting that LMAN neurons form a population that jointly encodes the features of song. In addition, the firing properties of single neurons in LMAN differed markedly across behavioral contexts. The firing rate of single neurons was typically greater in the undirected condition than in the directed condition, and the firing pattern during individual undirected trials was less tightly locked to song, varying from trial to trial. Spike count variability across repeated trials was greater during undirected song compared to that during directed song. Despite these differences in trial-by-trial variability, the average pattern of activity across many trials was similar for a particular neuron across behavioral contexts, in sharp contrast to the diverse firing patterns observed across different neurons.

LMAN activity in the two behavioral contexts differed greatly in the degree of bursting. LMAN neurons frequently produced bursts of spikes during undirected song and rarely during directed song. The number of spikes per burst varied from trial to trial, and bursts occurred at different times in the song across repeated undirected trials. If we retained only the first spike of each burst in our analysis, the spike count variability in the undirected condition was substantially reduced but still significantly greater than that in the directed condition. Thus, the context-dependent difference in variability was due, in part, to a difference in bursting. Our findings suggest that LMAN neurons may contribute to ongoing song production and plasticity in multiple ways: 1) the specific pattern of activity may serve as an instructive signal to guide changes in motor output; and/or 2) trial-by-trial variability in LMAN activity may introduce variability in motor output.

INTRODUCTION

Birdsong, like human speech, is a complex learned motor skill. Song learning occurs in two stages: 1) "sensory" learning, when a young bird listens to and memorizes the song of an adult tutor; and 2) "sensorimotor" learning, when the juvenile bird uses auditory feedback to gradually match its variable, immature vocalizations to the memorized song model (Konishi, 1965; Price, 1979). Once the song is learned, the ability to modify it remains important, either for feedback-based correction of errors or for continued motor exploration and optimization.

The neural substrate for song production and learning is a discrete series of forebrain and brainstem nuclei (Figure 2-1). This 'song system' includes a motor

pathway, which generates and coordinates the patterned breathing and vocal muscle activity necessary for song production throughout life (Nottebohm et al., 1976; Yu and Margoliash, 1996; Hahnloser et al., 2002; Leonardo and Fee, 2005), and an anterior forebrain pathway (AFP), a specialized basal ganglia–forebrain circuit (Reiner et al., 1998; Perkel, 2004) that is necessary for song plasticity but not for the production of stable adult song (Bottjer, et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991; Williams and Mehta, 1999; Brainard and Doupe, 2000). Anatomically, the AFP is well situated to influence the connectivity and ongoing activity in the motor pathway. The AFP output nucleus LMAN (lateral magnocellular nucleus of the anterior nidopallium) sends a direct excitatory projection to the premotor nucleus RA (robust nucleus of the arcopallium; Mooney and Konishi, 1991), where inputs from the two pathways converge.

Previous lesion studies have demonstrated that the AFP plays an important role in regulating vocal plasticity. In juvenile zebra finches, lesions of LMAN disrupt normal song development and induce premature stereotypy, significantly reducing the number of different song elements, or 'syllables', as well as the variability in the temporal structure of song (Bottjer, et al., 1984; Scharff and Nottebohm, 1991). Similarly, lesions of LMAN prevent the incorporation of new syllables in adult birds that have been experimentally manipulated to undergo late learning (Morrison and Nottebohm, 1993). In addition, lesions of LMAN in adult zebra finches prevent song plasticity induced by experimental manipulation of auditory and/or proprioceptive feedback (Williams and Mehta, 1999; Brainard and Doupe, 2000).

Consistent with a role in enabling vocal plasticity, neurons in LMAN increase their firing rate before singing begins and exhibit robust singing-related activity that is correlated with song structure (Hessler and Doupe, 1999a, b; Leonardo, 2002, 2005). In addition, microstimulation in LMAN in singing birds can induce specific changes in syllable structure on a moment-by-moment basis, consistent with a premotor role for LMAN in song production (Kao et al., 2005). Moreover, LMAN activity and song output are modulated by social context: multi-unit activity in LMAN and song structure are more variable when a male sings alone ('undirected' song) than when it sings to a female ('directed' song) (Sossinka and Böhner, 1984; Hessler and Doupe, 1999a; Kao et al., 2005). Lesions of LMAN eliminate the context-dependent modulation of song variability (Kao et al., 2005). Together, these findings suggest that a key function of the AFP in regulating vocal plasticity may be to modulate the ongoing activity in the premotor nucleus RA.

To investigate the contribution of LMAN activity to song production and plasticity, we recorded the activity of single LMAN neurons, both across sites in the nucleus and across behavioral contexts. We quantified the degree of reproducibility or variability in the spike trains of individual neurons. Comparison of the firing patterns across neurons in LMAN may help to elucidate how song is encoded in the AFP. In addition, variability in LMAN activity, either across neurons or across repeated trials for a particular neuron, may provide insight into the ability of LMAN neurons to reliably drive changes in the activity of postsynaptic neurons in the premotor nucleus RA.

We report here that different neurons in LMAN exhibit distinct patterns of firing during song. In addition, the firing properties of single LMAN neurons are strongly

modulated by social context. While individual neurons exhibit characteristic firing patterns during song, firing rate, burst rate, and trial-by-trial variability are greater during undirected song than during directed song. Such context-dependent differences in trial-by-trial variability are due, in part, to a difference in bursting. Together, these results suggest that activity in LMAN may contribute to song production and plasticity in several ways: 1) the pattern of activity of LMAN neurons may provide a signal to the premotor neurons in RA to guide the adaptive modification of song (Troyer and Doupe, 2000; Kao et al., 2005); and/or 2) trial-by-trial variability in the activity of LMAN neurons may introduce variability in RA activity and subsequent song output (Doya and Sejnowski, 2000; Kao et al., 2005).

MATERIALS AND METHODS

Subjects

Experiments were performed on adult male zebra finches (*Taeniopygia guttata*). Birds were selected on the basis of size and singing frequency and were then isolated in a small cage inside a sound-attenuating chamber (Acoustic Systems, Austin, TX). All procedures were performed in accordance with protocols approved by the UCSF Institutional Animal Care and Use Committee.

Surgical procedures

Surgery for the implantation of a small microdrive carrying tungsten electrodes was performed as described previously (Hessler and Doupe, 1999b). Briefly, birds were deprived of food and water for one hour and then anesthetized with an intramuscular

injection of equithesin (0.85 g chloral hydrate, 0.21 g pentobarbital, 0.42 g $MgSO_4$, 2.2 ml 100% ethanol, and 8.6 ml propylene glycol to a total volume of 20 ml with water). A lightweight microdrive (UCSF and Caltech Machine Shops) carrying two glass-coated platinum-plated tungsten electrodes (2-5 MΩ) (A. Ainsworth, Northhamptonshire, UK) (Merrill and Ainsworth, 1972) was positioned stereotaxically such that the electrode tips were ~700 μm dorsal to LMAN (5.0 mm anterior and 1.7 mm lateral to the posterior border of the divergence of the central sinus). A reference ground electrode (uninsulated tungsten electrode; A-M Systems, Calsborg, WA) was implanted in the contralateral hemisphere such that its tip was located within 2 mm of the targeted nucleus. The microdrive and connector socket (FHC, Bowdoinham, ME) were secured to the skull with epoxy (Devcon, Danvers, MA) and dental cement (Dentsply, Milford, DE), and a protective cap was fixed around the microdrive. All recordings were made from the right hemisphere.

Electrophysiology and data collection

During each experimental session, one end of a flexible lead terminating in a small operational amplifier circuit (Texas Instruments, Dallas, TX) was connected to the socket on the bird's head, and the other end was connected to a mercury commutator (Caltech Machine Shop). Electrodes were positioned at sites in LMAN where action potentials of single or multiple units could be clearly differentiated from the background neural activity. Spike amplitudes ranged from 500 μV to > 5 mV, peak to peak. The neural activity signal passed through the commutator to a differential amplifier (A-M Systems) and was filtered between 300 Hz and 10 kHz. The acoustic signal was recorded by a

small microphone (Countryman, Redwood City, CA) located above the birdcage and band-pass filtered between 200 Hz and 9 kHz (Krohn-hite, Avon, MA). The bird's behavior was monitored and recorded via a video camera inside the sound-attenuating chamber. Custom-written acquisition software (A. Leonardo, Caltech, and C. Roddey, UCSF) recorded the acoustic and neural signals before and after the sound amplitude crossed a threshold level.

Extracellular recordings were made at intervals of one day to several weeks, over a period of weeks to months. Typically, at the end of the recording session(s) on each day, the electrodes were retracted to a position above LMAN. Neural activity was recorded during non-singing and singing periods. Background, spontaneous activity was recorded during periods when the bird was silent; such periods were required to precede or follow any vocalization by at least 3 sec (Hessler and Doupe, 1999b). Recordings were made in two behavioral contexts: 'undirected', when the male was isolated in the sound-attenuating chamber, and 'directed', when the male sang to a female. To elicit directed song, one or more female zebra finches was presented in a separate cage to the male 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 ≤ 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 experimental sessions, which lasted between 30 minutes and several hours depending on singing frequency and the stability of the recording site.

At the conclusion of experiments in each bird, small electrolytic lesions (30μA for 10 sec) were made at previously recorded sites. Animals were deeply anesthetized with

Metofane (Schering-Plough, Union, NJ) and transcardially perfused with 0.9% saline, followed by 3.7% formalin in 0.025 M phosphate buffer. Electrode tracks and lesions were localized in 40 μm Nissl-stained sections. Locations of all sites were confirmed by their position relative to the depth of marker lesions.

Data analysis

Neural activity was analyzed offline, and single units were discriminated using Bayesian spike-sorting techniques (Lewicki, 1994; code originally written by M. Lewicki, Caltech; algorithm corrections and improvements by B. Wright, UCSF; user interface improvements by M. Kvale and B. Wright, UCSF). We verified the isolation of single units by visual inspection of the waveforms and by the presence of a refractory period in the interspike interval (ISI) histogram (refractory period ≤ 0.7 msec; $< 1\%$ ISI violations for each cell; $n = 23$ units in six birds). Only one unit was isolated from each electrode; second units on a single electrode were never discriminated either because they were not present or because we were less confident of their isolation.

Alignment of song and neural data. Zebra finch song can be classified into three levels of organization: 'syllables', which are individual song elements separated by silent intervals at least 5 msec in duration; 'motifs', which are stereotyped sequences of syllables, and 'bouts' of song, which are defined as periods of singing separated by silent intervals at least 2 sec in duration (Sossinka and Böhner, 1984). For each experiment, we analyzed the singing-related activity during repeated renditions of the song motif (repeated trials) shifted by an estimate of the premotor latency between neural activity in LMAN and song output. We chose a premotor latency of 40 msec based on previous

measurements of latency using brief electrical stimulation in LMAN during singing (~ 50 msec; Kao et al., 2005). In addition, previous recordings of multi-unit activity in LMAN found that the cross-covariance between LMAN activity and song was greatest when the activity was shifted forward by ~35 msec relative to the song output (Hessler and Doupe, 1999b).

The length of syllables and inter-syllable intervals varies from one rendition to the next, resulting in noise in the structure of spike trains if they are aligned only at the onset of each song motif (Chi and Margoliash, 2001). To compensate for differences in the duration of syllables and intervals across trials, we performed a piecewise linear time warp of each syllable and interval so that all of the motifs and spike trains were on a common-aligned time axis.

The alignment algorithm proceeded as follows. Song syllables were initially segmented automatically using a threshold crossing of the acoustic power (M. Brainard, UCSF) and were labeled manually ('a', 'b', 'c', etc.). The song motif with the median duration during a recording session was chosen as a reference, and we determined the onset time, offset time, and duration for each syllable and interval of this reference motif. Then, for each rendition of the motif, or trial, each syllable was linearly stretched or compressed to match the duration of the same syllable in the reference motif. Syllables were aligned at the onset of the reference syllables. The same procedure was applied independently to the intervals. Next, the spike train was projected onto the warped axis of the corresponding syllable or interval. By stretching or compressing the syllables and intervals in different trials and the associated neural activity to a common reference and

aligning their onsets, this algorithm substantially reduced the variability in the temporal registry of the spike trains.

For analyses of singing-related activity across multiple recording sessions, the same algorithm was applied to align the motifs and spike trains, but the reference motif was the motif with the median duration across all recording sessions in each bird (n = 2–8 recording sessions per bird in five birds).

Fano factor. To characterize trial-by-trial variability in neural activity, we computed the Fano factor (F), which is defined as the variance of the spike count within a window of size T divided by the mean spike count: $F(T, t) = \sigma^2/\mu$, where t is the time in the song where the onset of the window begins. Thus, F is a function of both the counting window T as well as the time in the motif, t. In all cases, we analyzed the singing-related activity associated with a motif (see above). We counted the number of spikes in a sliding window of width T (30 msec or 100 msec) for each motif rendition, or trial, evaluated at times t separated by 1 msec. The mean and variance of the spike count were computed for each window T across all trials in each behavioral condition. We emphasize that F is based on samples taken from the same window in the motif (same epoch in the peristimulus time histogram). For a Poisson process, $F(T, t) = 1$ for any window T at all times t, regardless of the fact that the firing rate varies with time within each trial.

Analysis of correlations in firing patterns. To quantify the degree of similarity in the firing pattern of a single unit in LMAN across behavioral contexts or in the firing patterns across pairs of LMAN neurons, we calculated the cross-covariance of the mean activity patterns during repeated motif renditions (i.e., cross-correlation after subtraction

of the mean firing rates). We used a time bin size of 10 msec and allowed for a shift in the peristimulus time histogram when computing the cross-covariance function to take into account the fact that the activity of different neurons could have different delays relative to the song output. We searched for the peak in the cross-covariance function between – 40 msec and + 40 msec. For within-site analyses across behavioral contexts, the peak in the cross-covariance function occurred at a time delay of 0 msec.

RESULTS

Extracellular recordings of 21 LMAN neurons from six adult male zebra finches during non-singing periods and during singing to a female ('directed' song) confirmed previous findings that neural activity in LMAN is strongly modulated during singing (Hessler and Doupe, 1999a; Leonardo, 2005). Neurons in LMAN typically increased their firing rate during directed singing compared to non-singing periods (21 of 23 neurons). The average spontaneous firing rate of LMAN neurons in awake birds when they were alone was 10.1 spikes per second (Hz) ± 5.6 (s.d.), and the average firing rate during directed singing was 22.7 Hz ± 12.4 ($p < 0.0001$; paired sign test). In addition, the firing pattern of single neurons was reliably locked to the acoustic structure of song during directed singing (Figure 2-2).

Correlation of the firing pattern of pairs of LMAN neurons

To compare the firing patterns across a population of LMAN neurons, we took advantage of the stereotyped sequence of syllables, or 'motif, in zebra finch song and aligned the activity of all single LMAN neurons recorded in an individual bird to a

common reference motif (see Materials and Methods; n = 8, 5, 3, 2, and 2 neurons in five birds). Recordings at different sites in the same bird were performed at intervals of one hour to several days, and alignment of the neural activity for each bird revealed that different neurons in LMAN exhibit different time-varying firing patterns during song. Figure 2-2 shows an example of the singing-related activity of eight LMAN neurons during repeated trials of the same motif (mean number of trials per neuron = 33 ± 15 (s.d.); range = 8 to 55). Individual neurons exhibited distinct patterns of firing tightly locked to the song (note the fine structure of the spike rasters across repeated trials), and the song-locked pattern varied across different neurons. For example, one neuron (*red*) did not fire during the interval between syllables 'c' and 'd' but consistently fired before syllable 'e'. In contrast, another neuron (*black*) fired two spikes consistently in the interval between 'c' and 'd' but was silent during the interval between 'd' and 'e'. To quantify the degree of similarity between activity patterns across neurons in the same bird, we calculated the cross-covariance of the mean activity patterns across repeated motif renditions between pairs of LMAN neurons (i.e., cross-correlation after subtracting the mean firing rates). For the example shown in Figure 2-2, the mean correlation was 0.09 ± 0.25 (s.d.). Across all birds, the mean correlation between firing patterns of pairs of LMAN neurons was 0.12 ± 0.26 (43 pairwise correlations in five birds; range = -0.45 to 0.55). Neurons often fired out of phase with one another, and only 2 of 43 pairwise correlations were > 0.5.

When multiple neurons are recorded sequentially, two explanations can account for observed differences in their firing patterns: 1) different neurons may exhibit unique firing patterns that are stable over time; or 2) all neurons may exhibit the same firing

pattern at any point in time, but the firing pattern changes over time so that the activity recorded at a later time point is different. To distinguish between these two alternatives, we first analyzed the activity patterns of neurons that were recorded for a period of three to four hours to determine the stability of the patterns over time. We compared the activity pattern during the first hour of recording with that in the last hour of recording (n = 4 neurons in two birds; at least nine trials per hour). Across all birds, the mean correlation was 0.80 ± 0.04 (s.d.) (range = 0.76 to 0.85). Thus, the pattern of activity of a single neuron remained stable over a period of several hours.

In addition, in a few experiments, two single neurons were recorded simultaneously on different electrodes ~300 μm apart (n = 2 pairs in two birds). In both experiments, the pair of neurons exhibited very different song-locked spiking patterns. Figure 2-3 illustrates one example of simultaneously recorded neurons that fired during different parts of song during undirected singing. One neuron (*red*) fired consistently during syllable 'a', while the other neuron (*blue*) fired reliably during syllable 'b'. The correlation between the activity patterns of these two neurons was low (0.28). Thus, neurons in LMAN do not all share the same firing pattern; rather, different neurons can exhibit distinct firing patterns during song. These findings are similar to a previous report that neurons in the premotor nucleus RA, which receives a direct excitatory projection from LMAN, generate unique bursts of activity during song (Leonardo and Fee, 2005).

Context-dependent differences in the firing properties of single neurons in LMAN

In addition to the different patterns of activity exhibited by different LMAN neurons, we found that the firing properties of single neurons were strongly modulated by social context, as observed previously (Hessler and Doupe, 1999b; Leonardo, 2002). Firing rate typically increased during singing compared to during non-singing periods (Figures 2-4, 2-5a), and the increase in firing rate was greater when a bird sang alone ('undirected' song) than when he sang to a female. Across all recordings, the firing rate was significantly greater during undirected song than during directed song (Figure 2-5b; $p < 0.0005$; paired sign test). This difference was significant in 10 of 12 single units. In addition, LMAN neurons frequently fired short bursts of action potentials during undirected song but rarely during directed song. Figure 2-4 illustrates a representative experiment in which the male sang > 50 renditions of the motif in each behavioral condition. Undirected and directed trials were interleaved during the experiment, and the firing properties changed within seconds after a female was presented or removed. In this example, a difference in bursting between the two conditions is apparent in the histograms of the inter-spike intervals (ISIs). There is a sharp peak in the ISI histogram before 5 msec (instantaneous firing rate $\geq$ 200 Hz) during undirected song but not during directed song. When we use a threshold instantaneous firing rate of 200 Hz to define burst onsets and offsets, the number of bursts per second was 3.9 $\pm$ 2.4 during undirected song and 0.4 $\pm$ 0.8 during directed song. Across all recordings, burst rate was significantly greater during undirected song compared to that during directed song ($p <$ 0.0005; paired sign test).

Trial-by-trial variability in the pattern of activity of single LMAN neurons was also modulated by social context. Across repeated undirected trials, the firing pattern was not very reproducible and did not appear to be consistently locked to the acoustic structure of song (e.g., Figure 2-4b, *top*). In contrast, during directed trials, the neuron's firing pattern was reliably locked to the song (e.g., Figure 2-4b, *middle*). Despite the greater trial-by-trial variability during undirected song, the average pattern of activity across many trials was similar across behavioral conditions (e.g., Figures 2-4c, 2-6a), as observed previously (Hessler and Doupe, 1999a). For the example in Figure 2-4, the correlation coefficient between the activity patterns in the two conditions was 0.74. Across all recordings, the mean correlation between the firing patterns of a single neuron across behavioral conditions was 0.70 ± 0.12 (range: 0.49 to 0.87). The similarity between the mean firing patterns of a single neuron across social context contrasts sharply with the different firing patterns exhibited by different LMAN neurons in the same bird (e.g., Figures 2-2, 2-3; $p < 0.0001$, Mann–Whitney U test). Thus, although there is variability in the pattern of activity across repeated trials, single neurons in LMAN appear to exhibit characteristic activity patterns associated with song.

Trial-by-trial variability of single neurons is greater during undirected song

To characterize the context-dependent differences in the trial-by-trial variability of single LMAN neurons, we computed the Fano factor (F), which is defined as the ratio of the spike count variance to the mean spike count. This measure has a value of 1 for a Poisson process, in which individual spikes are generated at random times according to a time-varying firing rate. Spike counts were obtained in a sliding 30 msec window for

each trial (see Materials and Methods), and the mean count and variance were calculated for each behavioral condition. We found that the Fano factor (F) varied as a function of time in the motif and was generally higher throughout the entire motif in the undirected condition compared to the directed condition.

Figure 2-6 shows representative examples of the modulation of firing rate and spike count variability during song for two LMAN neurons. The mean spike count per window represents a smoothed version of the PSTH (Figure 2-6a). Overall, variability was higher throughout the motif during undirected song. The average F for all 30 msec windows (time-averaged F) in the undirected and directed conditions was 2.03 versus 0.62 and 1.82 versus 0.67, respectively. Across the entire population, the time-averaged F during the motif was significantly greater in the undirected condition (Figure 2-6c; mean F: 1.97 versus 0.71; $p = 0.0005$; paired sign test). Similar results were obtained with 100 msec sliding windows (mean F: 2.4 versus 0.61; $p = 0.0005$; paired sign test; *data not shown*).

In addition, we found that trial-by-trial variability was inversely related to the firing rate in the directed condition: variability was lower when the firing rate was higher, and vice versa. For example, for the second neuron shown in Figure 2-6 ($LMAN_2$), the mean spike count changed from 0 to 2.7 spikes per 30 msec window (90 Hz) during directed song, and the variability reached a minimum F of 0.18 during times of maximum firing. In contrast, there was no obvious relationship between the firing rate and spike count variability in the undirected condition, suggesting that firing rate alone could not predict variability.

Firing rate does not account for context-dependent differences in trial-by-trial variability

In general, spike count variability is inversely related to firing rate: as firing rate increases, variability decreases because of the greater effect of refractory periods at higher firing rates (Berry et al., 1997; Berry and Meister, 1998; Kara et al., 2000). For LMAN neurons, however, we found that both firing rate and spike count variability were greater during undirected song than during directed song. To investigate whether the context-dependent differences in firing rate could account for the context-dependent differences in variability, we analyzed the variability of singing-related activity as a function of firing rate (spike count). For each neuron, we compared the variance in the spike count to the mean spike count in each window (30 or 100 msec) throughout the motif (Figure 2-7a). While this analysis does not take into account the temporal relationship between song and firing rate, it allows comparison of variability across behavioral conditions at a particular firing rate. In the directed condition, across a range of mean counts (firing rate), many windows had variance below the mean spike count ($F < 1$). In contrast, in the undirected condition, the variance generally exceeded the mean spike count ($F > 1$). At a given mean count (e.g., shaded region in Figure 2-7a), variability was greater in the undirected condition compared to the directed condition. For example, we averaged the Fano Factor for all 100 msec windows that had a mean spike count between one and two spikes (firing rate = 10–20 Hz). Even when the firing rate was the same, variability was greater in the undirected condition (Figure 2-7b; mean F at 10–20 Hz: 2.03 versus 0.58; $p = 0.0078$; paired sign test). Thus, firing rate alone could not account for the context-dependent differences in trial-by-trial variability.

Bursts contribute to context-dependent differences in trial-by-trial variability

Another conspicuous context-dependent difference in the activity of single neurons was the presence of bursts during undirected song (Figures 2-4, 2-5b). In other systems, it has been shown that spike count variability is substantially higher when cells fire bursts of spikes (Kara et al., 2000). We considered two ways in which bursts could contribute to the greater trial-by-trial variability in the undirected condition: 1) if the number of spikes within a burst was variable; and 2) if the bursts occurred at different times in the motif across trials. We found that the number of spikes per burst varied in the undirected condition, both within a trial and across trials (e.g., Figure 2-4b, *top*: mean number of spikes per burst was 3.1 ± 1.4; range: 2 to 8). In contrast, during directed song, if the neuron did fire a burst, the number of spikes per burst was typically two (Figure 2-4b, *bottom*: mean number of spikes per burst was 2.0 ± 0). Across all neurons, the mean number of spikes per burst was 3.0 ± 0.3 (s.d.) during undirected song and 2.1 ± 0.1 during directed song (Figure 2-8a; $p < 0.0005$; paired sign test). Moreover, variability in the number of spikes per burst was significantly greater during undirected song than during directed song (Figure 2-8b; mean coefficient of variation: 0.43 versus 0.09; $p < 0.0005$, paired sign test).

To investigate whether the timing of bursts varied across repeated trials of undirected song, we measured the onsets of bursts in each trial. Figure 2-9a illustrates the variable number and timing of bursts across repeated undirected trials for the LMAN neuron shown in Figure 2-4. Bursts did not occur reproducibly at particular times in the

motif (note the absence of peaks in the distribution of burst onsets, Figure 2-9b); rather, bursts occurred at different times in each trial.

To further examine the contribution of bursts to variability, we treated bursts as unitary events and retained only the first spike of each burst in our analysis. When we retained only the first spike per burst, trial-by-trial variability was significantly reduced during undirected song, and the context-dependent difference in variability was substantially reduced compared to the original data. Figure 2-10 illustrates the effect of treating bursts as unitary events for the neuron shown in Figure 2-4. In the absence of bursts, the firing rate was substantially reduced in the undirected condition but not in the directed condition (compare Figure 2-10b and Figure 2-6a, *top left*). In addition, the context-dependent difference in trial-by-trial variability was substantially reduced (Figures 2-10c, d). When we calculated the Fano Factor (F) in a 30 msec sliding window, in the undirected condition, many more windows had variance close to the mean count (time-averaged $F = 1.13$; Figures 2-10c, d) than in the original data (time-averaged $F = 2.0$; Figure 2-6b, *left*). In contrast, variability in the directed condition did not change substantially, given the small number of bursts in the directed condition (time-averaged F without bursts = 0.61; time-averaged F with bursts = 0.62).

For the population of neurons, the time-averaged F was significantly lower in the absence of bursts compared to the original data both in the undirected condition (means: 1.04 versus 1.97, respectively; $p = 0.0005$, paired sign test; compare Figures 2-11a and 2-6c) and in the directed condition (means: 0.67 versus 0.71; $p = 0.006$, paired sign test). Similar results were obtained when we calculated F with a 100 msec sliding window (undirected means: 1.19 versus 2.37; $p = 0.0005$, paired sign test; directed means: 0.56

versus 0.61; $p = 0.006$, paired sign test; *data not shown*). Thus, bursting contributed significantly to trial-by-trial variability.

Bursting alone, however, did not completely account for the context-dependent differences in variability. In the absence of bursts, context-dependent differences in variability, while substantially reduced, persisted. When we retained only the first spike per burst, the firing pattern across repeated trials was still more variable in the undirected condition and did not become reliably locked to the acoustic structure of song (e.g., Figure 2-10). Across all recordings, in the absence of bursts, variability was significantly greater during undirected song (Figure 2-11a; mean time-averaged F: 1.04 versus 0.67; $p < 0.0005$, paired sign test). Similarly, at a given firing rate, variability was significantly greater during undirected song, even in the absence of bursts (Figure 2-11b; mean F at 10-20 Hz: 1.23 versus 0.54; $p = 0.002$, paired sign test). Thus, although context-dependent differences in bursting contributed to the context-dependent differences in variability, bursting alone is not sufficient to account for the greater trial-by-trial variability during undirected song.

DISCUSSION

This study shows that social context can have a profound influence on the firing properties of single neurons in LMAN. During directed singing, LMAN neurons exhibited firing patterns that were reliably locked to the acoustic structure of song. Trial-by-trial variability was low and reliability was high compared to that of a Poisson process ($F < 1$; Figures 2-5–2-7). In contrast, during undirected singing, the firing rate was higher, and the firing pattern was not reliably locked to song. Trial-by-trial variability

during undirected song was high ($F \sim 2$; Figures 2-4, 2-6, and 2-7), in part due to the presence of bursts (Figures 2-8–2-10). Despite the socially driven differences in the level and variability of activity, the average pattern of activity for a particular neuron was similar across behavioral contexts (e.g., Figure 2-4). Thus, individual LMAN neurons exhibited characteristic patterns of activity correlated with song. By recording the activity of multiple single neurons in individual birds, we also show that different LMAN neurons can exhibit distinct firing patterns during song. Together, these findings are consistent with a premotor role for LMAN and suggest that activity in LMAN may modulate the ongoing activity in the premotor nucleus RA and subsequent song output.

Reproducibility and variability in the spike trains of single LMAN neurons

Our finding that single LMAN neurons exhibit reliable patterns of activity correlated with the acoustic structure of song during directed singing generally confirms and extends previous studies (Hessler and Doupe, 1999a; Leonardo, 2005). Consistent with previous reports, we found that spike count variability in LMAN was low across repeated trials of directed song, regardless of the size of the counting window ($F < 1$; e.g., Figure 2-4; Figures 2-6, 2-7). In addition, by recording from multiple single neurons in LMAN in individual birds and aligning their activity to a common reference motif, we found that different LMAN neurons exhibited different spiking patterns. Individual neurons turned off and on at different times in song in a predictable manner (e.g., Figures 2-2, 2-3). This result contrasts with a previous report that multi-unit activity in LMAN was very similar across different recording sites in the same bird (Hessler and Doupe, 1999b). While that study emphasized the similarity between the average activity patterns

across recording sites, we found that the mean correlation between the firing patterns of pairs of LMAN neurons was low (0.12 ± 0.26 (s.d.)).

Several factors may account for the overestimation of the similarity of activity patterns across recording sites in LMAN by Hessler and Doupe (1999b). First, the main difference between the two studies is that we recorded the activity of single LMAN neurons while they primarily characterized multi-unit activity in LMAN. Differences in the temporal resolution of the two studies may explain the different conclusions. Many of the neurons that we recorded in an individual bird exhibited similar patterns of activity that were slightly out of phase with one another (e.g., Figure 2-4). Combining the activity of multiple single neurons would tend to obscure the small differences in the timing of spikes across neurons, so that in general, the peaks and troughs in the average pattern of activity might appear to occur in similar locations in song across recording sites, as reported by Hessler and Doupe. Only when we compared the activity of single units did the differences in spike timing become apparent across neurons.

Second, Hessler and Doupe did not take into account the differences in the durations of syllables and intervals across repeated trials. In their study, the motif and associated neural activity were aligned by maximizing the overlap in the amplitude envelopes of the motif across repeated trials. Differences in the durations of syllables and intervals across trials persisted, resulting in variability in the aligned neural signals. Such variability in the neural signals is greater at time points farther from the points of alignment (see Chi and Margoliash, 2001) and may have obscured small differences in the timing of spikes across trials. In contrast, in this study, we performed a piecewise linear time warp to compensate for differences in syllable and interval durations across

trials (see Materials and Methods). This algorithm substantially reduced variability in the temporal registry of the spike trains and revealed reliable firing patterns across trials that were not always apparent in the raw data.

Comparison with the motor pathway

Comparison of the findings of this study with previous reports of the singing-related activity of single neurons in the premotor nucleus RA reveals several common features between the activity of LMAN and RA neurons. First, single neurons in both LMAN and RA exhibit firing patterns that are reliably locked to the acoustic features of song during directed singing (Yu and Margoliash, 1996; Leonardo, 2005; Leonardo and Fee, 2005). Second, in both nuclei, the pattern of singing-related activity differs across multiple neurons in the same bird (Leonardo and Fee, 2005). In both LMAN and RA, individual neurons in the same bird exhibit unique time-varying firing patterns; the mean correlation in the firing patterns across pairs of neurons in each nucleus is ~ 0 (LMAN: 0.12 ± 0.26 (s.d.); RA: 0.015 ± 0.12; Leonardo and Fee, 2005). Such similarities between the activity of neurons in LMAN and RA raise the possibility that LMAN neurons, like RA neurons, act jointly to encode the acoustic features of song (Leonardo and Fee, 2005). The different temporal patterns of spikes across multiple LMAN neurons may carry information beyond that encoded by the firing pattern of single neurons.

In contrast to the shared features in the activity of LMAN and RA neurons, comparison of singing-related activity across the two nuclei also reveals several differences. First, singing-related activity in RA in the directed condition is characterized by trains of short bursts of action potentials separated by periods of inhibition (Yu and

Margoliash, 1996; Leonardo and Fee, 2005). Burst onset times and the pattern of spikes within a burst are highly stereotyped in RA neurons across repeated directed trials. In contrast, LMAN neurons rarely fire bursts of action potentials during directed song (Figure 2-5b). Second, LMAN and RA neurons differ in the reproducibility of their firing patterns across repeated directed trials. In RA neurons, across repeated trials of directed song, the onset times of bursts and the pattern of spikes within a burst are reproduced with millisecond precision relative to the acoustic features of song (Chi and Margoliash, 2001; Leonardo and Fee, 2005). In contrast to the "machine-like" precision of RA activity, the exact timing and pattern of spikes of LMAN neurons are more variable across repeated directed trials (e.g., Figures 2-2, 2-3), in part because LMAN activity lacks the stereotyped burst structure of RA neurons. Such differences in the activity of RA and LMAN neurons may reflect the different contributions of the two nuclei to song production and plasticity. While RA is required throughout life to generate the motor commands to precisely coordinate the vocal and respiratory musculature for song, LMAN is necessary for song plasticity but not for the production of learned song.

Context-dependent differences in neural variability: potential sources and consequences

Previous recordings of the singing-related activity of single neurons in the motor pathway have focused primarily on directed song and neglected undirected song, in part because of the difficulty of holding single units for the time required to record spontaneous rather than female-triggered singing (Yu and Margoliash, 1996; Hahnloser

et al., 2002; Leonardo and Fee, 2005). Therefore, it is not known whether variability in the activity of single neurons in the premotor nuclei HVC and RA is modulated by social context.

A previous study of the singing-related expression of immediate early genes (IEGs) suggests that socially driven differences in LMAN activity do not originate in HVC (Jarvis et al., 1998). Both Area X and LMAN show much higher levels of the transcription factor *zenk* (also known as *zif*-268 and *egr*1) during undirected singing than during directed singing (Jarvis et al., 1998), consistent with more *zenk* being induced by the greater level of activity and degree of bursting during undirected singing. In contrast, *zenk* expression was not significantly different in HVC neurons across social context. This finding suggests that changes in AFP activity do not derive from socially driven alterations in the level of HVC activity. It remains to be determined, however, whether HVC neurons that project to the AFP exhibit differences in trial-by-trial variability, a property that cannot be determined from IEG expression.

Despite the absence of context-dependent differences in *zenk* expression in HVC, the level of *zenk* expression in RA was greater during undirected singing than during directed singing (Jarvis et al., 1998), suggesting that context-dependent differences in LMAN activity may drive changes in RA activity. Further elucidation of the origins and consequences of socially driven differences in LMAN variability will benefit from direct measurements of the variability of identified neurons at multiple levels of the song motor hierarchy.

Potential source of modulatory input to the AFP

Context-dependent differences in the firing properties of single LMAN neurons may derive from socially driven alterations in neuromodulatory inputs from outside of the song system. One candidate for the source of modulation is the midbrain nucleus VTA (ventral tegmental area), which sends a dense dopaminergic projection to the AFP (Lewis et al, 1981; Bottjer et al., 1989). Dopaminergic neurons are thought to participate in reward processing and reinforcement learning in vertebrates (for review, see Schultz, 2004), and dopamine is known to play a role in courtship and sexual behavior in birds (Balthazart et al., 1997, 2002) and in mammals (Hull et al., 2004). In addition, dopamine can alter the responsiveness of cells to extrinsic inputs through a number of mechanisms, including modulation of voltage-dependent conductances and synaptic transmission (for review, see Nicola et al., 2000). In other systems, dopamine has been shown to selectively filter cortical inputs to the striatum (Bamford et al., 2004) and to alter the signal to noise ratio for evoked activity in the striatum and nucleus accumbens (Nicola et al., 2000). Thus, dopamine is a likely candidate for mediating the socially driven differences in the excitability of LMAN neurons.

In songbirds, dopamine has been shown to directly modulate the synaptic inputs and excitability of neurons in the AFP (Ding and Perkel, 2002; Ding et al., 2003). In addition, preliminary *in vivo* extracellular recordings have found that the singing-related activity of VTA neurons is strongly modulated by social context (Yanagihara and Hessler, *personal communication*). VTA neurons that have low spontaneous firing rates increase their activity during directed singing but not during undirected singing, and VTA neurons with high spontaneous activity lower their firing rate only during directed

singing. Such socially driven differences in the activity of VTA neurons are consistent with the hypothesis that dopaminergic neurons may be the source of modulatory input to the AFP. Future experiments manipulating dopamine transmission in the AFP will be necessary to further clarify the possible role of this neurotransmitter in the social modulation of singing-related activity in the AFP.

Potential consequences of the social modulation of LMAN activity

The consequences of the socially driven differences in the variability of single LMAN neurons on the activity of their postsynaptic targets in RA remain unclear. One possibility is that activity in LMAN directly modulates the ongoing patterns of activity in the motor pathway. According to this hypothesis, greater variability in LMAN activity during undirected song should give rise to greater variability in RA activity. A variety of evidence is consistent with this hypothesis. First, manipulation of LMAN activity during singing induces specific changes in song in real time (Kao et al., 2005). Second, lesions of LMAN reduce the moment-by-moment variability in undirected song (Kao et al., 2005; see also chapter 4). Finally, preliminary recordings of single RA neurons suggest that the firing patterns of some RA neurons are less reliable during undirected song than during directed song (*unpublished data* cited in Chi and Margoliash, 2002; N. Hessler and M. Kao, *unpublished data*).

Elucidation of the consequences of LMAN activity on the ongoing activity in RA may require recordings in RA in birds of different ages because the susceptibility of the motor pathway to sources of perturbation is developmentally regulated (Sohrabji et al., 1990; Scharff and Nottebohm, 1991). Previous studies have demonstrated an age-dependent stabilization of song structure: song deterioration following deafening occurs

much more quickly in young birds than in older adult birds (Lombardino and Nottebohm, 2000; Brainard and Doupe, 2001). Similarly, context-dependent differences in song variability also decline with age (see chapter 4). These findings suggest that the sensitivity of the motor program to sources of perturbation, such as altered auditory feedback or context-dependent differences in LMAN activity, declines with age. If LMAN activity only weakly influences the ongoing patterns of activity of RA neurons in adult birds, then RA neurons in older birds might not exhibit context-dependent differences in activity despite the socially driven differences in LMAN activity.

An alternative hypothesis regarding the influence of LMAN activity on RA is that LMAN activity does not directly modulate ongoing activity in RA. Rather, signals from LMAN act on a slower timescale and affect the release of trophic factors that promote cell survival, synaptogenesis, and/or the incorporation of new neurons (Johnson et al., 1997; Kittelberger and Mooney, 1999). This hypothesis is consistent with LMAN's trophic support of RA during development (Akutagawa and Konishi, 1994; Johnson and Bottjer, 1994). Moreover, there is evidence that LMAN may provide brain-derived neurotrophic factor (BDNF) to RA (Johnson et al., 1997). In many systems, it has been demonstrated that the release of neuromodulators, such as peptides and neurotrophins, depends on the pattern of electrical stimulation. High frequency bursts of electrical stimulation are more effective than tonic depolarization or low frequency stimulation for eliciting the release of neurotrophins (Balkowiec and Katz, 2000). Thus, context-dependent differences in the firing properties of single LMAN neurons may result in the differential release of trophic factors.

These two hypotheses regarding the influence of LMAN on RA are not mutually exclusive. Neural activity in LMAN may influence the motor pathway on multiple timescales, and elucidation of the consequences of the socially driven differences in LMAN activity will ultimately benefit from simultaneous recordings of singing-related activity in RA and LMAN in juvenile and adult birds in different social contexts as well as from measurements of the activity-dependent release of endogenous neurotrophins.

Contributions of LMAN activity to social influences on song production and plasticity

Context-dependent differences in the firing properties of LMAN neurons may contribute to social influences on song production and plasticity. Numerous studies have shown that social factors can shape song learning and performance. Social interactions can influence which song models are learned and which song variants are retained (Nelson and Marler, 1994; West and King, 1988). In adult birds, social factors continue to influence song performance. For example, in several species, males often sing the song in their repertoire that best matches that of a neighboring conspecific ("song matching; Payne, 1983; Stoddard, 1992). Similarly, in adult zebra finches, song variability is acutely inhibited when a male sings to a female (Sossinka and Böhner, 1980; Kao et al., 2005).

The inhibition of song variability in the directed condition suggests that directed song, which is one component of courtship behavior, may reflect a state of performance in which male birds exploit what they have already learned to select patterns of motor activity to produce their "best" current version of song. Consistent with this hypothesis,

trial-by-trial variability in LMAN activity is low during directed song. Such reproducibility in the song-locked firing patterns of LMAN neurons may facilitate reproducibility in the pattern of activity of RA neurons. In this manner, LMAN activity may help to reduce variability in motor commands and subsequent song output. Furthermore, the specific pattern of activity of LMAN neurons during directed song may act as an instructive signal to bias the motor program towards a particular target (Troyer and Doupe, 2000; Kao et al., 2005). For example, given the observed precision of firing patterns in both HVC and LMAN across repeated trials of directed song (Hahnloser et al., 2002; Leonardo, 2005), coincident input from the two nuclei may result in the strengthening of a subset of synapses in RA. In this manner, the stereotyped pattern of activity during directed song may help to guide adaptive changes in the motor pathway.

In contrast, undirected song may reflect a state of motor practice (Jarvis et al., 1998; Kao et al., 2005). In the absence of a particular audience, male birds may produce a range of vocalizations and use auditory feedback to optimize and/or maintain the song. Trial-by-trial variability in motor output is an important component of feedback-based reinforcement learning. In order for song to change, it must vary from rendition to rendition so that evaluation mechanisms can selectively reinforce the patterns of motor activity that produce the desired behavior. In adult birds, variability in motor output remains important, either for continued motor exploration and optimization or for feedback-based correction of errors, which may arise from perturbations of the motor pathway, such as changes in hormone levels or the birth, death and incorporation of new neurons (Nordeen and Nordeen, 1988; Alvarez-Buylla et al., 1990; Scharff, 2000). Consistent with this hypothesis, greater trial-by-trial variability in LMAN activity may

contribute to plasticity in undirected song by generating variability in the ongoing patterns of motor activity in RA and subsequent song output (Doya and Sejnowski, 2000; Kao et al., 2005). Indeed, lesions of LMAN reduce the moment-by-moment variability in undirected song, suggesting that song variability is regulated in part by signals from the AFP (Kao et al., 2005; see chapter 4). In addition, LMAN bursts during undirected song may be an important mechanism for altering the ongoing patterns of activity in RA. By enhancing neurotransmitter release, bursts are more effective than single spikes at driving post-synaptic neurons (Lisman, 1997), and they are important for activity-dependent synaptic plasticity in many systems.

Thus, by affecting the format of information transmission from LMAN to RA (e.g., variable versus reliable patterns of activity and bursts versus single spikes), social context may influence song production and plasticity in multiple ways. Simultaneous measurement of the level and variability of activity across multiple levels of the song system are necessary in future studies to further elucidate the origins and extent of neural variability in the song system as well their consequences for song production and plasticity.

Figure 2-1 Schematic diagram of the song system. The 'motor pathway' (*gray*), which is required throughout life for normal song production, includes HVC, the robust nucleus of the arcopallium (RA), and the tracheosyringeal portion of the hypoglossal nucleus (nXIIts). The 'anterior forebrain pathway' (AFP, *black*) is necessary for vocal motor plasticity but is not required for song production. The AFP consists of Area X, the medial nucleus of the dorsolateral thalamus (DLM), and the lateral magnocellular nucleus of the anterior nidopallium (LMAN).

Figure 2-1

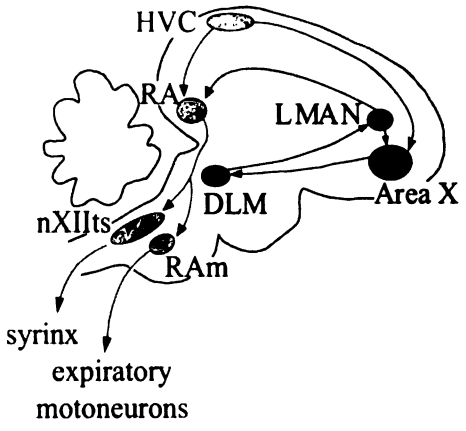

HVC
RA
LMAN
Area X
DLM
nXIIts
RAm
syrinx
expiratory
motoneurons

Figure 2-2 Activity of a population of LMAN neurons in one bird. *A*. Spectrogram (plot of frequency versus time) of the bird's motif ('abcdef'). *B*. Raster plot of the song-aligned activity of eight LMAN neurons during multiple renditions of the song motif. Each dot in the raster plot represents an action potential. Each row represents the singing-related activity during one motif, or trial. Different colors indicate different LMAN neurons, and the activity has been aligned to a common time axis using a reference song motif (see Materials and Methods). Vertical lines indicate syllable onsets. Songs and neural activity were collected when the male sang to a female (directed condition). Individual LMAN neurons exhibited different patterns of activity during the motif.

Figure 2-2

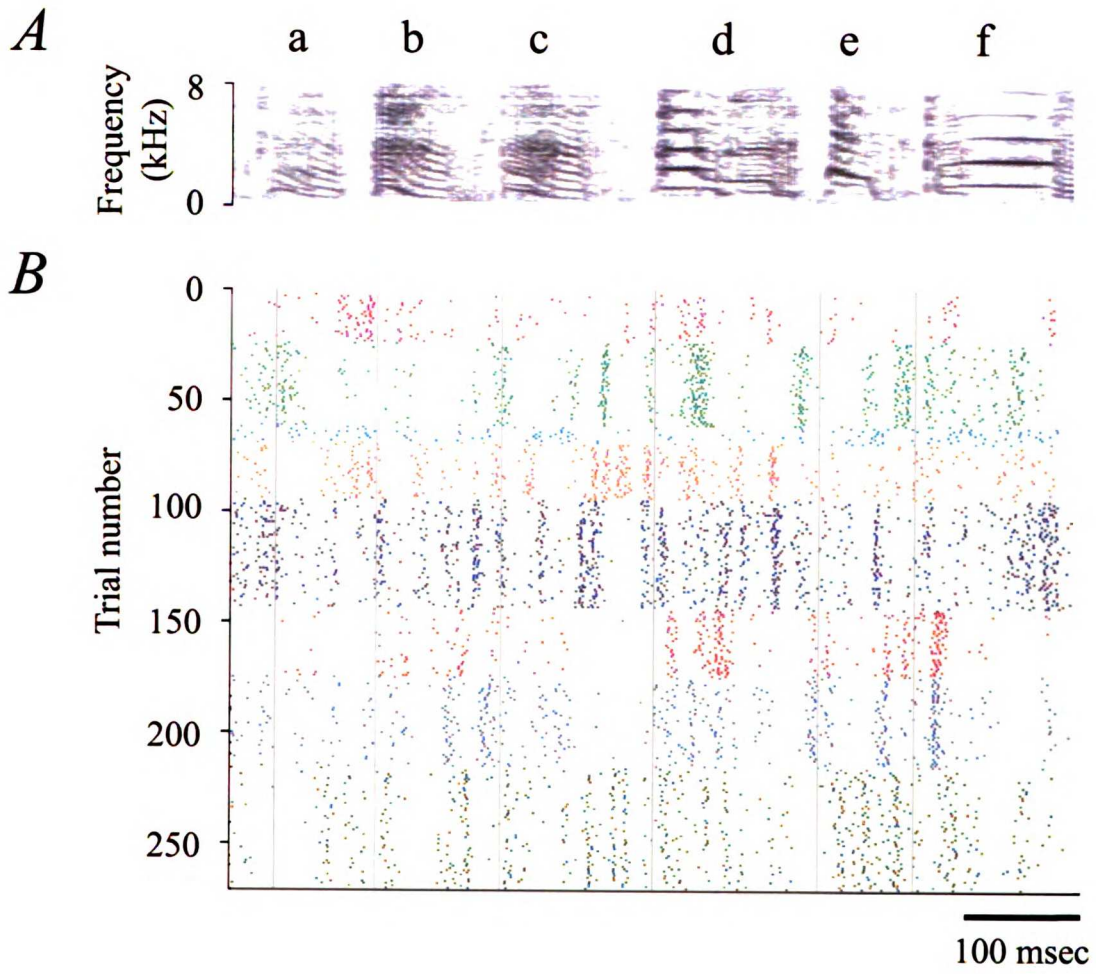

A
a
b
c
d
e
f
Frequency (kHz)
8
0
B
0
50
100
150
200
250
Trial number
100 msec

Figure 2-3 Different LMAN neurons exhibit distinct time-varying firing patterns. *A*. Spectrogram of a typical song motif for another bird ('ab'). *B*. Simultaneous recording of two LMAN neurons are shown for 15 renditions of the motif during undirected song (see Materials and Methods). Trials were chosen at random from the entire recording session (181 trials). Neurons were recorded on two electrodes ~300 μm apart, and they fired at different times in the motif. *C*. Peristimulus time histograms (PSTHs) for all 181 trials show that the two neurons fired out of phase from each other. The correlation coefficient for the PSTHs is 0.28.

Figure 2-3

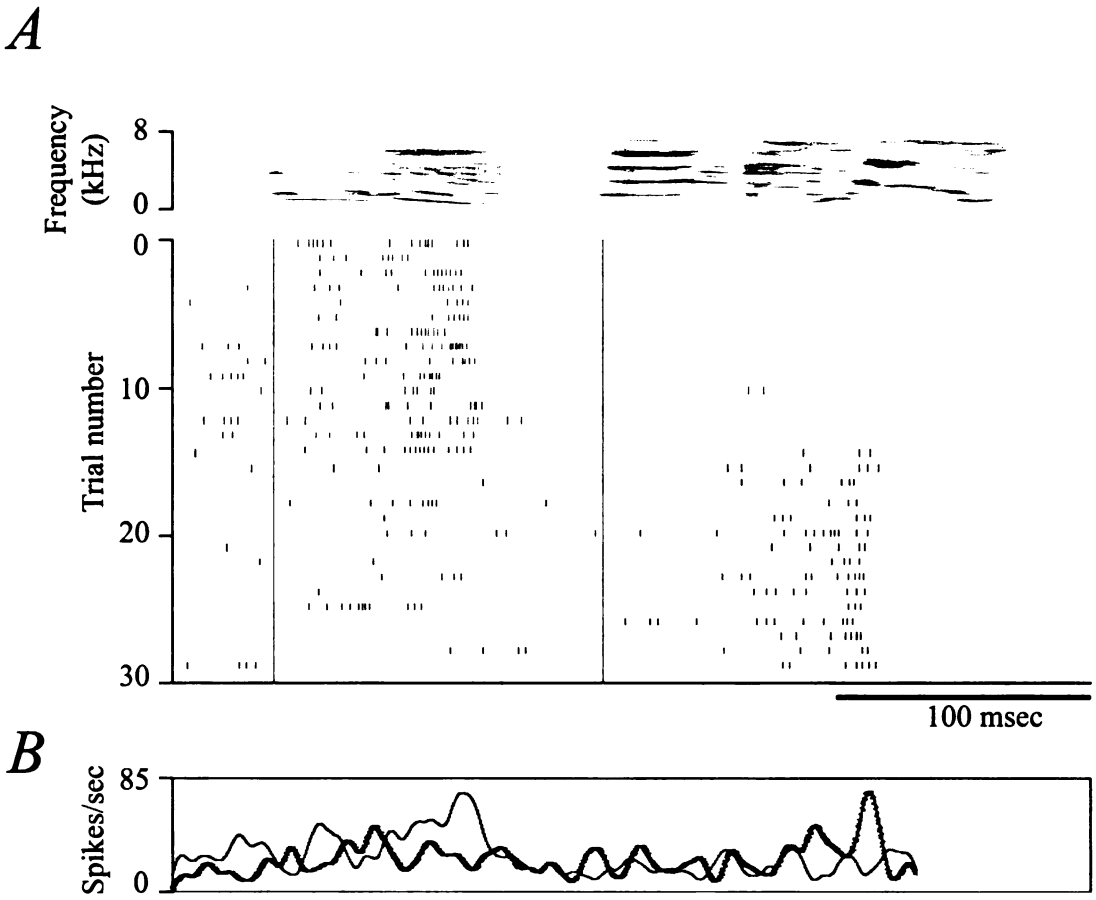

A
Frequency (kHz)
8
0
0
10
20
30
Trial number
100 msec
B
Spikes/sec
85
0

Figure 2-4 Single unit recording in LMAN during undirected and directed song. *A.* Spectrogram of a bird's motif ('abcde'). *B.* Singing-related activity of a single LMAN neuron during multiple renditions of the song motif in the undirected condition (*top*) and the directed condition (*middle).* Spontaneous activity of the neuron when the male was alone is plotted below (*bottom*). Histograms of the inter-spike intervals (ISIs) are shown for each condition (*right*). Firing rate increased during singing compared to the baseline spontaneous activity (mean spontaneous firing rate = 8.3 Hz ± 3.4 (s.d.); mean undirected firing rate = 25.4 Hz ± 5; mean directed firing rate = 23.9 Hz ± 5). The neuron frequently produced short bursts of action potentials in the undirected condition and rarely in the directed condition (note the peak in the ISI histogram from 0–5 msec in the undirected condition). In addition, the neuron's firing pattern was not very reproducible across repeated undirected trials. In contrast, the firing pattern was reliably locked to the acoustic structure of song in the directed condition. *C.* PSTHs of the same data are plotted below. The average pattern of activity of the neuron was similar across behavioral conditions (correlation coefficient = 0.74), despite the greater trial-by-trial variability in the undirected condition.

Figure 2-4

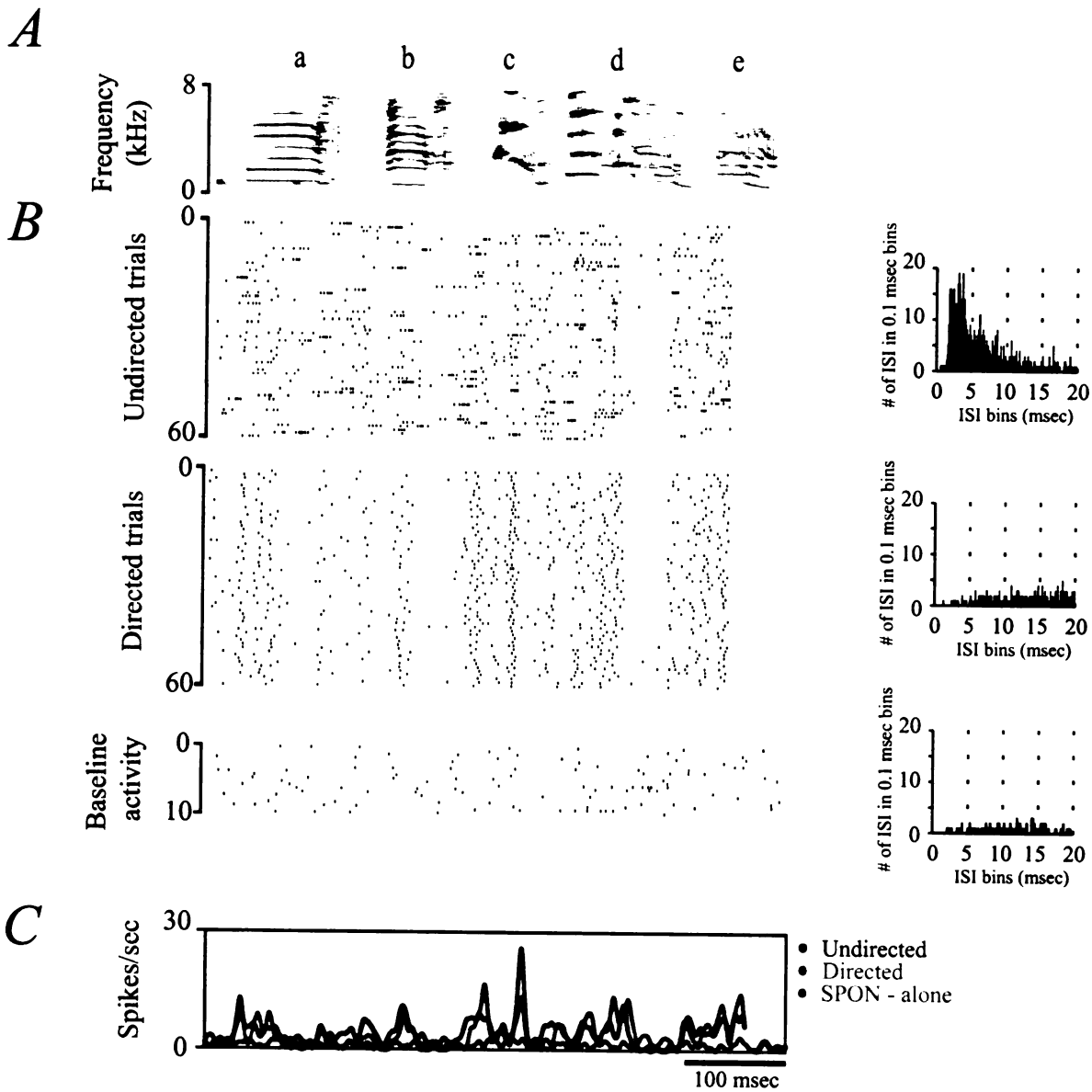

A
a
b
c
d
e
Frequency (kHz)
8
0
B
Undirected trials
0
60
Directed trials
0
60
Baseline activity
0
10
# of ISI in 0.1 msec bins
20
10
0
0 5 10 15 20
ISI bins (msec)
C
Spikes/sec
30
0
100 msec
Undirected
Directed
SPON - alone

Figure 2-5 Summary of the firing properties of single LMAN neurons in different behavioral contexts. *A.* The mean firing rate significantly increased during undirected singing ($p = .0005$, paired sign test) and during directed singing ($p = .006$, paired sign test) compared to baseline spontaneous activity. Across all neurons, the firing rate was significantly greater during undirected song compared to during directed song ($p = 0.0005$, paired sign test). Different symbols denote different neurons, and bars indicate the average for the population. For all subsequent figures, the same symbol is used for a particular neuron, double asterisks denote $p < 0.001$, and a single asterisk denotes $p < 0.01$. *B.* Burst rate was significantly greater during undirected singing compared to the spontaneous activity ($p = 0.0005$, paired sign test) and compared to directed singing ($p = 0.0005$, paired sign test).

Figure 2-5

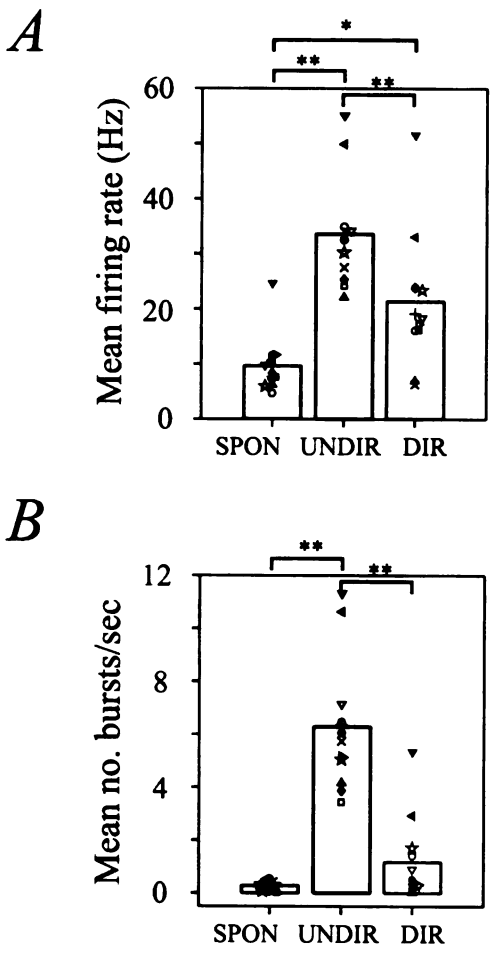

A
*
**
**
60
40
20
0
Mean firing rate (Hz)
SPON UNDIR DIR
B
**
**
12
8
4
0
Mean no. bursts/sec
SPON UNDIR DIR

Figure 2-6 Modulation of spike count variability by social context. *A*. Mean spike count (firing rate) in a sliding 30 msec window for two LMAN neurons during undirected (*black*) and directed (*red*) song. Each point represents the spike count in a 30 msec window, computed at times t separated by 1 msec. Data on the left are from the same cell shown in Figure 2-4. *B*. Fano factor (variance of the spike count divided by the mean spike count; *F*) for the same data through the course of the motif. Variability was generally higher throughout the motif during undirected song. During directed song, *F* was inversely related to the firing rate. There was no obvious relationship between firing rate and variability in the undirected condition. Data for a Poisson process would fall on the line of mean = variance ($F = 1$). *C*. The average *F* across all windows in a motif is plotted for each neuron. Bars indicate the mean time-averaged *F* across all neurons. The time-averaged *F* was greater during the undirected condition for all the neurons that we recorded ($p = 0.0005$, paired sign test).

Figure 2-6

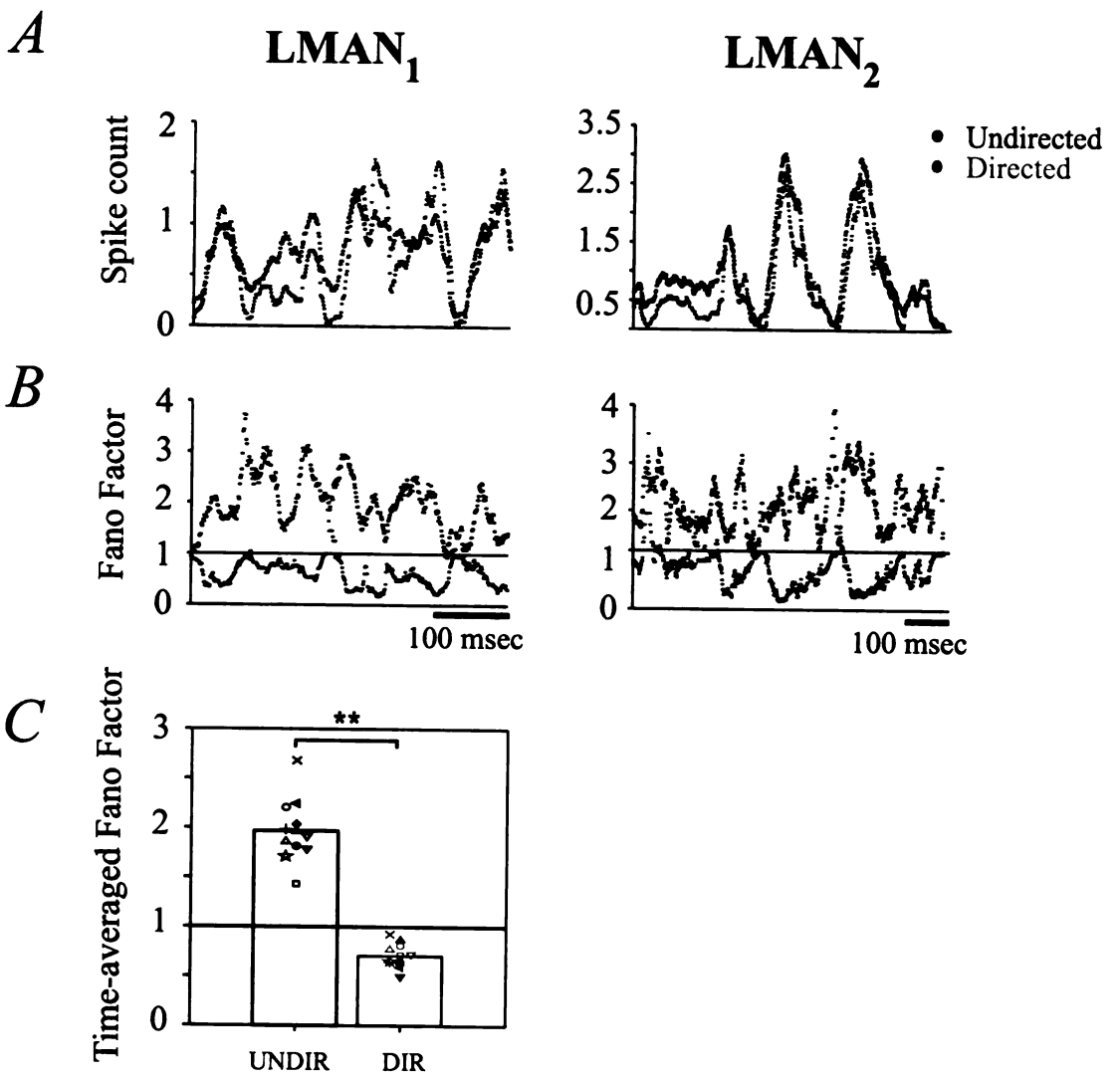

A
LMAN1
LMAN2
Spike count
2
1
0
3.5
2.5
1.5
0.5
Undirected
Directed
B
Fano Factor
4
3
2
1
0
100 msec
100 msec
C
Time-averaged Fano Factor
3
2
1
0
**
UNDIR
DIR

Figure 2-7 Firing rate alone cannot account for differences in trial-by-trial variability across behavioral context. *A*. Mean spike count versus variance for the same two cells shown in Figure 2-6. Each point represents the mean and variance of the spike count in a window of size T (*top*: 30 msec; *bottom*: 100 msec) at a fixed time t computed from all trials during undirected (*black*) and directed (*red*) song. Even when the firing rate is the same, variability is greater during undirected song. The data for a Poisson process would fall on the line of mean = variance ($F = 1$; diagonal line). The shaded region shows the range of spike counts in a 100 msec window (1–2 spikes) analyzed in (*B*). *B*. The mean F for all 100 msec windows that had between 1 and 2 spikes is plotted for individual neurons. Bars indicate the average for the population. Variability is greater during undirected song compared to during directed song, even when the firing rate is the same ($p = 0.008$, paired sign test).

Figure 2-7

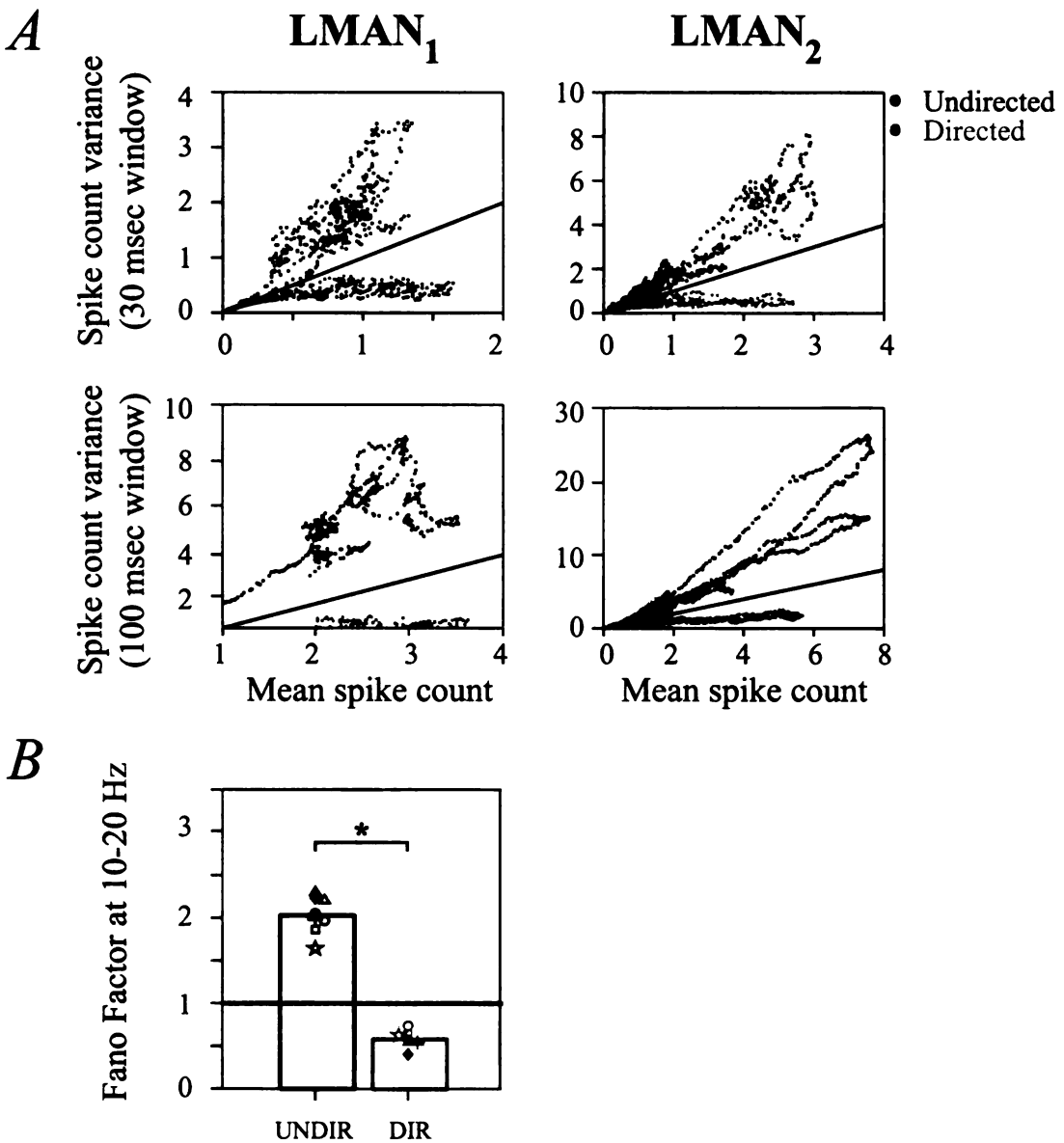

A
LMAN1
LMAN2
Undirected
Directed
Spike count variance (30 msec window)
Spike count variance (100 msec window)
Mean spike count
Mean spike count
B
Fano Factor at 10-20 Hz
UNDIR
DIR
*

Figure 2-8 Differences in bursting across behavioral contexts. *A*. The mean number of spikes per burst was significantly greater during undirected song ($p = 0.0005$, paired sign test). *B*. Variability in the number of spikes per burst was also greater in the undirected condition ($p = 0.0005$, paired sign test). The coefficient of variation (c.v.) of the number of spikes per burst is plotted for individual neurons, and bars indicate the mean c.v. across all neurons.

Figure 2-8

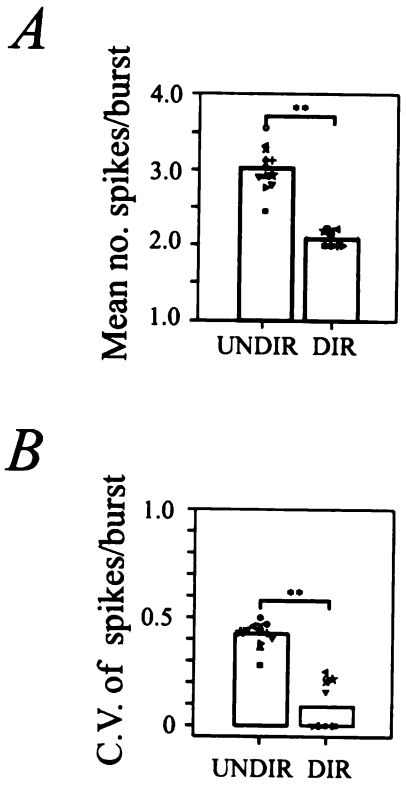

A
Mean no. spikes/burst
4.0
3.0
2.0
1.0
UNDIR DIR
B
C.V. of spikes/burst
1.0
0.5
0
UNDIR DIR

Figure 2-9 Bursts occurred at different times in each trial during undirected song.

A. Raster plot of bursts (instantaneous firing rate ≥ 200 Hz) during repeated trials for the cell shown in Figure 2-4. The timing of the bursts was not reliably locked across trials. *B.* Histogram of the burst onsets (1 msec bins) relative to the start of the motif for the same data. Burst onsets were evenly distributed across trials, except during periods when the cell was silent.

Figure 2-9

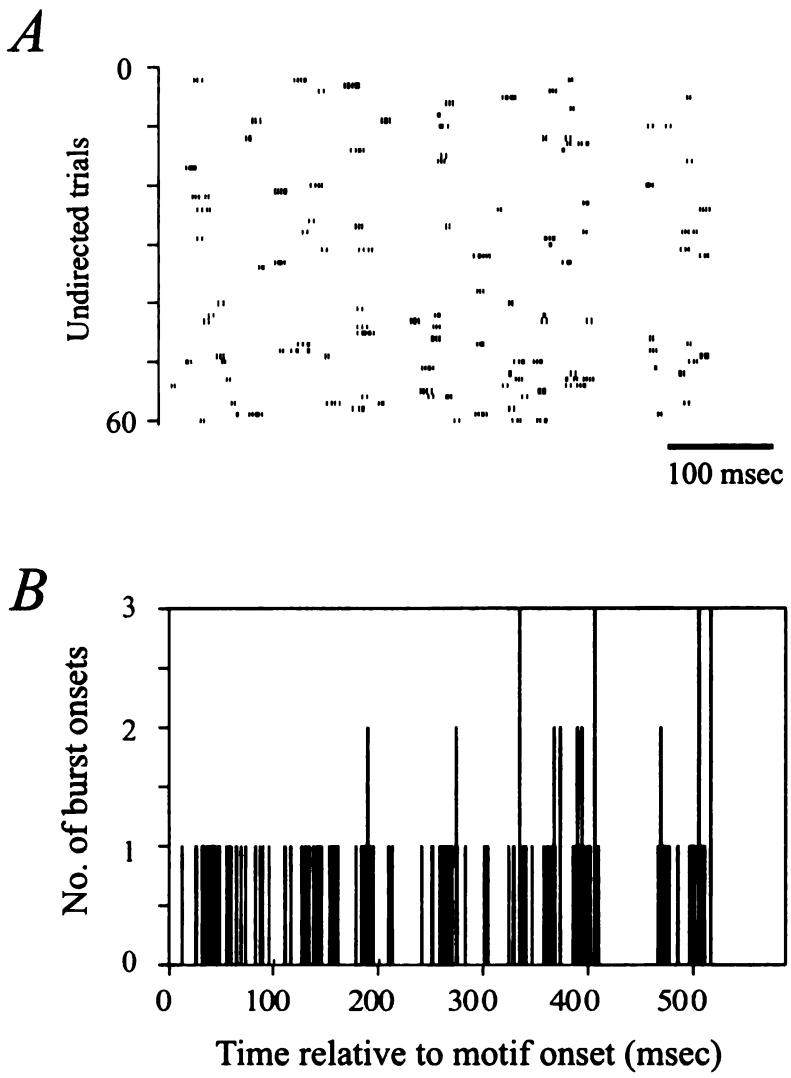

A
0
Undirected trials
60
100 msec
B
3
2
1
0
No. of burst onsets
0
100
200
300
400
500
Time relative to motif onset (msec)

Figure 2-10 Context-dependent differences in bursting contribute to greater trial-by-trial variability during undirected song. *A.* Singing-related activity when bursts are treated as unitary events and only the first spike per burst is retained. Data are from the same cell shown in Figure 2-4. The pattern of activity is more variable during undirected song (*middle*) compared to during directed song (*bottom*). *B.* Mean spike count (firing rate) in a sliding 30 msec window when only the first spike per burst is retained. *C.* Fano factor through the course of the motif for the same data. Context-dependent differences in the variability are still present but reduced compared to the original data (compare to Figure 2- 6b, *left*). Spike count variance is close to the mean count in the undirected condition. In the directed condition, many windows had variance below the mean count. *D.* Mean spike count versus variance in windows of size T (30 msec or 100 msec) when only the first spike per burst is retained. At the same firing rate, variability is greater in the undirected condition. Compared with the original data, however, many points lie along the line of mean = variance ($F = 1$) in the undirected condition (compare with Figure 2-7a, *left*).

Figure 2-10

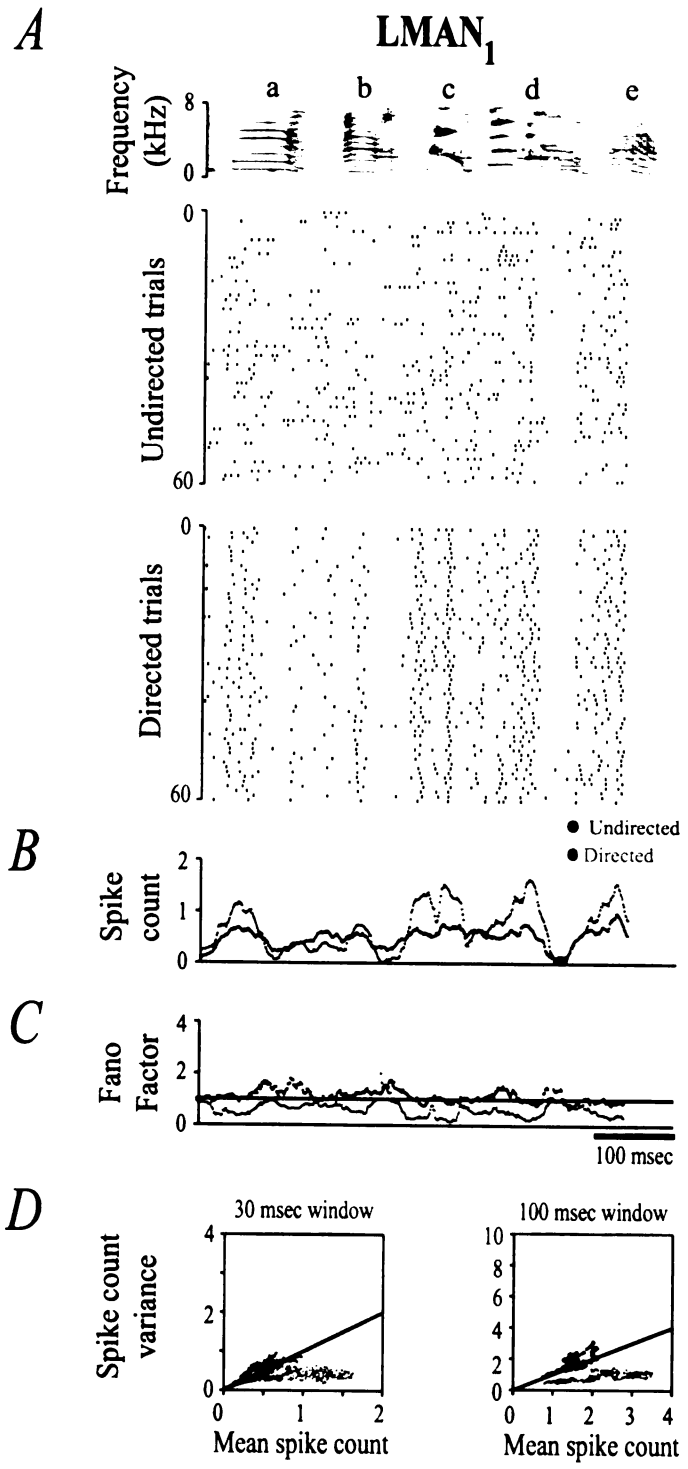
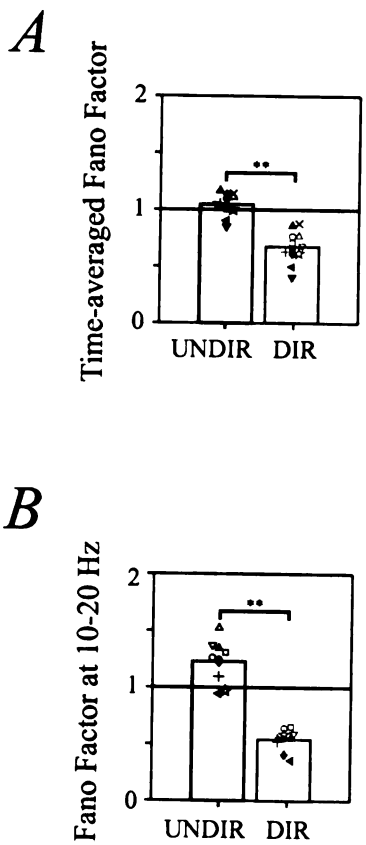

A
LMAN1
a
b
c
d
e
Frequency (kHz)
8
0
Undirected trials
0
60
Directed trials
0
60
B
Undirected
Directed
Spike count
2
1
0
C
Fano Factor
4
2
0
100 msec
D
30 msec window
100 msec window
Spike count variance
4
2
0
0 1 2
Mean spike count
10
8
6
4
2
0
0 1 2 3 4
Mean spike count

Figure 2-11 Context-dependent differences in trial-by-trial variability persist in the absence of bursting. *A.* The time-averaged *F* for each cell is plotted when bursts are treated as unitary events, and bars indicate the average for the population . *F* is significantly greater during undirected song, even in the absence of bursts ($p = 0.0005$, paired sign test). *B.* The mean *F* for all 100 msec windows that had between 1 and 2 spikes is plotted for individual cells when only the first spike per burst is retained. When the firing rate is the same, variability is significantly greater in the undirected condition ($p = 0.002$, paired sign test).

Figure 2-11


A
Time-averaged Fano Factor
2
1
0
**
UNDIR
DIR
B
Fano Factor at 10-20 Hz
2
1
0
**
UNDIR
DIR

Chapter 3: Contributions of an avian basal ganglia–forebrain circuit to real-time modulation of song

ABSTRACT

Cortical-basal ganglia circuits play a critical role in motor control and motor learning (Graybiel et al., 1994). In songbirds, the anterior forebrain pathway (AFP) is a basal ganglia-forebrain circuit required for song learning and adult vocal plasticity but not for production of learned song (Bottjer et al., 1984; Scharff and Nottebohm, 1991; Williams and Mehta, 1999; Brainard and Doupe, 2000). Here, we investigate functional contributions of this circuit to the control of song, a complex, learned motor skill. Specifically, we test the hypothesis that neural activity in the AFP of adult birds can direct moment-by-moment changes in primary motor areas responsible for generating song. We report that song-triggered microstimulation in the output nucleus of the AFP induces acute and specific changes in learned parameters of song (Tchernichovski et al., 2001; Williams et al., 1989). Moreover, under both natural and experimental conditions, variability in the pattern of AFP activity is associated with variability in song structure. Finally, lesions of the output nucleus of the AFP prevent naturally occurring modulation of song variability. Together, these findings demonstrate a previously unappreciated capacity of the AFP to direct real-time changes in song. More generally, they suggest that frontal cortical and basal ganglia areas may contribute to motor learning by biasing motor output towards desired targets or by introducing stochastic variability required for reinforcement learning.

INTRODUCTION

Song is a complex, learned motor skill that involves the precise coordination of vocal and respiratory musculature in order to produce highly stereotyped renditions of a

memorized song model. Two pathways have been identified in the songbird forebrain that contribute to this behaviour (Figure 3-1a): 1) a motor pathway that is required throughout life for normal song production (Nottebohm, et al., 1976) and 2) a basal ganglia-forebrain circuit (Perkel, 2004) the 'anterior forebrain pathway' (AFP) – that is necessary for song learning and plasticity but not for the production of adult song (Bottjer et al., 1984; Scharff and Nottebohm, 1991; Williams and Mehta, 1999; Brainard and Doupe, 2000). The AFP converges with the motor pathway at the premotor nucleus RA (robust nucleus of the arcopallium). Although lesion studies have demonstrated that the AFP is required for vocal motor plasticity (Bottjer et al., 1984; Scharff and Nottebohm, 1991; Williams and Mehta, 1999; Brainard and Doupe, 2000), its function in motor control and learning remains unclear.

Previous modelling work has hypothesized that a critical contribution of the AFP to song plasticity relies on its capacity to modulate ongoing song (Doya and Sejnowski, 2000; Troyer and Doupe, 2000). Such a modulatory influence might serve to introduce into song the stochastic variability that is necessary for reinforcement learning (Doya and Sejnowski, 2000), or to systematically bias song towards desired targets in a manner that eventually becomes encoded in the song motor pathway (Troyer and Doupe, 2000). Consistent with this hypothesis, the AFP exhibits patterned neural activity in singing birds (Hessler and Doupe, 1999a, b). However, previous studies have failed to demonstrate any direct contribution of the AFP to the production of adult song. In adult zebra finches, lesions of LMAN (lateral magnocellular nucleus of the anterior nidopallium), the output nucleus of the AFP (Figure 3-1a), have not been reported to affect ongoing song. Moreover, while stimulation in the motor pathway of quiescent

birds can elicit vocalizations (Vu et al., 1994; Vicario and Simpson, 1995), comparable microstimulation in LMAN does not. Finally, a previous study did not report any gross effects of LMAN microstimulation on song (Vu et al., 1994). These findings indicate that LMAN is not an obligatory premotor structure for song, but they leave unresolved whether, and how, patterned activity from the AFP modulates song production.

MATERIALS AND METHODS

Subjects

Juvenile (> 80 days) and adult (> 125 days) male zebra finches (*Taeniopygia guttata*) were used for experiments. Birds were selected on the basis of size, singing frequency, and song complexity, and were isolated in a sound-attenuating chamber. All procedures were approved by the UCSF Institutional Animal Care and Use Committee.

Physiological recording

Surgical procedures were performed as described previously (Hessler and Doupe, 1999b). Briefly, a lightweight microdrive (UCSF and Caltech Machine Shops) carrying two metal electrodes (2-5 MΩ) was positioned stereotaxically above LMAN in the right hemisphere. A reference electrode was implanted within 2 mm of LMAN, and the microdrive and a connector socket were secured to the bird's skull.

During experimental sessions, a flexible lead terminating in an operational amplifier was connected to the socket on the bird's head, and the other end was connected to a commutator (Hessler and Doupe, 1999b). Neural signals were amplified and filtered between 300 and 10,000 Hz. Acoustic signals were recorded with a small

microphone above the birdcage and filtered between 200 and 9000 Hz. Custom-written acquisition software (C. Malek and A. Leonardo, Caltech, and C. Roddey, UCSF) recorded the acoustic and neural signals before and after the sound amplitude crossed a threshold level, and the bird's behaviour was monitored by a video camera.

Electrodes were positioned either at control sites 400-1000 μm dorsal to LMAN (n = 3 birds) or at sites in LMAN selected on the basis of their characteristic singing-related activity (n = 5 birds; Hessler and Doupe, 1999b). At sites at least 400 μm dorsal to LMAN, there was no conspicuous change in multi-unit firing during singing when compared to the spontaneous neural activity during non-singing periods. At the conclusion of experiments, of site locations were confirmed in 40 μm Nissl-stained brain sections by their position relative to the depth of marker lesions.

Song-triggered microstimulation

To deliver electrical stimuli reproducibly during specific parts of song, custom-written software (J. Houde and C. Roddey, UCSF) compared the bird's vocalizations with pre-defined spectral templates for targeted song elements in real time. Detection triggered unilateral microstimulation via the same electrodes used for recording activity. For both fixed current amplitude and variable current amplitude experiments, control 'catch trials', in which no stimulation was delivered, were randomly interleaved with stimulation trials. Electrical stimuli consisted of 25-550 msec trains of biphasic current pulses at 400 Hz (0.4 msec per phase; 2.5 msec between phases, Vu et al., 1994). Current amplitudes varied between 10 and 100 μA. Microstimulation was applied only in the 'undirected' condition (see below, n = 5 birds) and never evoked vocalization in quiescent animals.

Lesions

Electrolytic lesions of LMAN were performed and evaluated as previously described (Brainard and Doupe, 2000). The percentage of LMAN that was removed bilaterally ranged from 50% to 100%.

Behavioural analysis

'Undirected' song was recorded when the bird was alone. To elicit 'directed' song, one or more female zebra finches was presented in a separate cage. Each female presentation lasted ≤ 2 minutes, and songs were classified as 'directed' only when the male faced the female(s). Female presentations were interleaved with bouts of undirected singing.

Analysis of neural signals

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

Song analysis

To characterize differences in syllable structure, we measured fundamental frequency. For a particular syllable, we calculated the autocorrelation of a segment of the sound waveform that has constant frequency components (median segment: ~50% of the total

syllable duration; range: 20–90%). The fundamental frequency was defined as the distance, in frequency, between the zero-offset peak and the highest peak in the autocorrelation function (ACF). To improve the resolution of the estimates, we performed a parabolic interpolation of the ACF's peak.

This algorithm was applied to syllables with clear harmonic structure and a well-defined fundamental frequency or a high frequency, band-limited element. In stimulation experiments, fundamental frequency was measured for at least eight renditions in each condition. To investigate natural differences between directed and undirected songs, fundamental frequency was measured for at least 19 renditions in each context.

To characterize the effects of microstimulation on amplitude, the sound waveform was filtered between 300 and 8000 Hz, rectified, and smoothed with a 2 msec window. Syllables were segmented using an amplitude threshold, and amplitude was quantified by measuring the area under the rectified waveform from syllable onset to syllable offset.

Statistics

Comparisons of effects across different experimental conditions were made using the nonparametric Mann-Whitney U test (for within syllable changes) and paired sign test (for group changes). Comparisons of variability in fundamental frequency in different experimental conditions were made using the F-test for equality of variance. In all cases, the minimum significance level was set at $p < 0.05$.

RESULTS

Here, we more closely examine the hypothesis that neural activity in the AFP contributes to vocal control by using microstimulation triggered by real-time song to manipulate activity in LMAN during precisely targeted parts of song (Figure 3-1b). We report that artificially altering the pattern of activity in LMAN induces acute changes in the structure of individual song elements, or 'syllables', without altering the order or structure of ensuing syllables. Such changes include increases and decreases in sound frequency (a learned parameter of song; Tchernichovski et al., 2001), as well as increases and decreases in sound amplitude (a parameter of song that is precisely controlled; Williams et al., 1989; Brumm and Todt, 2004). Figure 3-1c illustrates a representative experiment. Every time the bird sang a rendition of a stereotyped sequence of syllables, or 'motif' ('abcdef'), syllable 'b' was detected using a real-time template-matching algorithm. After detection, microstimulation was delivered in LMAN on randomly selected trials (in this case, during the first motif rendition, but not the second). In this experiment, LMAN stimulation caused a systematic downward shift in the fundamental frequency of syllable 'c'. Figure 3-1d illustrates a second experiment using the same paradigm in which LMAN microstimulation caused a systematic decrease in the loudness of the targeted syllable. Such acute, stimulation-induced changes in syllable structure were observed for 18 of 20 sites in LMAN of five birds (Supplementary Tables 3-1, 3-2).

Evoked changes in syllable structure were tightly locked to the delivery of stimuli. In cases where latency could be assessed, the mean latency between the onset of a stimulus and an effect on syllable structure was 50 msec but could be as short as 35 msec (see Supplementary Figure 3-1). In addition, the effects of stimulation typically terminated within 60–70 msec after the end of the stimulus train (e.g., Figure 3-1d).

LMAN projects directly to the song motor nucleus RA, which is thought to provide motor commands that control the precise structure of individual song elements with a latency of 40–45 msec (Vu et al., 1994; Yu and Margoliash, 1996). Although the exact mechanisms and pathways underlying the influence of LMAN on song remain to be determined, both the rapidity with which LMAN stimulation could drive changes in syllable structure and the rapid termination of its effects are consistent with a direct modulation of RA by LMAN.

In a given experiment, stimulation in LMAN typically induced systematic changes in syllable structure, rather than a general degradation of song structure or an enhancement of song variability. When stimulation elicited a significant shift in the mean fundamental frequency of a syllable, it had no effect on the variability of the fundamental frequency in 59% of cases (e.g., note the standard deviations of the histograms in Figure 3-2b), increased the variability in 30% of cases (e.g., Figure 3-1c), and reduced the variability in the remaining 11% of cases. Thus, the predominant effect of artificially imposing a fixed pattern of activity in LMAN during singing was to systematically shift the mean value of syllable parameters, rather than to grossly disrupt song.

There was significant specificity to the changes elicited by microstimulation in LMAN. For 13 of 18 sites, a fixed pattern of stimulation had qualitatively different effects when delivered during different syllables (Supplementary Table 3-2). Figure 3-2a shows a case in which stimulation delivered at a fixed site in LMAN caused an increase in the amplitude of one syllable, a decrease in the amplitude of a second syllable, and little change to a third syllable. Moreover, when we explicitly varied the site of

stimulation in LMAN, while holding all other parameters constant, we could elicit qualitatively different effects on a given syllable. Figure 3-2b shows a case where stimulation at a dorsal site in LMAN caused a significant increase in the mean fundamental frequency, while stimulation at a more ventral site in LMAN caused a significant decrease in the fundamental frequency of the same syllable. These results suggest that LMAN may be functionally compartmentalized, consistent with known topographic projections from LMAN to RA (Johnson et al., 1995).

The observed changes in syllable structure were restricted to stimulation in LMAN. Stimulation that induced significant shifts in frequency when delivered in LMAN was ineffective when applied at control sites 400–1000 μm dorsal to LMAN (Figure 3-2c). At even higher current intensities, stimulation outside of LMAN rarely caused significant changes in syllable structure (Figure 3-2c). In contrast, we found that stimulation both within LMAN and at control sites dorsal to LMAN with higher current intensities could induce the suspension of ongoing motifs, as seen previously with stimulation of the premotor nucleus HVC (Vu et al., 1998). Song suspensions occurred at a median current intensity that was 233% of that required to elicit significant effects on syllable structure (range: 200%–300%, $n = 8$). Because song suspensions could be elicited from control sites outside of LMAN, they are not likely to reflect the specific activation of RA, and instead may result from antidromic activation of nucleus HVC (Figure 3-1a), which sends projections through the anterior forebrain (Vu et al., 1994; Mooney, 2000).

The acute changes in syllable structure induced by artificially altering activity in LMAN indicate that neural activity in LMAN can modulate ongoing motor performance

on a moment-by-moment basis. These results raise the question of whether the natural pattern of activity in LMAN modulates the motor pathway and subsequent song. To examine this possibility, we compared song produced by birds in two behavioural conditions in which the neural activity in the AFP is known to differ; when a male bird sings alone ('undirected' song), activity in LMAN is greater in magnitude and more variable in pattern across renditions than when it sings to another bird ('directed' song) (Figures 3-3a–c, e; Jarvis et al., 1998; Hessler and Doupe, 1999a). We found that the mean fundamental frequency of syllables did not differ systematically between these two conditions, but that the variability in the fundamental frequency was significantly greater during undirected song (Figures 3-3d, f). Moreover, across birds, there was a significant correlation between the magnitude of changes in LMAN variability and the magnitude of changes in song variability ($R^2 = 0.83$; Supplementary Figure 3-2). The observed correspondence between variability in LMAN activity and variability in syllable structure (Figures 3-3e, f) is consistent with a direct modulatory influence of LMAN on song under natural conditions.

To test explicitly whether trial-by-trial variability in LMAN activity can give rise to variability in motor output, we altered the pattern of LMAN activation across song renditions by varying the current intensity. Stimulation with a fixed current intensity tended to elicit a systematic change in fundamental frequency (e.g., Figures 3-1c, 3-2b); however, varying the activity of LMAN neurons at a single site across motif renditions caused a significant increase in the variability of the fundamental frequency (Figures 3-4a, b; Supplementary Table 3-3). Thus, the artificial introduction of variability in LMAN

activity was sufficient to recapitulate one natural difference between directed and undirected song (compare Figure 3-3f and Figure 3-4b).

If activity from LMAN is indeed responsible for the difference in song variability between behavioural contexts, then removing this activity should eliminate the observed difference. We tested this by measuring the variability of syllable structure in directed and undirected songs of five birds before and after bilateral lesions of LMAN. Before lesions, for each bird, the variability in the fundamental frequency was significantly greater during undirected song (Figure 3-4c, 2–5 syllables per bird). Immediately after the lesions, this context-dependent difference in song variability was eliminated (1–3 days after lesions; Figure 3-4c). Thus, LMAN is necessary for modulating naturally occurring differences in song variability.

DISCUSSION

Our results suggest that a critical contribution of the AFP to song plasticity may derive from its previously unappreciated role in acutely modulating activity in the premotor nucleus RA. Neural activity in LMAN could contribute to the adaptive modification of song in juvenile and adult birds in at least two ways (Brainard and Doupe, 2000). One possibility is that LMAN provides an instructive signal to RA that systematically biases the motor pathway towards a particular goal (Scharff and Nottebohm, 1991; Brainard and Doupe, 2000; Troyer and Doupe, 2000). Consistent with this idea, we found that for a given syllable, a fixed pattern of LMAN activation typically induced specific changes in syllable structure. This specificity suggests that the AFP has the capacity to selectively bias independent components of song towards desired targets.

An analogous instructive role has been postulated to be one function of descending inputs from frontal cortical and striatal regions in other vertebrate systems (Miller, 2000; Hikosaka et al., 2002).

A second possibility is that neural activity in LMAN contributes to plasticity by generating variability in the motor pathway and subsequent song output (Scharff and Nottebohm, 1991; Doya and Sejnowski, 2000). Consistent with this idea, we found that both naturally occurring and experimentally induced variability in LMAN activity is associated with variability in song output. Moreover, although LMAN is not required for the production of learned song, it is necessary for the state-dependent changes in the variability of syllable structure. In the context of feedback-based reinforcement learning, trial-by-trial variability in motor output is required in order for evaluation mechanisms to selectively reinforce the patterns of motor activity that produce the desired behaviour (Doya and Sejnowski, 2000; Troyer and Bottjer, 2001). Once a behaviour is learned, the ability to modify it remains important, both for feedback-based correction of errors that may result from changes in the periphery (e.g., altered muscle tone or innervation) and for continued motor exploration in order to optimize the behaviour.

These two models for the contribution of LMAN to vocal plasticity are not mutually exclusive. Rather, the influence of the AFP will depend on its actual pattern of activity during singing. When AFP activity is stereotyped from one song rendition to the next (e.g., during directed song), our results suggest that it should systematically bias vocal production. In contrast, when AFP activity is variable across motif renditions (e.g., during undirected song), it should contribute to variability in motor output, which may be a critical component of motor exploration during the process of vocal learning and/or

maintenance. According to both of these models, the absence of patterned activity from the AFP accounts for the elimination of long-term vocal plasticity by lesions of LMAN (Williams and Mehta, 1999; Brainard and Doupe, 2000).

The anatomical substrates for the influence of LMAN activity on the motor pathway are individual premotor neurons in RA that receive inputs from both HVC and LMAN (Mooney and Konishi, 1991). This convergence of inputs on the same RA neurons provides a cellular locus where LMAN activity can influence synaptic connections and/or transmission in RA (Kittelberger and Mooney, 1999). Moreover, LMAN input to RA is unusual in that it is mediated predominantly by NMDA receptors (NMDAR) (Mooney and Konishi, 1991; Stark and Perkel, 1999), which are known to be important for structural and/or functional plasticity. NMDAR-mediated synaptic responses evoked by LMAN terminals in RA are well situated for modulating the gain of active synapses in RA during singing. Modulation of ongoing motor activity has been observed in other systems (Komatsu and Wurtz, 1989; Tanaka and Lisberger, 2001), and may provide a general mechanism for enabling plasticity in motor circuits.

Several lines of evidence suggest that cortical–basal ganglia circuits contribute to the selection and sequencing of behaviour during the learning and performance of motor skills. Disorders of the basal ganglia can be characterized, in part, as either resulting in too much movement (e.g., Huntington's disease) or in too little movement (e.g., Parkinson's disease), suggesting that activity in cortical–basal ganglia circuits adjusts the gain of motor output. Moreover, altered patterns of activity in the basal ganglia (either experimentally-induced or in disease states) can affect the degree of stereotypy versus variability in motor performance (Canales and Graybiel, 2000; Matsumoto et al., 1999).

We suggest that in songbirds, the AFP can modulate ongoing motor activity and drive specific changes in motor output on a moment-by-moment basis, perhaps by adjusting the gain of active synapses in the motor pathway. In addition, we have shown that introducing variability in the activity of LMAN, the output nucleus of the AFP, can drive variability in motor performance. Finally, we found that lesions of LMAN eliminate the naturally occurring context-dependent modulation of song variability. We suggest that a key contribution of frontal–basal ganglia circuits to motor learning and performance relies on the capacity of these circuits to bias motor output towards specific targets and to introduce variability in motor output.

Figure 3-1 Song-triggered microstimulation in LMAN elicits acute changes in learned parameters of syllable structure. *A.* 'Song system'. The motor pathway includes HVC and RA. The AFP consists of Area X, the medial portion of the dorsolateral thalamus (DLM), and LMAN. *B.* Experimental design. 'i' indicates an introductory element that usually occurs at the start of a song bout before the motif(s) ('abcdef'). *C.* Stimulation-induced shift in frequency. *Top:* Spectrogram illustrating two trials. LMAN was stimulated (30 μA; red bar) during syllable 'c' on random trials. *Bottom:* Fundamental frequency for 'c' for control (blue) and stimulation trials (red). Stimulation caused a 10% decrease in fundamental frequency (means (circles) ± s.d. (bars); $p<0.0001$). *D.* Stimulation-induced change in amplitude. *Top:* Spectrogram of another bird's song. *Middle:* Mean amplitude waveforms (±s.e.m.) for control (blue) and stimulation (red) trials. *Bottom:* Stimulation (40 μA) significantly reduced the amplitude of 'c' (~27%; $p < 0.0001$), but not that of the next syllable ($p = 0.74$).

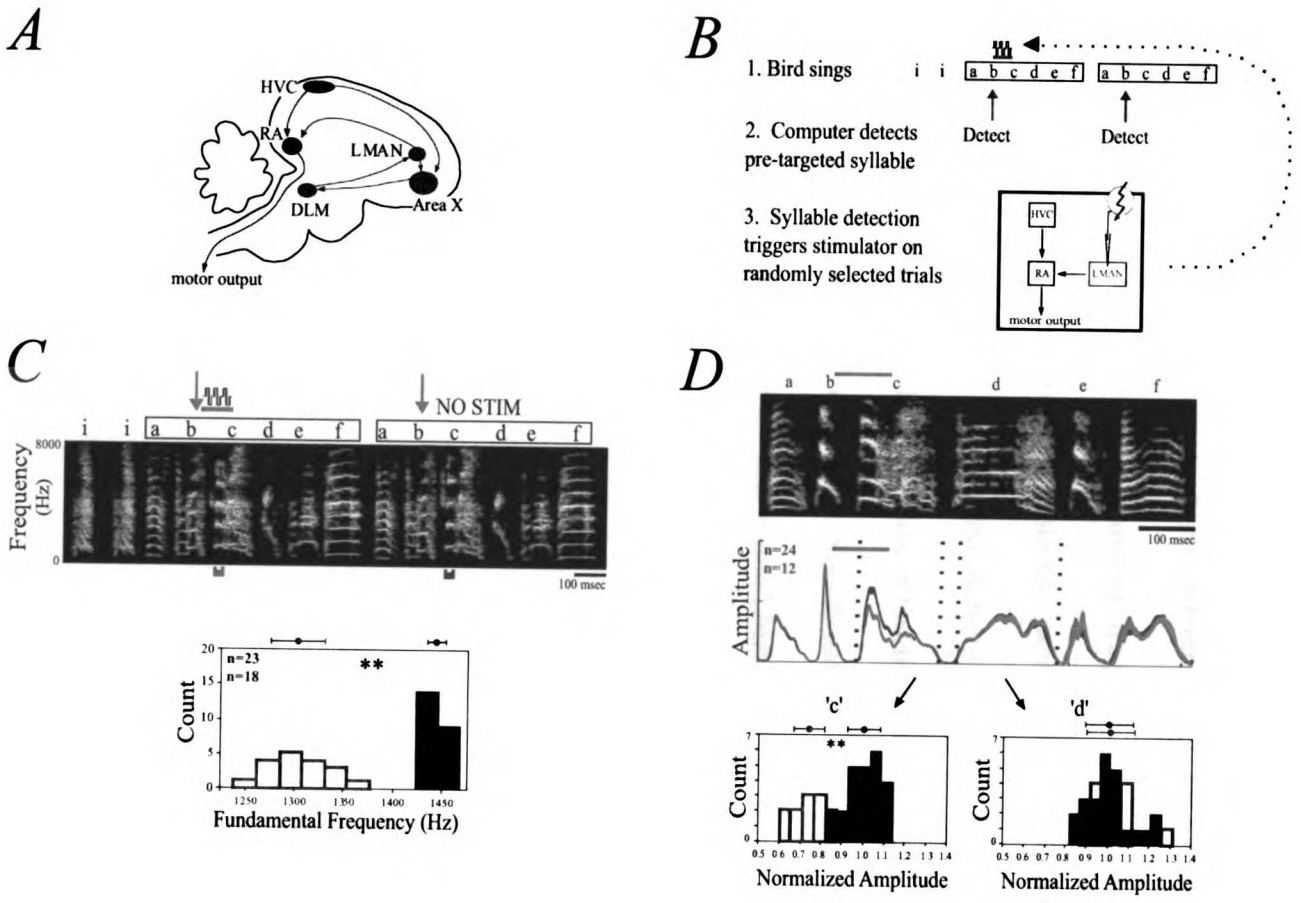

A
HVC
RA
LMAN
DLM
Area X
motor output
B
1. Bird sings
i i
a b c d e f
a b c d e f
Detect
Detect
2. Computer detects pre-targeted syllable
3. Syllable detection triggers stimulator on randomly selected trials
HVC
RA
LMAN
motor output
C
NO STIM
i i
a b c d e f
a b c d e f
Frequency (Hz)
8000
0
100 msec
n=23
n=18
**
Count
Fundamental Frequency (Hz)
D
a b c d e f
100 msec
n=24
n=12
Amplitude
'c'
'd'
**
Count
Normalized Amplitude
Count
Normalized Amplitude

Figure 3-1

Figure 3-2 Specificity of stimulation. *A.* Stimulation at one site could elicit qualitatively different effects on different syllables. A fixed pattern of stimulation (30 μA; red bar) increased the amplitude of 'c', decreased the amplitude of 'f', and had little effect on other syllables (e.g., 'd'). Red, stimulation; blue, control. *B.* Stimulation at different sites in LMAN could induce qualitatively different effects for a given syllable. At one site, stimulation (20 μA) increased fundamental frequency (means (circles) ± s.d. (bars); $p < 0.0001$), whereas at a more ventral site, the same stimulation decreased the fundamental frequency ($p < 0.0001$). *C.* Evoked changes were specific to stimulation in LMAN. Lines connect data points where the same stimulation was delivered inside (red circles) and outside (green triangles) of LMAN. Significant changes of fundamental frequency (filled symbols) were observed only inside LMAN. When current intensity was increased at control sites (green diamonds), significant effects were elicited in one of seven cases.

Figure 3-2

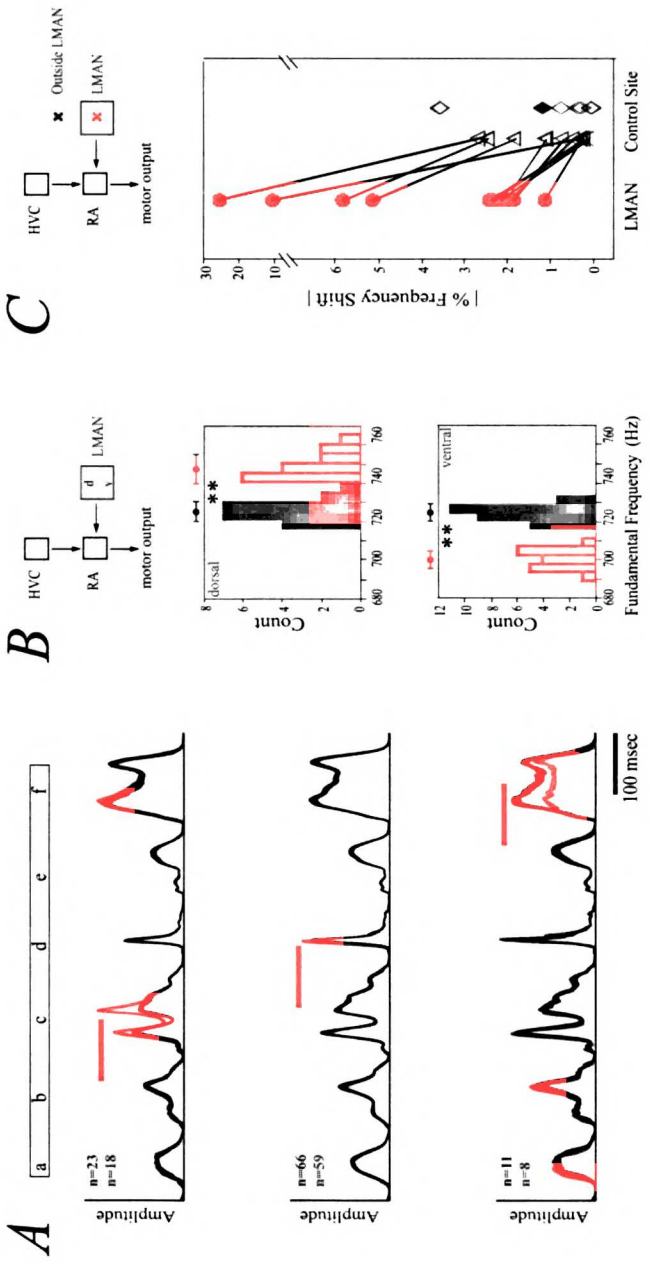

A
a
b
c
d
e
f
n=23
n=18
n=66
n=59
n=11
n=8
Amplitude
100 msec
B
HVC
RA
LMAN
motor output
dorsal
ventral
Count
Fundamental Frequency (Hz)
C
HVC
RA
LMAN
Outside LMAN
motor output
% Frequency Shift
LMAN
Control Site

Figure 3-3 Context-dependent changes in variability. *A*. Spectrograms of the motif ('abcd') during directed (*left*) and undirected (*right*) singing. *B.* LMAN multi-unit activity during three renditions of the motif. *C.* LMAN activity waveforms for ten renditions of the motif. Note that the singing-related activity (normalized against background activity level) is greater than the background activity level; the dotted line represents the mean background activity level during non-singing periods. *D.* Histograms and Gaussian fits of fundamental frequency for syllable 'a'. Mean fundamental frequency (arrows) did not differ (497.4 & 496.7 Hz; $p = 0.54$), but variability in fundamental frequency was greater in undirected song (s.d.: 4.17 and 8.10; $p = 0.004$). The Gaussian fit for directed song is overlaid in gray on the Gaussian fit for undirected song (*right*). E. Variability in activity was greater in undirected song ($n = 29$ sites, 11 birds; $p < 0.0001$). *F.* Variability in fundamental frequency was greater in undirected song (50 of 53 syllables, 18 birds; $p < 0.0001$).

Figure 3-3

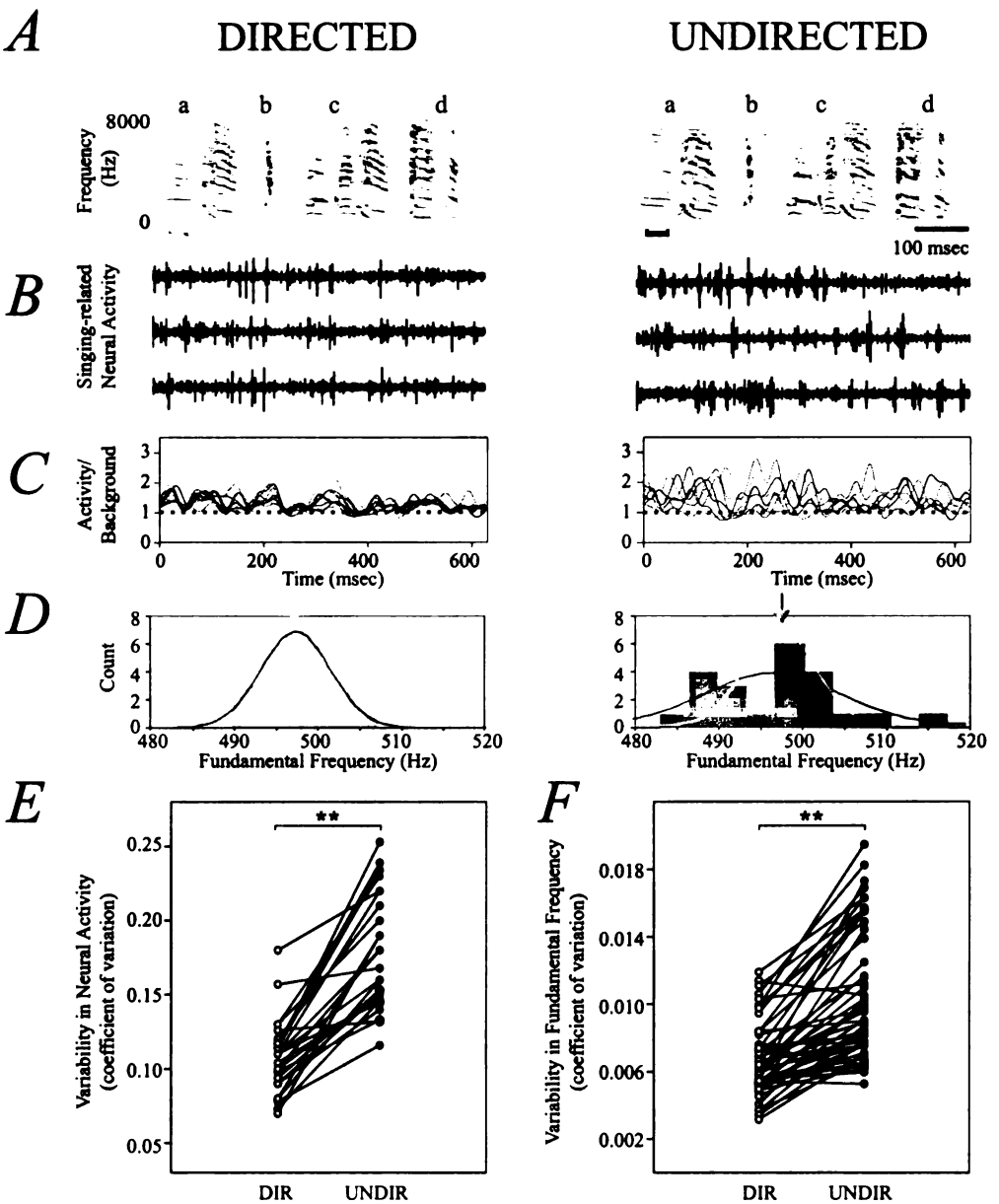
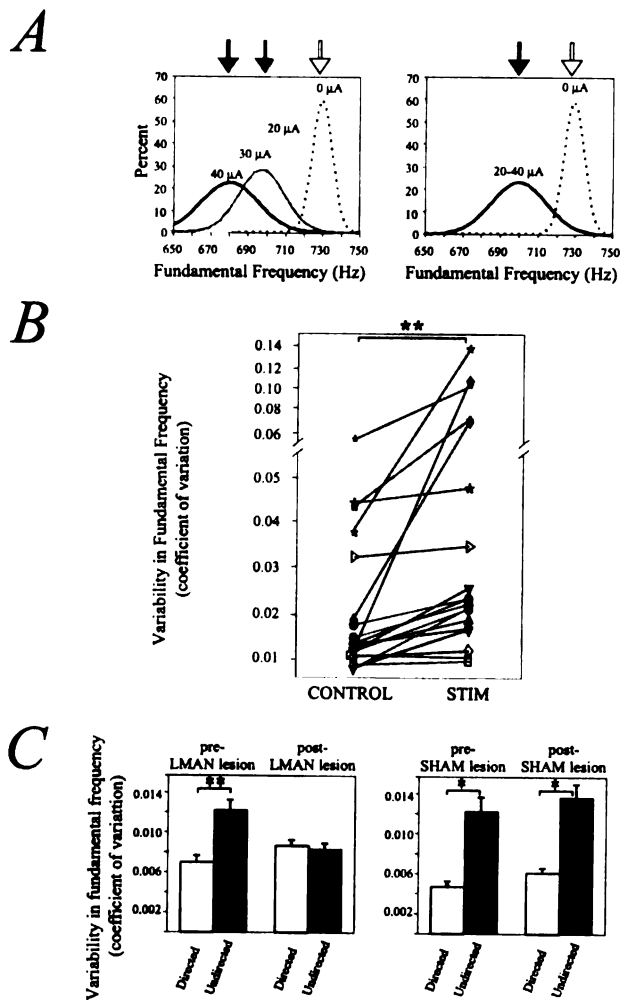

A
DIRECTED
UNDIRECTED
a
b
c
d
8000
Frequency (Hz)
0
100 msec
B
Singing-related Neural Activity
C
Activity/ Background
3
2
1
0
0
200
400
600
Time (msec)
D
Count
8
6
4
2
0
480
490
500
510
520
Fundamental Frequency (Hz)
E
F
**
Variability in Neural Activity (coefficient of variation)
0.25
0.20
0.15
0.10
0.05
DIR
UNDIR
Variability in Fundamental Frequency (coefficient of variation)
0.018
0.014
0.010
0.006
0.002

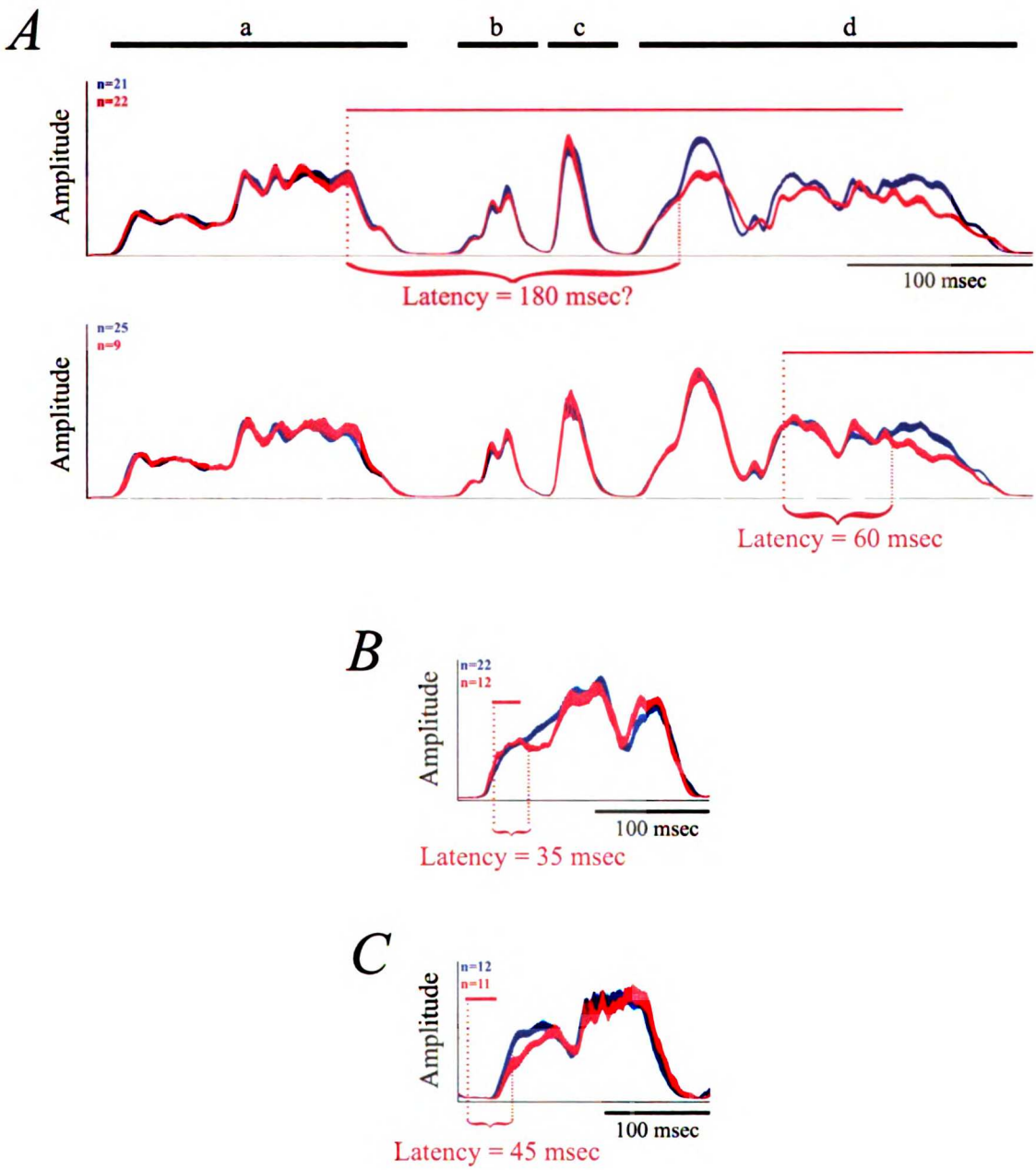
Figure 3-4 Contributions of the AFP to real-time song modulation. *A. Left.* Gaussian fits of fundamental frequency for one syllable for control (dotted) and stimulated trials of different current intensities. *Right:* Varying stimulation across trials increased the variability in the fundamental frequency relative to controls ($p = 0.022$). *B.* Variability in fundamental frequency for control and STIM trials in which different current intensities were delivered at the same site. Symbols denote syllables (1–3 per bird). Differential activation of LMAN increased the variability in the fundamental frequency in 18 of 19 cases ($p < 0.0001$). *C.* LMAN lesions eliminate context-dependent differences in song variability. Bars indicate the mean coefficient of variation of fundamental frequency (±s.e.m.) for syllables during directed and undirected song before and after LMAN lesions (19 syllables, five birds; pre: $p < 0.0001$; post: $p = 0.167$) or sham lesions (six syllables, two birds; pre: $p = 0.03$; post: $p = 0.03$).

Figure 3-4


A
Percent
0 μA
20 μA
30 μA
40 μA
Fundamental Frequency (Hz)
0 μA
20-40 μA
Fundamental Frequency (Hz)
B
Variability in Fundamental Frequency (coefficient of variation)
CONTROL
STIM
C
Variability in fundamental frequency (coefficient of variation)
pre-LMAN lesion
post-LMAN lesion
pre-SHAM lesion
post-SHAM lesion
Directed
Undirected

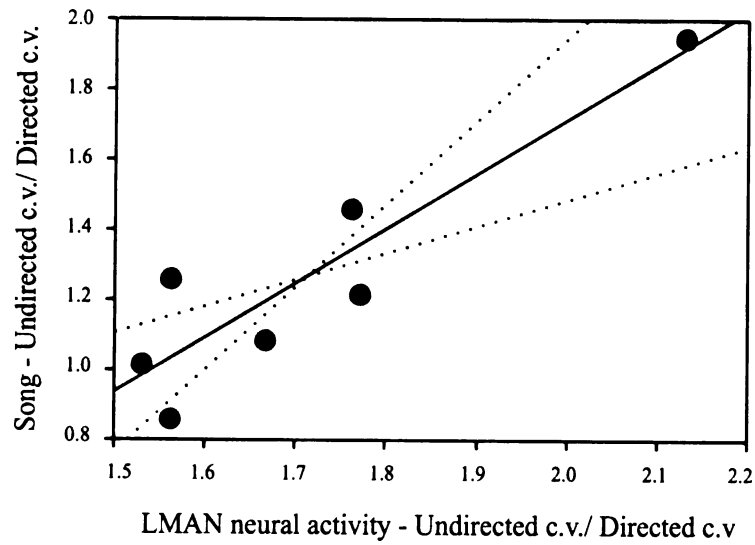
Supplementary Figure 3-1 Calculation of latency. *A.* Because the effects of stimulation at a given site could be specific to a subset of syllables, the determination of latency could be complicated. For example, stimulation at a given site might only affect syllable 'd' of the sequence 'bcd' (Panel a, *top*) and not affect syllables 'b' or 'c', regardless of how far in advance of syllable 'd' the stimulation was initiated. In such cases, the measured latency could potentially over-estimate how long it takes for altered signals from LMAN to drive changes in song. In contrast, application of the same stimulus with a delay so that it was delivered only during the affected syllable elicited the same change in syllable structure (decrease in sound amplitude) with a shorter latency (Panel a, *bottom*). Thus, latency could only be measured accurately when the stimulus was applied close to the onset of an effect. *B–C.* We explicitly tested the latency of stimulation-induced effects for a subset of syllables (six syllables in four birds) that exhibited robust stimulation-induced changes. We applied short trains of stimuli (20–30 msec) near the onset of each targeted syllable and measured the latency for a significant deviation from control of the amplitude or frequency of that syllable. Panels b–c show examples of two experiments in which latencies were determined in this fashion. In cases such as these, where latencies could be determined confidently, values ranged from 35–70 msec, with an average of 50 msec. In addition, the effects of stimulation typically terminated within 60–70 msec after the end of the stimulus train (e.g., Fig. 3-1d) but could terminate as quickly as 40 msec after the end of the stimulus train (e.g., Panel b).

Supplementary Figure 3-1



Supplementary Figure 3-2 Correlation between the magnitude of changes in LMAN variability and the magnitude of changes in song variability. The variability of neural activity recorded at single sites in LMAN was consistently greater during undirected singing than during directed singing (see Figure 3-3e). Likewise, the variability of syllable structure was consistently greater during undirected singing (see Figure 3-3f). To investigate whether the magnitude of neural changes could predict the magnitude of song changes, we examined data from seven individual birds where we could derive global measures of both neural and song variability. We characterized global changes in LMAN variability for each bird as the average of the ratio of undirected to directed variability (c.v.) across all recording sites from that bird (1–4 sites per bird). We characterized global changes in song variability for each bird as the average of the ratio of undirected to directed variability in fundamental frequency (c.v.) across all syllables that had clearly defined harmonic structure (1–4 syllables per bird). These data are plotted below with a least squares regression fit to the data ($y = -1.4 + 1.6x$, R-squared = 0.83) and 95% confidence limits for the slope of the fit. There was a significant positive correlation between the magnitude of changes in neural and song variability, indicating that birds which exhibited large context-dependent changes in the variability of neural activity in LMAN also exhibited large context-dependent changes in song variability. This bird-by-bird correspondence further supports the possibility that changes in syllable structure are driven by changes in neural activity in LMAN under natural conditions

Supplementary Figure 3-2



Supplementary Table 3-1 – Summary of the effects of microstimulation in LMAN for each syllable in five birds. Pt. 1

Bird ID	*Syllable or note*[a]	# of sites with significant effects/ Total # of sites tested	Current intensities applied[b] (μA)	Evoked change in amplitude[c] (# of sites)	Evoked change in FF[c, d] (# of sites)	% change in FF (significance)[e]
1	a	0/1	40	none	none	n/a
1	b2	2/2	**40**, 80	↓(1)	↑ (1) ↓ (1)	2.42% ($p < 0.0001$)
1	c1	5/8	**30**, 40, 80, 100	↑ (1)	↑ (4) ↓ (2)	-9.6% ($p < 0.0001$)
1	c2	4/8	**30**, 40, 80, 100	↑ (1)	↑ (1) ↓ (3)	-13% ($p < 0.0001$)
1	d	4/8	**30**, 40, 80, 100	↑ (1)	↑ (4)	-8.8% ($p < 0.0001$)
1	e	0/6	30, 40, 80	none	n/a[f]	n/a[f]
1	f	4/6	**30**, 40, 50,80, 100	↓(1)	↑ (1) ↓ (3)	-4.2 ($p = 0.0002$)
2	a	1/1	**20**, 30	↓ (1)	↑ (1)	1.5% ($p = 0.0007$)
2	b	1/1	20, 30, 40, 50	none	n/a[f]	n/a[f]
2	c	1/1	20, **30**, 40, 50		↑ (1)	3.7% ($p = 0.0045$)
2	d1	1/1	**20**, 30, 40, 50	↓(1)	↑ (1) ↓ (1)	2.0% ($p < 0.0001$)
2	d2	1/1	**20**, 30, 40, 50	↓ (1)	↑ (1) ↓ (1)	2.4% ($p < 0.0001$)
2	e1	1/1	**20**, 30, 40, 50	↓ (1)	none	n/a
2	e2	1/1	**20**, 30, 40, 50	↓ (1)	↑ (1)	2.6% ($p = 0.0001$)
2	f	1/1	**20**, 30, 40, 50	↓ (1)	↑ (1)	2.5% ($p = 0.0218$)
3	a	0/1	40	none	none	
3	b	2/2	20, **40**	↓ (1)	↑ (1)	3.1% ($p = 0.0007$)
3	c1	2/3	**20**, 40	↓ (1)	↓ (1) ↑ (2)	15.1% ($p = 0.0002$)
3	c2	2/2	**20**, 40	↓ (2)	↓ (2)	-12.4% ($p = 0.0003$)
4	i	n/a[g]	n/a[g]			
4	a	n/a[g]	n/a[g]			
4	b	2/2	10, **20**, 40, 60	↓ (2)	↑ (2)	1.9% ($p < 0.0001$)
4	c	5/5	**10**, 20, 40, 60	↓ (5)	↓ (4) ↑ (3)	-25.4% ($p < 0.0001$)

4	d1	3/3	**10**, 20, 30, 40	↓ (2)	↓ (1) ↑ (1)	-7.7% ($p < 0.0001$)
4	d2	3/3	**10**, 20, 30, 40	↓ (2)	↓ (4) ↑ (3)	-6.7% ($p = 0.0002$)
5	a	n/a[g]	n/a[g]			
5	b	n/a[g]	n/a[g]			
5	c	2/2	10,15, **20**, 40	↓ (2)	none	n/a
5	d	4/4	10,**15**, 20, 40, 50, 60	↓ (3)	↓ (1) – (4)[h]	2.2% ($p < 0.0001$)
5	e	½	15, **20**, 40, 50, 60	↓ (1)	↓ (1)	-3.3% ($p = 0.0006$)
5	f	1/1	**20**, 40	↓ (1)	↑ (1)	5.5% ($p < 0.0001$)

[a] For syllables that contain more than one harmonic stack or high frequency component, we measured the fundamental frequency(FF)for each component separately.
[b] The lowest current intensity for evoking changes in syllable structure at any site in LMAN is bold-faced.
[c] Arrows indicate the direction of the stimulation-induced change – increase (↑) or decrease (↓).
[d] The number of sites listed as having an evoked change in FF could exceed the total number of sites tested because at some sites, stimulation with different current intensities elicited both increases and decreases in fundamental frequency (FF).
[e] The largest observed change in the FF is listed for all sites tested.
[f] FF could not be measured for this syllable.
[g] Stimulation was not applied during this syllable.
[h] Stimulation elicited complex changes in the structure of this syllable at all sites tested, including the suppression or enhancement of harmonically related frequencies.

Supplementary Table 3-2 – Summary of the effects of stimulation in LMAN for each bird by site. Pt. II

Bird ID.site	# of syllables tested/ total # of syllables	Lowest current intensity that elicited changes in syllable structure[a] (μA)	Duration of stimulus trains[b] (msec)	# of syllables affected by threshold current intensity/Total # of syllables tested
1.1	4/6	30	15-400	4/4[c]
1.2	4/6	40	50-250	3/4
1.3	4/6	40	50-250	3/4
1.4	3/6	40	60-250	2/3[d]
1.5	6/6	40	60-250	1/6
1.6	4/6	40	50-550	2/4
1.7	4/6	n/a	50-550	0/4
1.8	2/6	n/a	50-250	0/2
2.1	6/6	20	30-400	4/6[d]
3.1	2/3	20	105-300	1/2[d]
3.2	3/3	40	110-400	2/3
4.1	1/5	20	20-300	1/1
4.2	2/5	10	10-300	2/2
4.3	2/5	10	10-300	2/2
4.4	2/5	20	30-300	2/2
4.5	2/5	20	35-300	2/2[c]
5.1	3/6	20	10-250	3/3[c]
5.2	2/6	15	10-250	1/2
5.3	2/6	15	10-250	1/1
5.4	2/6	40	5-250	1/2

[a] The lowest (threshold) current intensity for evoking changes in any syllable at this site.
[b] For each syllable tested, the duration of stimulation was defined as the time from the onset of stimulation to the end of the syllable. For syllables that were affected, the nature and magnitude of the effect were typically consistent across all stimulus durations.
[c] At the threshold current intensity, qualitatively different effects were elicited for different syllables at this site (e.g., either the direction of the effect or the parameter affected was different across the different syllables; see Figure 3-2a).
[d] At these sites, stimulation in LMAN with a supra-threshold current intensity evoked changes in one additional syllable.

Supplementary Table 3-3 – Summary of the effects of differential activation of LMAN neurons at a particular site.

Bird #	Site #	Syllable or note[a]	Current intensities (μA)	Control variability (c.v. of FF)	STIM variability (c.v. of FF)[b]	*p* value
1	1	b	40-80	0.012	0.018	$p = 0.0001$
1	1	c1	40-80	0.013	0.023	$p < 0.0001$
1	1	c2	40-80	0.014	0.022	$p < 0.0001$
2	1	e	20-60	0.013	0.016	$p = 0.0221$
3	1	a	20-30	0.010	0.011	$p = 0.4807$
3	1	c	20-40	0.032	0.035	$p = 0.5588$
3	1	d1	20-40	0.011	0.021	$p < 0.0001$
3	1	d2	20-40	0.017	0.023	$p = 0.0216$
4	1	c1	20-40	0.012	0.107	$p < 0.0001$
4	1	c2	20-40	0.018	0.070	$p < 0.0001$
5	1	c	10-20	0.037	0.136	$p < 0.0001$
5	1	d2	10-20	0.007	0.016	$p < 0.0001$
5	2	c	10-20	0.043	0.072	$p = 0.0145$
5	2	d1	20-40	0.011	0.025	$p < 0.0001$
5	2	d2	20-40	0.007	0.021	$p < 0.0001$
5	3	b	10-40	0.010	0.010	$p = 0.7979$
5	3	c	10-40	0.044	0.047	$p = 0.3818$
5	4	b	20-40	0.008	0.009	$p = 0.3205$
5	4	c	20-40	0.058	0.103	$p < 0.0001$

[a] For syllables that contain more than one harmonic stack or high frequency component, we measured the fundamental frequency (FF) for each component separately.
[b] The variability in the FF across all current intensities applied. For 47% of these cases, there was a significant increase in the variability of the FF at the highest current intensity tested. Hence, in these cases, the greater variability in the FF observed across all current intensities could derive from a combination of the greater variability in the FF elicited by the highest current intensity and the variability in the magnitude of the change in the mean FF elicited by the different current intensities (see Figure 3-4a).

Chapter 4: Lesions of an avian forebrain nucleus prevent variability of learned vocalizations

ABSTRACT

Trial-by-trial variability is an important component of feedback-based motor learning. Variation in motor output enables evaluation mechanisms to differentially reinforce those patterns of motor activity that produce the desired behavior. Vocal learning in songbirds exhibits these features: young birds learn to sing by using auditory feedback to gradually match their vocalizations to a memorized song model. Once the song is learned, variability in its performance remains important for continued motor exploration, optimization, and/or song maintenance. Here, we investigate the role of the anterior forebrain pathway (AFP), an avian basal ganglia–forebrain circuit that is required for song learning and plasticity, in the regulation of song variability.

Previous studies have shown that variability in AFP activity and variability in the structure of individual song elements, or 'syllables', are greater when a bird sings alone ('undirected' song) compared to when it sings to another bird ('directed' song) (Hessler and Doupe, 1999a; Kao et al., 2005). Moreover, the artificial introduction of variability into AFP activity induces greater variability in syllable structure (Kao et al., 2005). To test the hypothesis that a critical function of the AFP is to introduce variability into the ongoing patterns of motor activity, we characterized songs produced in the two social contexts before and after lesions of the output nucleus of the AFP. We show that removal of signals from the AFP eliminates context-dependent differences in syllable variability by reducing the moment-by-moment variability in undirected song to the level present in directed song. The effect of the lesions is immediate and long lasting. In addition, we show that the AFP is also required for the enhanced variability of syllable structure that follows deafening. Finally, we report that the stabilization of syllable

structure by lesions of the AFP output nucleus is similar to a natural stabilization of song that occurs with aging. Trial-by-trial variability in syllable structure during undirected song declines with age, reminiscent of the artificial stabilization induced by AFP lesions. In contrast to the necessity of the AFP for changes in the variability of syllable structure, we find that the AFP is not required for the social modulation of higher-order features of song, such as the number of repetitions or the number of introductory elements, or other motor aspects of courtship, such as the dance movements that accompany song, suggesting that distinct brain regions mediate different motor aspects of courtship behavior. Together, our findings suggest that a critical contribution of the AFP to song performance and plasticity is to modulate moment-by-moment variability in the structure of individual song elements, which is a requisite component of feedback-based motor learning.

INTRODUCTION

Birdsong, like human speech, is a complex, learned vocal behavior that is critical for social interaction. Song development includes two stages: 1) "sensory" acquisition, when juvenile birds memorize the song of an adult tutor; and 2) "sensorimotor" learning, when birds use auditory feedback to gradually match their developing vocalizations to the tutor song model (Konishi, 1965; Price, 1979). Previous work has suggested that song development may occur via reinforcement learning: variability in motor output results in a range of vocalizations, and auditory feedback is used to evaluate the degree of match to the tutor song to selectively reinforce the motor commands that produce the desired behavior (Doya and Sejnowski, 1999; for review, see Troyer and Bottjer, 2001 and

Brainard, 2004). Once the behavior is learned, the ability to modify it remains important, and adult birds regulate variability in song performance depending on social context (Sossinka and Böhner, 1980; Payne, 1983; West and King, 1988; Kao et al., 2005).

The neural substrate for song production, learning, and maintenance is a discrete series of forebrain and brainstem nuclei (Figure 4-1). This "song system" is comprised of two pathways: 1) a motor pathway which includes nuclei that exhibit neural activity tightly locked to song (Yu and Margoliash, 1996; Hahnloser et al., 2002) and are necessary throughout life for normal song production (Nottebohm et al., 1976); and 2) an anterior forebrain pathway (AFP), a basal ganglia–forebrain circuit (Reiner et al., 1998; Perkel, 2004) that is necessary for song learning and plasticity but not for the production of adult song (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991; Williams and Mehta, 1999; Brainard and Doupe, 2000). The AFP output nucleus LMAN (lateral magnocellular nucleus of the anterior nidopallium) projects directly to the premotor nucleus RA (robust nucleus of the arcopallium), where inputs from the two pathways converge.

Previous lesion studies have demonstrated that the AFP is critical for trial-by-trial variability in song production and for feedback-based vocal plasticity (Bottjer et al., 1984; Scharff and Nottebohm, 1991; Williams and Mehta, 1999; Brainard and Doupe, 2000; Kao et al., 2005). Lesions of LMAN in juvenile zebra finches disrupt song development and induce premature stereotypy, resulting in highly repetitive, simplified songs that consist of a few abnormally structured elements (Bottjer et al., 1984; Scharff and Nottebohm, 1991). In adult zebra finches, lesions of LMAN prevent song plasticity induced by experimental manipulation of auditory and/or proprioceptive feedback

(Williams and Mehta, 1999; Brainard and Doupe, 2000). Consistent with a role in regulating song plasticity, LMAN neurons exhibit robust singing-related activity that is correlated with song structure (Hessler and Doupe, 1999b; Leonardo, 2005; see chapter 2). 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 the synaptic connections in the motor pathway (Scharff and Nottebohm, 1991; Kittelberger and Mooney, 1999; Kao et al., 2005). Such a modulatory influence could serve to introduce variability in the motor pathway and subsequent output, which are required for reinforcement learning (Doya and Sejnowksi, 1999; Kao et al., 2005).

To examine the hypothesis that neural activity in LMAN regulates plasticity by introducing variability into the patterns of activity in the motor pathway, we focused on natural differences in the pattern of singing-related activity in LMAN. Song serves as a courtship signal in zebra finches, and previous studies have shown that singing-related activity in LMAN is strikingly sensitive to social context: when a male zebra finch sings alone ('undirected' song), neural activity in LMAN is greater in magnitude and more variable in pattern across repeated renditions than when it sings to another bird ('directed' song) (Jarvis et al., 1998; Hessler and Doupe, 1999; Kao et al., 2005). Subtle aspects of song structure also vary with social context (Sossinka and Böhner, 1980; Kao et al., 2005). We have shown previously that context-dependent variability in LMAN neural activity is associated with variability in syllable structure (Kao et al., 2005). Moreover, the artificial introduction of variability in LMAN activity can drive variability

in song performance (Kao et al., 2005). These findings suggest that greater trial-by-trial variability in LMAN activity during undirected singing may generate variability in the ongoing activity in RA and subsequent song output (Figure 4-2). In contrast, stereotyped patterns of LMAN activity during directed song might facilitate reproducibility in RA activity and song output. This hypothesis predicts that lesions of LMAN should eliminate socially driven differences in song variability (Kao et al., 2005).

Here, we extend our previous findings and show that LMAN is a source of the variability in syllable structure. Lesions of LMAN cause a reduction in the moment-by-moment variability in syllable structure during undirected song to the level present during directed song, thus eliminating context-dependent differences in song variability. This effect is immediate and long lasting, consistent with the hypothesis that a critical contribution of the AFP to song plasticity is to modulate variability in ongoing activity in the motor pathway. Similarly, we show that lesions of LMAN also prevent an increase in the variability of syllable structure that is induced by deafening. Finally, we report that the lesion-induced stabilization of song is similar to a natural stabilization of syllable structure that occurs with aging. In contrast to the requirement of LMAN for the modulation of syllable structure, input from LMAN is not required for the social modulation of higher-level features of song, such as the number of introductory elements and the number of repetitions, or for other motor aspects of courtship. Male zebra finches continue to orient towards, approach, and solicit females following lesions of LMAN, suggesting that modulation of the courtship display is mediated by structures other than the AFP. Taken together, our findings suggest that a key function of LMAN in

regulating vocal plasticity is to modulate ongoing, moment-by-moment variability in the structure of individual song elements.

MATERIALS AND METHODS

Subjects

Juvenile (> 80 days) and adult (> 125 days) male zebra finches (*Taeniopygia guttata*) were used for experiments (n = 39 birds). All birds were raised in individual breeding cages with their parents and siblings until at least 60 days of age. Birds were selected on the basis of their size, singing frequency, and song complexity, and were then isolated in a small cage in a sound-attenuating chamber (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 $MgSO_4$, 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 five 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 50% to 100%. "Sham"

lesions were entirely dorsal to LMAN. In all birds, the medial magnocellular nucleus of the anterior nidopallium (MMAN) remained intact. Lesions were made in birds between 101 and 123 days (n = 11 birds).

Chronic implants. Electrodes were implanted chronically as described previously (Hessler and Doupe, 1999b). Briefly, a lightweight microdrive (UCSF and Caltech Machine Shops) carrying two tungsten electrodes (2-5 MΩ) 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 ~700 μm above LMAN. A reference ground electrode (uninsulated tungsten electrode; A-M Systems, Carlsborg, WA) was implanted such that its tip was located within ~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.

Deafening. Birds were deafened by bilateral cochlear removal between 100 and 125 days (n = 10 birds; Konishi, 1965). The extracted cochleae were examined under a dissecting microscope to ensure that the entire structure had been successfully removed.

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% formalin 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 or reacted with an antibody to calcitonin gene-related peptide (CGRP; Sigma, St. Louis, MO; Bottjer, et al., 1997).

Sound recording

During each experimental session, acoustic signals were recorded by a small microphone above the birdcage and filtered between 200 and 9000 Hz (Krohn-hite, Avon, MA). Custom-written acquisition software (M. Brainard, UCSF; C. Malek and A. Leonardo, Caltech, and C. Roddey, UCSF) recorded the acoustic signals before and after the sound amplitude crossed a threshold level. For experiments in which song was recorded in different behavioral contexts, 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 frequency of undirected song was often greater when the experimental bird could hear the calls of other birds outside of 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 an experimental session. Experimental sessions lasted between 30 minutes and several hours depending on singing frequency (n = 29 birds).

For experiments examining the effects of lesions of LMAN on context-dependent differences in song, for each bird, interleaved bouts of directed and undirected song were recorded once a week for 2–3 weeks preceding surgery, on the day before bilateral lesions, and immediately following lesions. Birds were recorded every day following the lesions until enough songs were collected in both social conditions on a particular day (1–3 days) and then once a week for three 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). Two age-matched control birds were also recorded weekly. The range of ages of the birds at the first recording was 88–102 days.

To investigate the effect of age on context-dependent differences in song structure, we recorded from six birds that were greater than four years old and 11 birds less than six months old.

For experiments examining the effects of deafening on song structure, undirected songs were recorded one day prior to deafening and ~30 days following deafening (n = 10 birds).

Physiological recording

During each experimental session, a flexible lead terminating in a small operational amplifier circuit (Texas Instruments, Dallas, TX) 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,

1999b). The neural activity signal passed through the commutator to a differential amplifier (A-M Systems, Carlsborg, WA) 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 before and after the sound amplitude crossed a threshold level, and the bird's behavior was monitored and recorded by a video camera.

Recordings were made at intervals of one day 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 (see above). 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 msec in duration; 'motifs', which are stereotyped sequences of syllables, and 'bouts' of song, which are defined as periods of singing separated by silent intervals at least 2 sec 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).

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, and 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. To improve the resolution of the frequency estimates, we performed a parabolic interpolation of the peak of the ACF (de Cheveigné and Kawahara, 2000).

This algorithm was applied to syllables that had either clear harmonic structure with a well-defined FF or a high frequency, band-limited element. 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.

For experiments investigating the effects of deafening on syllable structure, the songs used for our analysis were selected based on the following criteria: 1) the sequence of syllables was the same pre- and post-deafening; and 2) the spectral structure of individual syllables was not abnormal by visual inspection. All syllables had clearly defined harmonic structure and did not exhibit any conspicuously upwardly sweeping frequency components, which become more common as song deteriorates after deafening (Brainard and Doupe, 2001). These restrictions ensured that the same feature of syllable structure was measured before and after deafening.

Analysis of higher-order features of song. To quantify context-dependent differences in higher-order features of song, we counted the number of introductory elements and the number of motifs per song bout. Song bouts were separated by ≥ 2 sec

of silence (Sossinka and Böhner, 1980). 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 msec 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 across different birds. For the number of motifs per bout, we counted all motifs in which at least 50% of the motif was sung because zebra finches often truncate the last motif in a song bout (i.e., if the canonical motif was 'abcdef', all motifs that included at least 'abc' were counted).

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 sec) recorded 1 day pre-lesion and 7 days post-lesion 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 all 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 (Sossinka and Böhner, 1980; Kao et al., 2005). 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 song elements, or 'motif', per bout, and is delivered at a faster tempo than when the male sings alone or to no particular audience ('undirected song'; Figure 4-3a). In addition, stereotypy in the structure of individual song elements, or 'syllables', is greater during directed song than during undirected song (Figure 4-3b). In the following sections, we systematically investigate the contribution of LMAN to each of these context-dependent features of song.

Context-dependent differences in 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 song elements, or '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 (see Figure 4-3b). We chose to quantify fundamental frequency (FF) because it is a learned parameter of song that is precisely controlled (Tchernichovski et al., 2001) and robust to differences across recording conditions. Moreover, 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).

We first investigated the stability or persistence of context-dependent differences in the variability of syllable structure over time. We found that variability in FF was consistently greater during undirected song than during directed song across multiple recording sessions of the same birds over a period of three weeks (Figures 4-4a, b, *open symbols*; Figures 4-5a, b, *left*; $n = 34$ syllables in 9 birds; age at first recording ranged from 88–102 days; $p \leq 0.001$ for each week; paired sign test). Variability in the spectral structure of individual syllables is illustrated with scatterplots comparing the variability in the FF in the two social contexts for all weekly recordings (Figures 4-4a, b). In normal birds, in the absence of experimental manipulation (*open symbols*), the majority of syllables lie above the diagonal line, indicating that variability in FF is consistently greater during undirected song.

Next, we investigated whether input from LMAN is responsible for the observed context-dependent difference in the variability of syllable structure. If the greater trial-by-trial variability in the pattern of neural activity in LMAN during undirected song generates variability in the motor pathway and subsequent song output, then removing LMAN activity should eliminate the observed difference in syllable structure (Figure 4-2). We found that lesions of LMAN eliminate context-dependent differences in the variability of syllable structure by reducing the moment-by-moment variability in FF during undirected song to the level present during directed song. This is illustrated in Figure 4-4a by the downward shift in the population of syllables to the diagonal line, which indicates that variability in FF is equal across social context. In contrast, greater variability in syllable structure during undirected song persisted in control birds with matched weekly recordings (Figure 4-4b, *open symbols*; nine syllables in two birds; Figure 4-5b, *right*). Moreover, the lesion-induced reduction in the absolute level of variability during undirected song did not reflect a general effect of surgery. Greater trial-by-trial variability in FF during undirected song persisted in birds that received sham lesions for up to 8 weeks post-surgery (Figure 4-4b, *filled symbols*; six syllables in two birds; Figure 4-5b, *right*). Thus, elimination of the context-dependent difference in the variability of syllable structure was the result of the stabilization of syllable structure in the undirected condition, not an enhancement of variability in the directed condition or a general degradation of syllable structure. These findings support the hypothesis that LMAN actively introduces variability into ongoing motor activity and subsequent song output.

In contrast to the stable, context-dependent differences in the variability of syllable structure pre-lesion, socially driven differences in syllable structure were absent immediately following lesions of LMAN (in the first recordings 1–3 days post-lesion; Figure 4-5a). Moreover, variability in FF during undirected song was significantly lower in the first recording post-lesion compared to that one day pre-lesion ($p = 0.002$; Student-Newman-Keuls test). Finally, the effects of the lesions were long lasting. Context-dependent differences in the variability of syllable structure remained absent for up to 8 weeks following lesions of LMAN (Figure 4-5a).

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 following the lesions (Figures 4-4a, 4-5a). 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, may represent 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. For example, the birth, death, and incorporation of new neurons in the motor pathway may contribute to variability in syllable structure following lesions of LMAN (Nordeen and Nordeen, 1988; Alvarez-Buylla et al., 1990; Scharff, 2000).

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? The finding that lesions of LMAN did not affect the level of variability in syllable structure during directed singing suggests that lesions of LMAN do not affect courtship behavior. To examine this issue directly, we scored video clips of directed and undirected bouts of song recorded 1 day pre-lesion and ~7 days post-lesion. 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 Zann, 1996; Williams, 2001).

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. Figure 4-6 illustrates the difference in the behavior of male zebra finches in different 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 (Figure 4-6 *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 (Figure 4-6, *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.

Higher-order features of song: context-dependence and effects of lesions of LMAN

Since lesions of LMAN specifically affect context-dependent differences in song output and not other motor aspects of courtship, we next asked whether the lesions affect other, higher-order features of song structure. Previous studies have characterized several context-dependent differences in song structure: directed songs contain more introductory elements and more motifs per bout than undirected songs and are sung at a slightly faster rate (Figure 4-3a; Sossinka and Böhner, 1980). 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).

To assess the contribution of LMAN to socially driven differences in higher-level features of song, we focused our analysis on the number of introductory elements and the number of motifs per bout because context-dependent differences in song tempo are less robust and often inconsistent across multiple recordings of an individual bird (*data not shown*). Consistent with previous reports, we found that the number of introductory elements was significantly greater during directed song in all birds prior to lesions of LMAN (Figure 4-7a, *left*; $p < 0.0005$; Mann–Whitney U test for comparisons within one recording session for each bird; $n = 5$ birds). In four of five birds, the number of motifs per bout was also significantly greater during directed song (Figure 4-7b, *left*; $p < 0.005$, M–W U test for comparisons within one recording session for each bird). One week after

lesions of LMAN, these context-dependent differences persisted. In four of five birds, the number of introductory elements and the number of motifs per bout were significantly greater during undirected song than during directed song (Figures 4-7a, b, *right*; $p <$ 0.0005, M–W U test for comparisons within one recording session for each bird). Thus, in contrast to the requirement of LMAN for the social modulation of syllable structure, modulation of higher-level features of song by social context did not require input from the AFP, suggesting that different parameters of song may be independently regulated by distinct brain regions.

Trial-by-trial variability: a strategy for the adaptive modification of song?

Trial-by-trial variability in motor output is an important component of feedback-based motor learning. In order for song to change, it must vary across trials so that evaluation mechanisms can selectively reinforce patterns of motor activity that produce the desired behavior and/or punish motor patterns that result in worse performance. To further investigate the hypothesis that LMAN regulates vocal plasticity by modulating variability in motor output, we examined the contribution of LMAN to the modification of song in birds under conditions of increased plasticity (deaf birds) and under conditions of reduced plasticity (older birds).

Increased variability in syllable structure following deafening requires LMAN

In the context of reinforcement learning, variability in song output should depend on the current status of motor performance. When the song is close to the desired target, as in adult birds, a low degree of variability ensures that each rendition is near the target.

In contrast, when song is far from the desired target, greater variability is appropriate in order to coarsely explore the motor space in search of better solutions.

To test this model of song learning, we deafened adult birds (102-124 days) and characterized the nature of the changes to syllable structure, focusing on variability in the spectral structure of syllables across repeated renditions (Figures 4-8a, b). The model predicts that trial-by-trial variability in syllable structure should increase as a result of the mismatch between auditory feedback and the desired target, thereby enabling the search for changes that might lead to an improvement. When we compared the undirected songs of individual adult birds recorded prior to deafening with those recorded approximately one month after deafening, we found that prior to changes in the temporal order of syllables, deafening induced a significant increase in the variability of syllable structure (Figure 4-8; $p = 0.0039$; paired sign test; nine of nine syllables in five birds, including one bird with sham lesions). The increase in the variability of fundamental frequency (FF) following deafening was significant in five of nine harmonic stacks (F-test for equality of variance across conditions for each syllable). We did not observe, however, a systematic change in the mean FF of syllables one month after deafening, consistent with previous reports (*data not shown*; Nordeen and Nordeen, 1992; Brainard and Doupe, 2001).

These findings demonstrate that the degree of variability in syllable structure present during undirected song under normal conditions does not reflect the maximum level of variability possible in adult birds. Even in the undirected condition, adult males may restrict the variability of syllable structure in order to ensure that each rendition is

near the desired target. Only when there is a gross mismatch between auditory feedback and the target does the latent variability become manifest.

To examine whether input from LMAN contributes to the deafening-induced increase in the variability of syllable structure, we also analyzed the songs of birds that received bilateral lesions of LMAN one day prior to deafening. Figure 4-8c shows that at one month after deafening, variability in syllable structure did not increase in birds with lesions of LMAN (*right*; $n = 7$ syllables in four birds; $p > 0.9999$; paired sign test). Moreover, variability in FF was significantly lower in lesioned–deafened birds compared with that of age-matched deafened birds (Figure 4-8c; $p = 0.023$; M–W U test). Thus, lesions of LMAN prevent deafening-induced increases in the variability of syllable structure, which precede the gross deterioration of song that normally follows deafening (Nordeen and Nordeen, 1998; Lombardino and Nottebohm, 2000; Brainard and Doupe, 2000, 2001). Together, these results provide further evidence to support the hypothesis that a critical contribution of LMAN to song plasticity is to introduce variability into motor pathway (Doya and Sejnowski, 1999; Kao et al., 2005).

Stabilization of syllable structure by age

In contrast to the high variability expected when motor output is far from the desired target, lower variability is expected when song approaches the desired target because it ensures that each rendition is close to the target. In adult birds that have learned the memorized song model, only a low degree of variability should be necessary to maintain the song. Moreover, stereotypy in song output may facilitate the recognition of individuals. To further explore the hypothesis that variability in song output depends

on the current state of motor performance, we examined whether and how variability in syllable structure changes with age.

Previous studies have demonstrated a natural stabilization of song structure that occurs with aging (Lombardino and Nottebohm, 2000; Brainard and Doupe, 2001). Consistent with these reports, we found that context-dependent differences in the variability of syllable structure declines with age (Figure 4-9a). In young adults (< 6 months old), variability in FF was significantly greater during undirected song than during directed song (Figure 4-9a; *left*; 28 of 29 syllables in 11 birds; $p < 0.0001$; paired sign test). These differences were significant in 19 of 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 (Figure 4-9a, *right*; $n = 13$ syllables in six birds; $p = 0.5811$; paired sign test). The lack of context-dependent differences in syllable variability in older birds was the result of a reduction in the absolute level of variability in FF during undirected song to the level present during directed song (Figure 4-9a). As predicted by the model, variability in FF during undirected song was significantly lower in older birds than in young adults (Figure 4-9a; $p = 0.0015$, M–W U test), reminiscent of the lesion-induced stabilization of syllable structure (compare Figures 4-5a and 4-9c).

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 become hard-wired, the ability of extrinsic signals to modulate ongoing motor patterns and subsequent song output may decline with age (Lombardino and Nottebohm, 2000).

To distinguish between these possibilities, we compared the singing-related activity in LMAN in birds over a wide range of ages (< 6 months to > 4 years old). Consistent with previous reports, we found that trial-by-trial variability in the pattern multi-unit activity in LMAN was significantly greater during undirected song than during directed song in young adult birds (< 6 months old; Figure 4-9b, *left*; 10 of 10 sites in five birds; $p = 0.002$; paired sign test). Similarly, in older adults (> 4 years old), variability in LMAN activity was also greater during undirected song (Figure 4-9b, *right*, nine of nine sites in two 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 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 (Figure 4-9b). This age-dependent decline in the absolute level of LMAN variability was apparent in both behavioral contexts (Figure 4-9b; 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 may contribute to the age-dependent stabilization of syllable structure: 1) the susceptibility of the motor pathway to

sources of perturbation, such as context-dependent differences in LMAN activity, may decline with age; and 2) the factors that promote variability in motor activity may decline with age.

DISCUSSION

This study generally confirms and extends previous reports of the striking modulation of behavior by social context (Sossinka and Böhner, 1980; Kao et al., 2005). In addition to the context-dependent differences in higher-level features of song, such as the number of introductory elements and the number of motif repetitions, we show that there is a stable, robust difference in the variability of syllable structure across social context (Figures 4-4a, b; *open symbols*; Figures 4-5a, b; *left*). Moreover, we demonstrate that input from the AFP is required for the context-dependent differences in syllable variability. Lesions of LMAN specifically reduce the moment-by-moment variability in syllable structure during undirected song to the level present in directed song (Figures 4-4a, 4-5a), but do not affect other socially driven differences in song (Figure 4-7). In addition, input from LMAN is also required for the increased variability in syllable structure that is induced by deafening and precedes the gross deterioration of song following deafening (Figure 4-8). Together, these results suggest that a critical contribution of LMAN to vocal plasticity is the modulation of variability in motor output.

Contributions of LMAN to song plasticity

Signals from LMAN could influence variability in the motor pathway on multiple timescales. On a rapid timescale, neural activity in LMAN may modulate the ongoing

patterns of activity in the premotor nucleus RA. Consistent with this idea, we have shown previously that microstimulation in LMAN during singing can induce changes in syllable structure on a moment-by-moment basis (Kao et al., 2005). Moreover, the artificial introduction of variability to ongoing activity in RA can drive increased variability in syllable structure (Kao et al., 2005). In this study, we provide further evidence that LMAN activity may act on a fast timescale. We show that lesions of LMAN cause a rapid reduction in the trial-by-trial variability in syllable structure during undirected song that is apparent in the first recordings post-lesion.

Alternatively, or in addition, other signals from LMAN could influence variability in the motor pathway on a slower timescale. For example, trophic factors from LMAN may introduce variability in song output by promoting synaptogenesis, the maintenance of weak connections in RA, and/or the incorporation of new neurons (Scharff and Nottebohm, 1991; Johnson et al., 1997; Kittelberger and Mooney, 1999, 2005). Indeed, previous studies have demonstrated that LMAN provides trophic support to RA during development (Akutagawa and Konishi, 1994; Johnson and Bottjer, 1994). While we cannot rule out the role of trophic factors from LMAN on the generation of variability in the motor pathway, the rapid changes in song structure elicited by microstimulation in LMAN (Kao et al., 2005) and the rapid effects of lesions of LMAN are consistent with an acute modulation of activity in the motor pathway.

Our findings, however, do not preclude other contributions of neural activity in LMAN to the adaptive modification of song. Previous work has suggested that LMAN activity may contribute to song plasticity by providing an instructive signal to bias the motor pathway towards a particular target (Troyer and Doupe, 2000; Brainard and

Doupe, 2000). Consistent with this idea, we previously demonstrated that a fixed pattern of activation in LMAN typically induced systematic changes in song structure, not a general enhancement of song variability (Kao et al., 2005).

Determining the functional contribution(s) of the AFP to song plasticity (instructive signal versus generation of variability) may be facilitated by finer analyses of the songs of juvenile birds following lesions of LMAN. If neural activity in LMAN acts by biasing patterns of motor activity towards a particular target (i.e., the memorized song model), then removal of this instructive signal during sensorimotor learning should result in songs that are farther from the target than the pre-lesion songs. In addition, comparison of LMAN activity in juvenile birds during "good" and "poor" renditions of song may be informative. If LMAN is a source of variability to the motor pathway, then the degree of variability in LMAN activity across repeated trials should depend on the current state of motor performance. When song is far from the desired target (i.e., the memorized song model), greater variability in LMAN activity should introduce variability in RA activity and subsequent song output, facilitating the search for changes that might lead to an improvement. In contrast, during "good" renditions, when the song is close to the desired target, greater stereotypy in LMAN activity should help to reduce variability in RA activity and ensure a good match to the target.

Possible functions of different song types

In the context of reinforcement learning, trial-by-trial variability in motor output is required in order for evaluation mechanisms to differentially reinforce those patterns of motor activity that produce the desired behavior and/or to punish the motor commands

that result in worse performance. After the behavior is learned, the ability to modify it remains important in adult birds, either for continued motor exploration and optimization or for correction of errors to maintain song. Adult birds tightly regulate song variability depending on the intended audience. For example, in several species of birds, males often sing the song in their repertoire that best matches the song sung by a nearby conspecific ("song matching"; Payne, 1983; Stoddard, 1992). Similarly, the response of females may determine the song variant that a male sings (West and King, 1988). In zebra finches, song variability is actively inhibited when a male sings to a female (Sossinka and Böhner, 1980; Kao et al., 2005).

Variability in motor output, however, may continue to be important in adult birds in order to compensate for perturbations in the motor program, which may arise from changes in hormone levels, the birth, death and incorporation of new neurons in the motor pathway, and changes in the periphery (e.g., muscle tone and innervation) (Nordeen and Nordeen, 1988; Alvarez-Buylla et al., 1990; Scharff, 2000). Under such circumstances, the ability to modify song is critical for feedback-based correction of errors and song maintenance.

While the reasons for the striking modulation of song variability by social context remain unclear, one 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 (Jarvis et al., 1998). In contrast, the acute inhibition of variability in the directed condition suggests that directed song may reflect a state of motor performance in which individuals exploit what they

have already learned and consistently select the patterns of motor activity that produce their "best" current version of song (Sutton and Barto, 1998).

Previous studies have shown that female preference is affected by features of song that may reflect the outcome of learning, such as song complexity, rate, quantity, and vocal performance (reviewed by Nowicki and Searcy, 2004). It has been postulated that such features indicate male quality because of the high cost of developing a neural substrate for song learning and production (Nowicki et al., 1998; Buchanan et al., 2003). According to this hypothesis, features of song specific to directed singing might serve as indicators of male quality. In zebra finches, preliminary studies using a two choice behavioral paradigm suggest that both mated and naive females have a preference for directed song over undirected song (S. C. Woolley and A. Doupe, *unpublished observations*). While it is known that female zebra finches respond more to higher song rates and longer durations (Clayton and Prove, 1989; Collins et al., 1994), two features of directed song, it remains to be determined whether variability in syllable structure also affects female preference. Further studies using the directed songs of males before and after lesions of LMAN may help to elucidate those features of directed song that are important to females because lesions of LMAN specifically affect variability of syllable structure without affecting context-dependent differences in the number of introductory notes and motifs renditions (i.e., song duration).

Possible mechanisms underlying song stabilization

Consistent with previous reports, we found that both age and lesions of LMAN cause an increase in the stereotypy of song (Bottjer et al., 1984; Scharff and Nottebohm,

1991; Williams and Mehta, 1999; Lombardino and Nottebohm, 2000; Brainard and Doupe, 2000). We report that context-dependent differences in the variability in syllable structure decline with age as the moment-by-moment variability in fundamental frequency is reduced in undirected song to the level present during directed song (Figure 4-9a). This age-dependent stabilization of song structure coincides with an age-dependent reduction in the sensitivity of song to deafening, perhaps reflecting a decline in the susceptibility of the motor pathway to sources of perturbation, such as altered auditory feedback or context-dependent differences in the activity of the AFP (Lombardino and Nottebohm, 2000; Brainard and Doupe, 2001). Indeed, we found that context-dependent differences in the singing-related activity in LMAN are present in older adult zebra finches (> 4 years old). Regardless of age, variability in the pattern of LMAN activity was greater during undirected song (Figure 4-9b). These results suggest that the ability of the AFP to modulate the motor pathway declines with age.

A decline in the sensitivity of neural circuits to extrinsic signals has been shown in many systems. Numerous forms of learning, including vocal learning, are subject to 'sensitive' or 'critical' periods during which experience is crucial for shaping the function and connectivity of neural circuits. Once the sensitive period is closed, however, the susceptibility of the nervous system to the same inputs declines.

In addition to a possible decline in the susceptibility of the motor pathway to persistent context-dependent differences in LMAN activity, we also found that the absolute level of variability in LMAN activity was significantly lower in older birds than in young birds in both social contexts (Figure 4-9c). These results suggest that stabilization of song by age is due, in part, to a decline in the factors from the AFP that

promote variability in the motor pathway. Indeed, other sources of perturbation to the motor pathway have been shown to decline with age, such as the production or early survival of new neurons in HVC (Wang et al., 2002). Thus, stabilization of song by age may derive from a decline in the sensitivity of the motor pathway to sources of perturbation as well as a decline in the sources of perturbation themselves.

Separate neural circuits for different aspects of courtship behavior

In contrast to the requirement of LMAN for the social modulation of syllable structure, modulation of other features of song and courtship did not require input from the AFP. These findings suggest that distinct brain regions may mediate different parameters of song and courtship. This idea is consistent with the anatomy of the song system: neurons in LMAN project directly to the premotor nucleus RA, which is thought to provide the motor commands the control the precise structure of individual song elements (Vu et al, 1994; Yu and Margoliash, 1996). In contrast, other components of the song system may govern higher-level features of song, such as the temporal pattern of syllables, the rate of song delivery, and the number of introductory elements and motif repetitions. Indeed, microstimulation in the motor nucleus HVC has been shown to cause interruption of singing, restarting of song, and changes in the temporal pattern (Vu et al., 1994). In addition, estrogen receptors are present in HVC in zebra finches (Metzdorf et al., 1999), and estrogen has been shown to stimulate males to increase song rate and the number of songs per bout in the directed condition (Walters et al., 1991).

In addition, song is only one element in the courtship repertoire of birds. In zebra finches, directed song is often accompanied by a rhythmic dance that involves

approaching the female, hopping to and fro, swinging the body from side to side, and changing head positions (reviewed in Zann, 1996). These body and dance movements are coordinated with song during courtship (Williams, 2001) and have been shown to be susceptible to alteration of the level of neuromodulators, such as dopamine (Harding, 2004). Despite the strong dopaminergic input to the AFP from the avian ventral tegmental area (Lewis et al., 1981), we found that lesions of LMAN did not prevent the courtship dance that accompanies directed song (Figure 4-6). Following lesions of LMAN, males continued to orient towards, approach, and solicit females, suggesting that the social modulation of the courtship display is mediated by structures other than the AFP.

Figure 4-1 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 (nXIIts). 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).

Figure 4-1

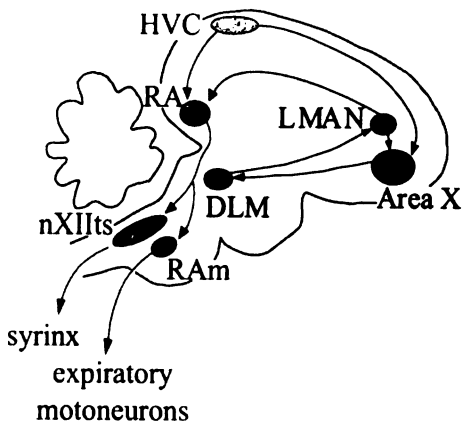

HVC
RA
LMAN
DLM
Area X
nXIIts
RAm
syrinx
expiratory
motoneurons

Figure 4-2 Hypothetical model for the contribution of LMAN activity to social influences on song production and plasticity. *A.* During undirected song, trial-by-trial variability in the patterns of LMAN activity introduces variability into the activity in the premotor nucleus RA, resulting in variable song output. In contrast, during directed song, stereotyped patterns of LMAN activity facilitate reproducibility in the activity of RA neurons and subsequent song output. *B.* Lesions of LMAN eliminate socially driven differences in song variability.

Figure 4-2

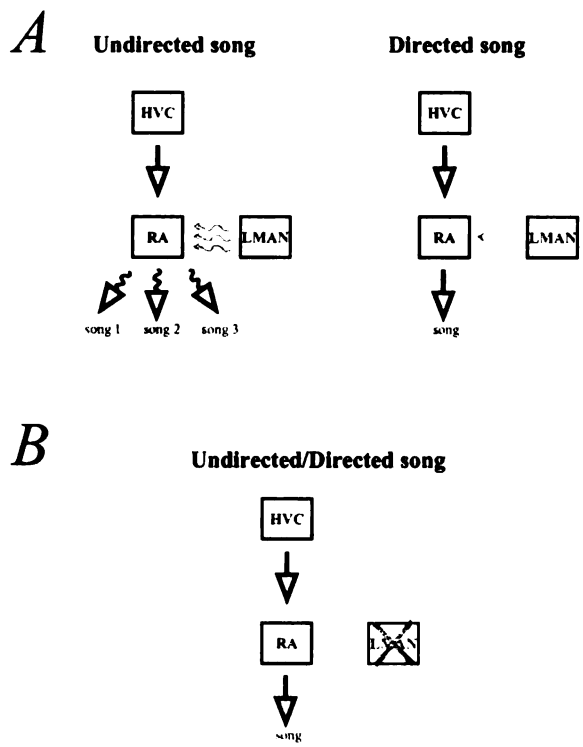

A
Undirected song
Directed song
HVC
RA
LMAN
song 1
song 2
song 3
song
B
Undirected/Directed song
HVC
RA
LMAN
song

Figure 4-3 Context-dependent differences in song output. *A.* Spectrogram (plot of frequency versus time) of one bout of song recorded when a male is alone ('undirected' song; *top*) and when it sings to a female ('directed' song; *bottom*). Song bouts consist of a series of introductory elements ('i') followed by a variable number of repetitions of a stereotyped sequence of syllables, or 'motif' (indicated by bars). The number of introductory elements and motifs per bout is lower on average during undirected song than during directed song. *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' (*left*) and 'c' (*right*). Variability in the FF is greater during undirected song than during directed song (syllable 'a': s.d.: 8.8 versus 3.4; $p < 0.0001$, F-test for equality of variance; syllable 'c': s.d.: 7.2 versus 2.5; $p < 0.0001$; F-test for equality of variance).

Figure 4-3

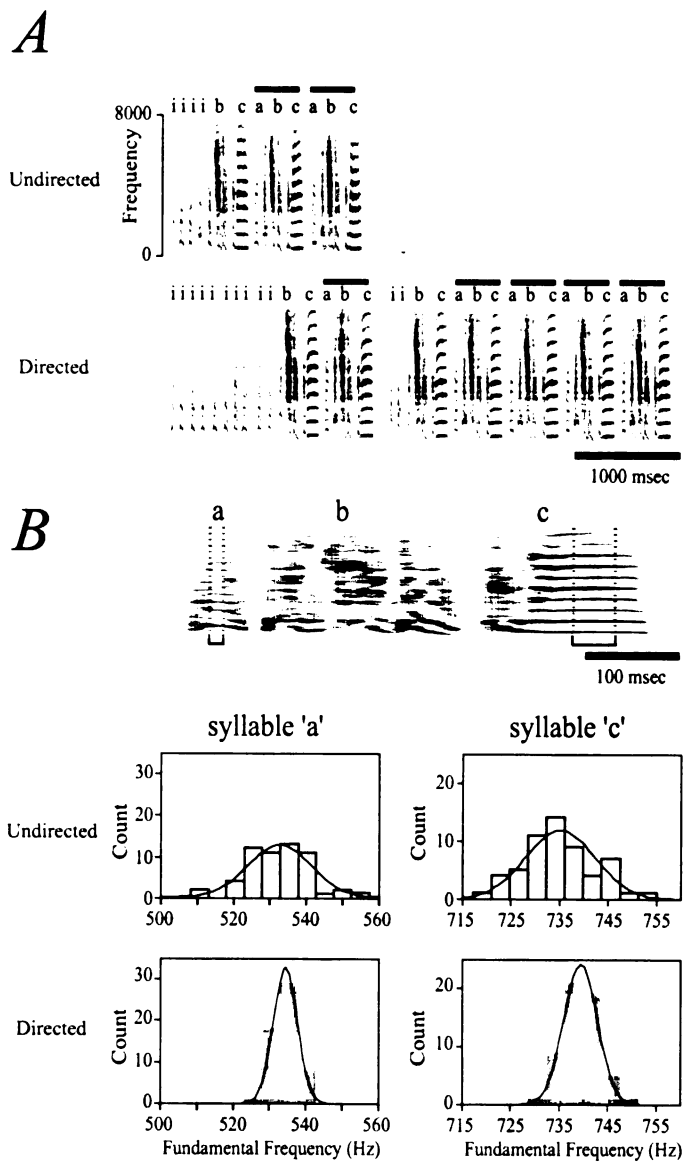
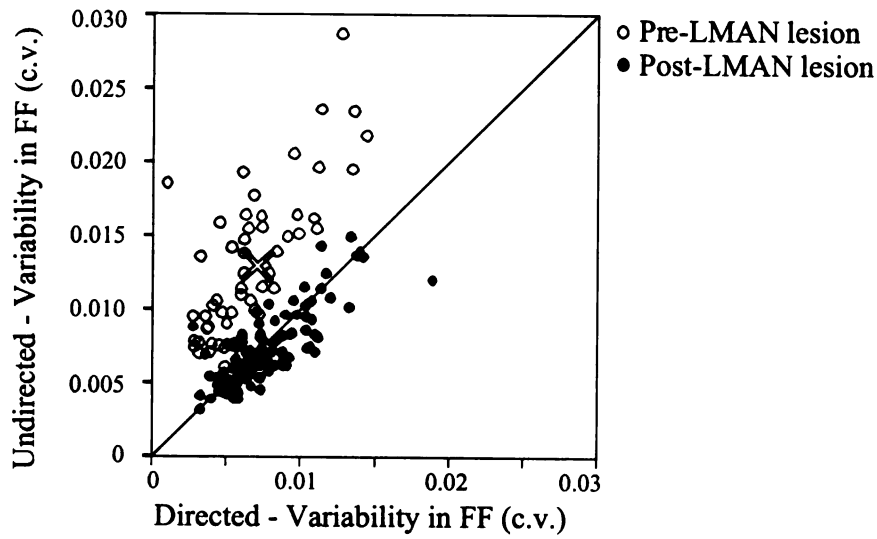

A
8000
Frequency
0
Undirected
Directed
1000 msec
B
a
b
c
100 msec
syllable 'a'
syllable 'c'
Undirected
Directed
Count
30
20
10
0
500
520
540
560
715
725
735
745
755
Fundamental Frequency (Hz)

Figure 4-4 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 c.v. of the FF for one syllable in both social contexts during one recording session. *A.* Data are plotted for 19 syllables for all recordings before (*open symbols*) and after (*filled symbols*) lesions of LMAN. Crosses denote the means for the populations. 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). *B.* Context-dependent differences in FF persisted in birds with sham lesions (*triangles*; $n = 6$ syllables in two birds) and in control birds (*open black circles;* $n = 9$ syllables in two birds).

Figure 4-4

A


0.030
0.025
0.020
0.015
0.010
0.005
0
0
0.01
0.02
0.03
Undirected - Variability in FF (c.v.)
Directed - Variability in FF (c.v.)
Pre-LMAN lesion
Post-LMAN lesion

B

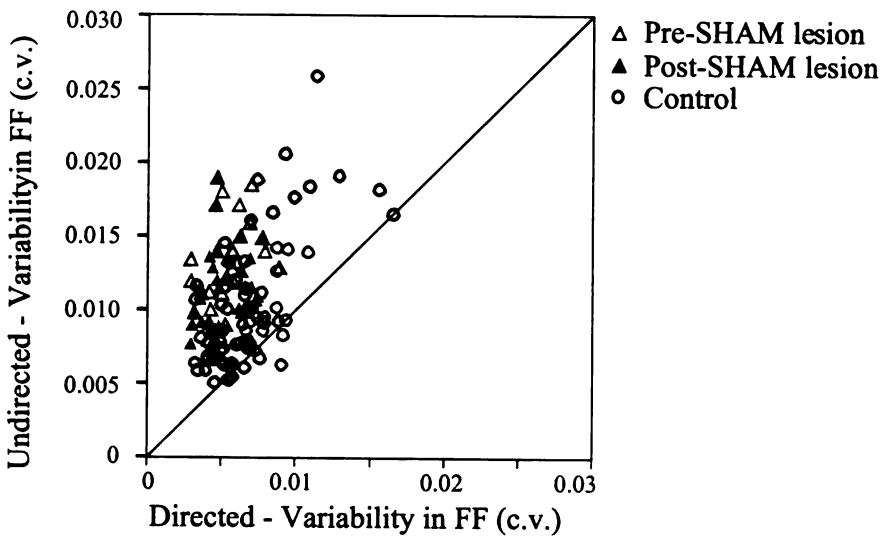

0.030
0.025
0.020
0.015
0.010
0.005
0
0
0.01
0.02
0.03
Undirected - Variabilityin FF (c.v.)
Directed - Variability in FF (c.v.)
Pre-SHAM lesion
Post-SHAM lesion
Control

Figure 4-5 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 (*white)* and directed song (*gray*). *A.* Summary for all syllables before and after lesions of LMAN. Bilateral lesions of LMAN abolish the socially driven differences in song variability (19 syllables in five birds; $p < 0.0001$ for each recording pre-lesion, paired sign test) by reducing variability in the undirected condition. Variability in FF during undirected song was significantly lower in the first recording post-lesion compared to that one day pre-lesion ($p = 0.002$; Student-Newman-Keuls test). *B.* Context-dependent differences in song variability persist in control birds and birds with sham lesions ($n = 15$ syllables in four birds; $p < 0.05$ for each recording, 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 $p < 0.01$.

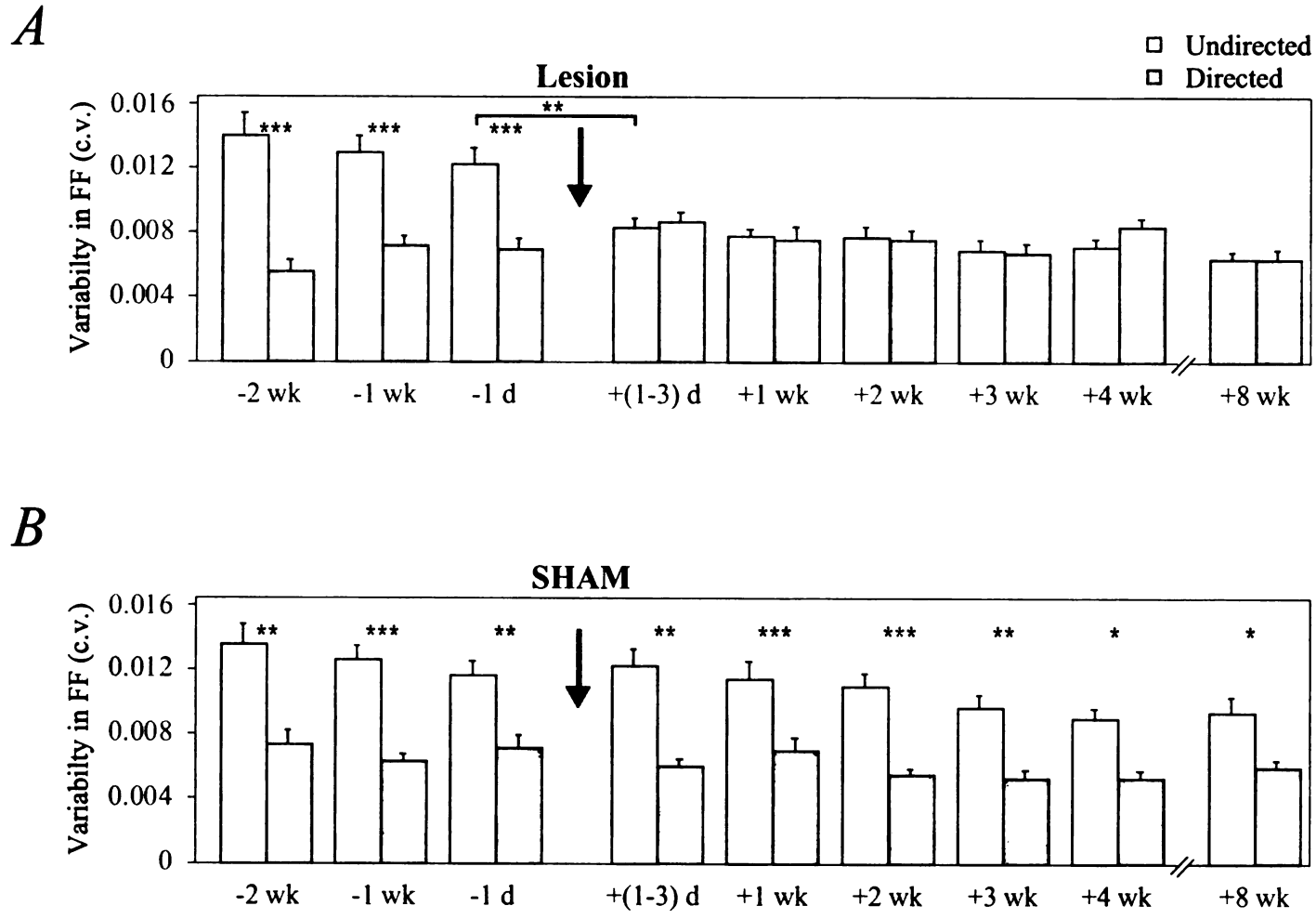

A
Lesion
Undirected
Directed
Variabilty in FF (c.v.)
0.016
0.012
0.008
0.004
0
***
***
***
**
-2 wk
-1 wk
-1 d
+(1-3) d
+1 wk
+2 wk
+3 wk
+4 wk
+8 wk
B
SHAM
Variabilty in FF (c.v.)
0.016
0.012
0.008
0.004
0
**
***
**
**
***
***
**
*
*
-2 wk
-1 wk
-1 d
+(1-3) d
+1 wk
+2 wk
+3 wk
+4 wk
+8 wk

Figure 4-5

Figure 4-6 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 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.

Figure 4-6

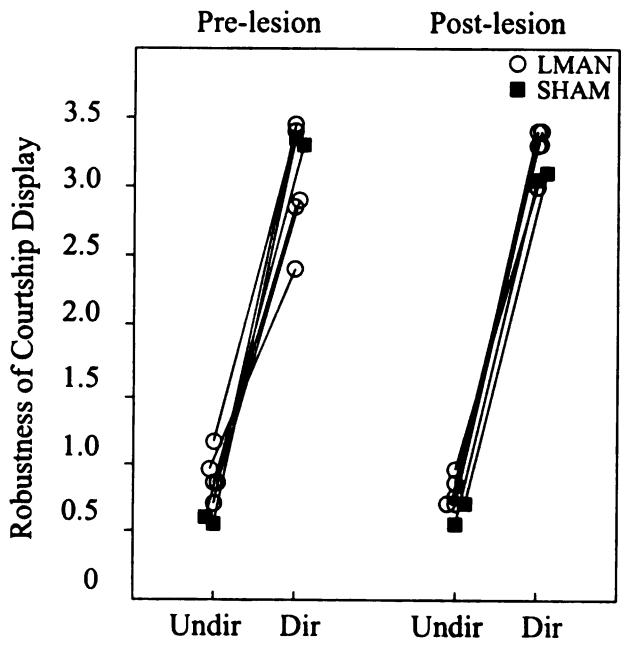

Pre-lesion
Post-lesion
LMAN
SHAM
Robustness of Courtship Display
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
Undir
Dir
Undir
Dir

Figure 4-7 Context-dependent differences in the global structure of song are not affected by lesions of LMAN. The number of introductory elements and the number of motifs per bout were higher during directed song than during undirected song before and after lesions of LMAN. Each point represents the average number of introductory elements per song bout (*A*) or the average number of motifs per bout (*B*) for one recording session, and lines connect data points for the same bird across behavioral contexts. Bars indicate group means; error bars indicate the s.e.m.

Figure 4-7

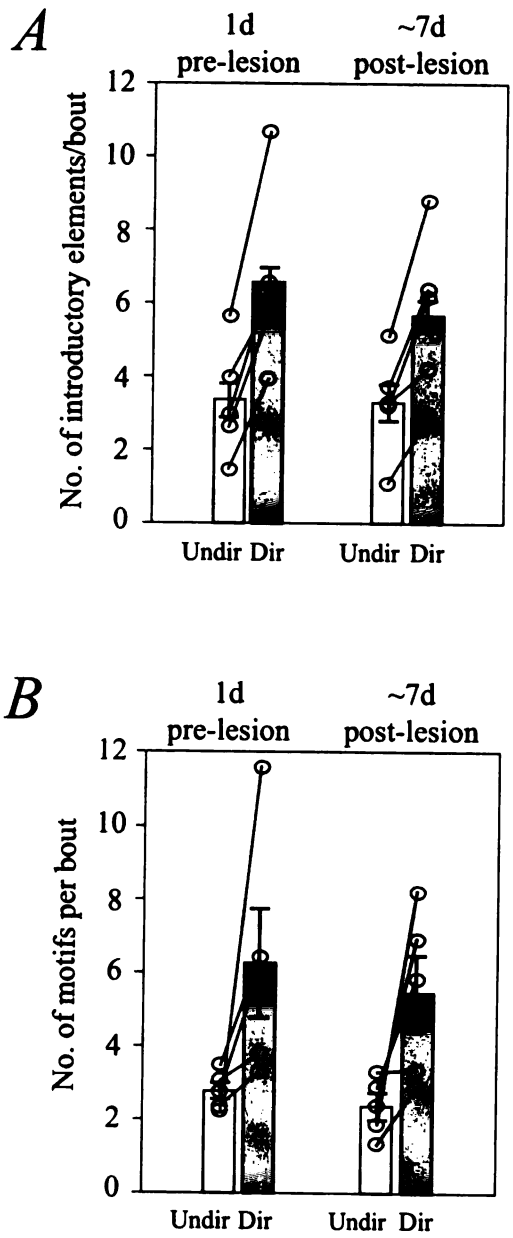

A
1d
pre-lesion
~7d
post-lesion
No. of introductory elements/bout
12
10
8
6
4
2
0
Undir Dir
Undir Dir
B
1d
pre-lesion
~7d
post-lesion
No. of motifs per bout
12
10
8
6
4
2
0
Undir Dir
Undir Dir

Figure 4-8 Deafening-induced increase in the trial-by-trial variability in syllable structure requires LMAN. *A*. Spectrograms illustrating changes to song for one bird ~30 days after deafening. Deafening induced a speeding up of the motif ('abcd') and an increase in the variability in syllable structure even when the sequence of syllables did not change (see Materials and Methods). Dotted red lines indicate the portion of the syllable for which FF was measured in (*B*). *B*. Histograms of FF for syllable 'c' before (*top*) and after deafening (*bottom*). Variability in the FF increased following deafening (s.d.: 9.6 versus 15.7). *C*. Summary of syllable stability following deafening in intact birds and birds with lesions of LMAN. Each symbol indicates the c.v. of the FF for one syllable, and lines connect data points for the same syllable before and after experimental manipulation. Deafening induced a significant increase in the variability in syllable structure (nine of nine syllables in five birds; $p = 0.004$, paired sign test). Birds that received lesions of LMAN prior to deafening did not exhibit an increase in the variability in syllable structure following deafening (7 syllables in 4 birds; $p > 0.9999$, paired sign test). Variability in FF was significantly lower in lesioned–deafened birds than in age-matched deafened birds ($p = .023$; Mann–Whitney *U* test).

Figure 4-8

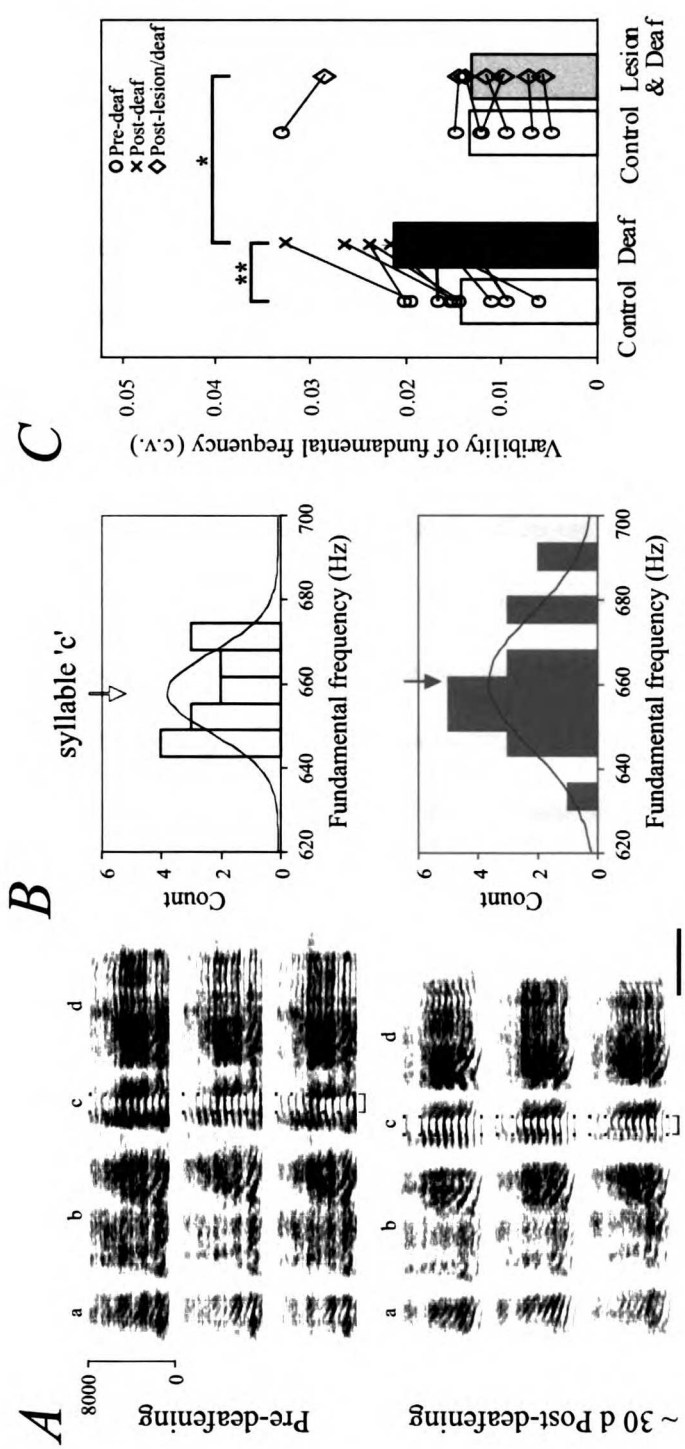
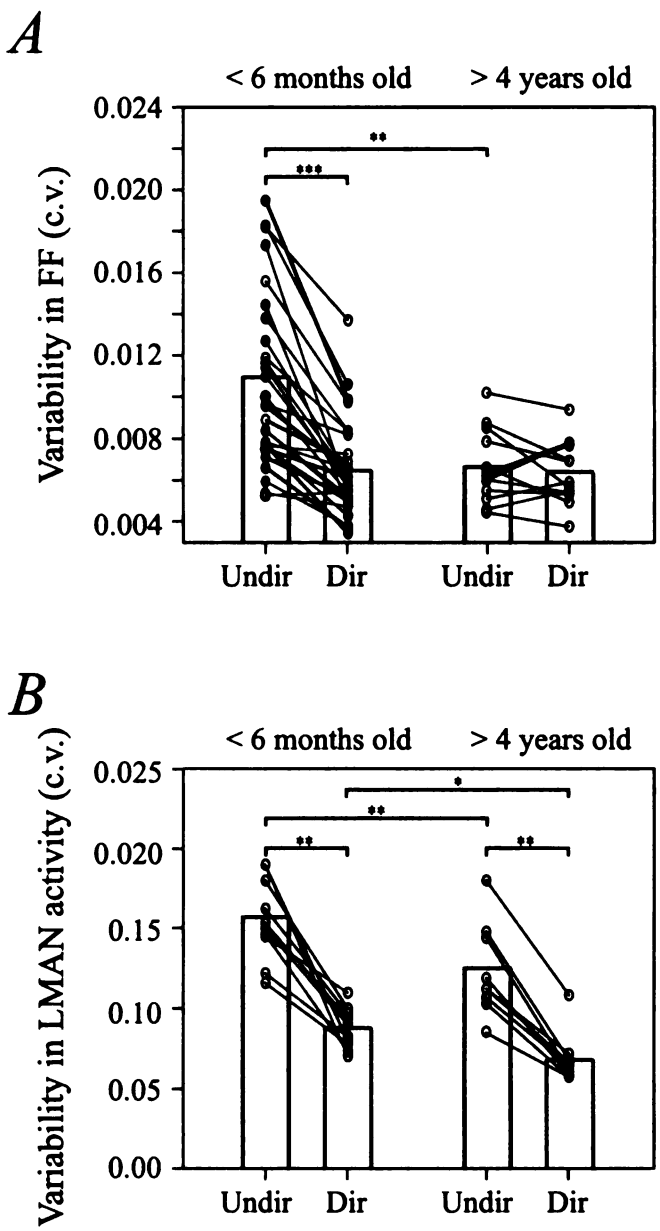

A
Pre-deafening
~ 30 d Post-deafening
8000
0
a
b
c
d
100 msec
B
syllable 'c'
Count
Fundamental frequency (Hz)
620
640
660
680
700
0
2
4
6
C
Variability of fundamental frequency (c.v.)
Pre-deaf
Post-deaf
Post-lesion/deaf
**
*
Control Deaf
Control Lesion & Deaf
0
0.01
0.02
0.03
0.04
0.05

Figure 4-9 Age-dependent stabilization of syllable structure despite persistent context-dependent differences in LMAN activity. *A*. Context-dependent differences in trial-by-trial 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. Trial-by-trial variability in FF was significantly greater during undirected song than during directed song in birds less than six months old (*left*: 28 of 29 syllables in 11 birds; $p < 0.0001$, paired sign test) but not in older birds (*right*: > 4years old; 13 syllables in six birds; $p = 0.5811$, paired sign test). Variability in FF during undirected song decreased significantly with age ($p = 0.0015$, Mann–Whitney *U* test) to the level present during directed song. *B*. Context-dependent differences in LMAN activity are present in birds across a range of ages. Variability in LMAN activity was significantly greater during undirected song than during directed song in birds of different ages (*left*: < 6 months old: 10 of 10 sites in five birds; $p = 0.002$, paired sign test; *right*: > 4 years old: nine of nine sites in two birds; $p = 0.004$, paired sign test). Although context-dependent differences were present in older birds, the absolute level of variability in LMAN activity was significantly lower in older adults in both behavioral contexts (undirected c.v.: 0.155 versus 0.123; $p = 0.01$, Mann–Whitney *U* test; directed c.v.: 0.087 versus 0.068; $p = 0.004$, Mann–Whitney *U* test).

Figure 4-9


A
< 6 months old
> 4 years old
Variability in FF (c.v.)
0.024
0.020
0.016
0.012
0.008
0.004
**
***
Undir
Dir
Undir
Dir
B
< 6 months old
> 4 years old
Variability in LMAN activity (c.v.)
0.025
0.020
0.15
0.10
0.05
0.00
*
**
**
**
Undir
Dir
Undir
Dir

Chapter 5: Future directions

Dissociation of sensory and motor roles of LMAN

Neural activity in LMAN carries both sensory and motor-related signals. Studies in anesthetized birds have characterized neural responses to playback of song stimuli: LMAN neurons respond more strongly to playback of the bird's own song (BOS) than to conspecific songs (Doupe and Konishi, 1991). Moreover, this "song selectivity" emerges during development (Doupe, 1997; Solis and Doupe, 1997), suggesting that neurons in the AFP process auditory feedback of BOS during learning. In awake birds, some neurons in LMAN have been shown to respond to playback of song stimuli, while others do not exhibit auditory responses (Hessler and Doupe, 1999b). In addition to the sensory signals, recordings of LMAN activity in awake, behaving birds have revealed a premotor role for LMAN in song production. Individual neurons in LMAN exhibit distinct, time-varying firing patterns that are correlated with song. Moreover, manipulation of LMAN activity during singing can induce specific changes in learned parameters of song in real-time. It remains unclear, however, whether the same neurons in LMAN carry both sensory and motor signals and how these signals are related to one another.

To dissociate the sensory and motor components of LMAN activity, it is necessary to record from single neurons during singing and during playback of song stimuli. Comparison of the firing pattern of a single LMAN neuron during directed singing, when trial-by-trial variability is low, with the firing pattern during playback of BOS will help to elucidate the sensory versus motor signals in LMAN and their relationship. For example, recordings in the premotor nucleus RA have demonstrated that neurons that are active during singing also exhibit selective auditory responses during sleep (Dave and Margoliash, 2002). Moreover, the pattern of activity evoked by

playback of BOS is similar to the singing-related activity. Playback of a segment of song triggers an auditory response that resembles the premotor activity for the next syllable, suggesting that the auditory response is a prediction of the motor command for the next syllable. It remains to be determined whether a similar sensory-motor correspondence exists in LMAN.

In addition, characterization of the activity of multiple single units in LMAN in the same bird during singing and during playback can help inform our understanding of the function of the different components of LMAN activity. Recordings of multiple single neurons in LMAN in the same bird have shown that different neurons in LMAN exhibit unique patterns of activity during singing. This is consistent with the known topographic projections from LMAN to RA (Johnson and Bottjer, 1995) and suggests that LMAN neurons act jointly to encode the acoustic features of song. It is not known, however, whether different neurons in LMAN exhibit different firing patterns in response to playback of BOS. One possibility is that all neurons in LMAN exhibit a similar response to playback of BOS. Indeed, in anesthetized birds, different LMAN neurons in the same bird seem to exhibit similar patterns of activity in response to playback of BOS (Doupe, 1997; S. Kojima, *personal observations*), suggesting that LMAN sends a global signal to RA. Alternatively, individual neurons in LMAN may exhibit distinct responses to playback of song, consistent with the idea that different neurons may encode different features of song. To investigate whether neural responses differ across the nucleus and whether they are organized topographically will require extensive sampling in LMAN, which may be accomplished by simultaneous recordings with small electrode arrays.

Finally, another way to identify and characterize the sensory versus motor components of LMAN activity is to compare the singing-related activity in LMAN under conditions of normal and altered auditory feedback. If neurons in LMAN are sensitive to auditory feedback, then the firing pattern should differ when the bird is producing the same song but the auditory feedback is perturbed. A previous study in adult zebra finches found little change in the singing-related firing patterns of single LMAN neurons, even when altered auditory feedback prevented the birds from hearing their own vocalizations (Leonardo, 2005). However, the susceptibility of zebra finches to perturbations of auditory feedback has been shown to decline with age (Lombardino and Nottebohm, 1999; Brainard and Doupe, 2001), so similar studies in other species that are more dependent on auditory feedback, such as Bengalese finches, may be more suitable for identifying and characterizing the sensory component of LMAN activity.

The role of the AFP during song learning in juvenile birds

The contribution of neural activity in LMAN to song learning and plasticity in juvenile birds remains unclear. One possibility is that patterned neural activity in LMAN acts as an instructive signal to RA to guide adaptive changes in the motor pathway so that song progresses towards the memorized song model (Troyer and Doupe, 2000; Kao et al., 2005; reviewed in Brainard, 2004). Alternatively, or in addition, neural activity in LMAN may provide factors that are required for changes in the motor program but do not directly guide such changes ("permissive role"; reviewed in Brainard, 2004). Previous studies have suggested that a critical contribution of LMAN to song plasticity may be the introduction of variability into the patterns of activity in the motor pathway (Doya and

Sejnowski, 2000; Kao et al., 2005). Trial-by-trial variability in motor output is an important component of reinforcement learning because it allows evaluation mechanisms to selectively reinforce the patterns of motor activity that produce the desired behavior (i.e., a good match to the memorized song model) and/or punish the motor commands that result in worse performance.

Chronic recordings of LMAN activity in juvenile birds would help to elucidate the functional contribution(s) of LMAN activity to song learning and plasticity. During sensorimotor learning, juvenile birds produce a range of vocalizations and use auditory feedback to gradually match their vocalizations to the memorized song model. Comparison of the activity of LMAN neurons in juvenile birds during "good" and "poor" renditions of song may provide insight into the nature of the signals that LMAN sends to RA. For example, in the context of reinforcement learning, if LMAN is a source of variability to the motor pathway, then the degree of variability in LMAN activity across repeated trials should depend on the current status of the motor output. During "good" renditions, when song is close to the memorized model, low variability in LMAN activity should facilitate stereotypy in RA activity and ensure a good match to the target. In contrast, when song is far from the memorized model (i.e., during "poor" renditions), high variability in LMAN should introduce variability into RA activity and subsequent song output, thereby facilitating motor exploration and the search for changes that might lead to an improvement.

Alternatively, or in addition, if the specific pattern in LMAN acts as an instructive signal to guide changes in the motor pathway, then the firing pattern should differ between "good" and "poor" renditions of song. The firing pattern could merely reflect

the degree to which the current rendition deviates from the memorized model or it could indicate how the song deviates from the model.

The contribution of LMAN activity to song learning and plasticity can be tested directly by chronic manipulation of LMAN activity during sensorimotor learning. We have shown the microstimulation in LMAN in adult birds can induce specific changes in learned parameters of song on a moment-by-moment basis (Kao et al., 2005). These findings raise the possibility that chronic manipulation of LMAN activity during sensorimotor learning, when song has not yet stabilized, may cause permanent changes in song output. For example, if LMAN activity provides an instructive signal, chronic stimulation with a fixed pattern of activity during specific parts of song may drive permanent changes in the morphology of individual syllables and/or changes in the sequence of syllables. In contrast, microstimulation in LMAN with variable patterns of activity across repeated trials may delay or prevent the stabilization of song.

Combining such manipulations of LMAN activity with chronic recordings in RA would greatly augment our understanding of the influence of LMAN activity on song learning and plasticity. Recordings of the activity of RA neurons with and without manipulation of LMAN activity would reveal whether and how the activity of neurons in RA are affected by changes in the pattern of activity of LMAN neurons. One possibility is that activity in LMAN directly modulates the ongoing pattern of activity of RA neurons. Alternatively, or in addition, signals from LMAN may act on a slower timescale and affect the release of trophic factors that promote cell survival, synaptogenesis, and/or the incorporation of new neurons (Johnson et al., 1997;

Kittelberger and Mooney, 1999). In this case, manipulation of activity in LMAN may not result in immediate changes in the ongoing patterns of activity in RA.

In addition, recordings of RA activity during singing would allow the stimulation to be triggered off of particular patterns of RA activity. Since neurons in RA fire stereotyped bursts of spikes that are precisely correlated with song output, this would allow fine control over the manipulation of activity in LMAN and characterization of its effects on RA activity.

Contributions of different components of the song system

Recordings of neurons in different nuclei in the song system have revealed several shared properties across multiple levels of the song system. For example, auditory responses selective for the bird's own song have been described in all three nuclei of the AFP as well as in the premotor nuclei HVC and RA (Margoliash, 1983; Doupe and Konishi, 1991; Doupe, 1997; Solis and Doupe, 1999; Janata and Margoliash, 1999). Similarly, neurons in both Area X and LMAN exhibit robust singing-related activity that is modulated by social context (Hessler and Doupe, 1999a). The purpose of a string of three nuclei in the anterior forebrain and the contribution of each component to song production and plasticity, however, remain unclear.

Simultaneous recordings of activity in multiple song nuclei may help to elucidate whether and how activity is transformed by processing in the anterior forebrain circuit. For example, it is not known whether activity in the motor pathway is modulated by social context. Previous studies of singing-related activity in HVC and RA focused primarily on directed song and neglected undirected song, in part because of the difficulty

of holding single units for the time required to record spontaneous rather than female-triggered singing. Thus, it is not known whether socially driven differences in trial-by-trial variability emerge in the AFP or if such differences affect ongoing motor activity in RA. Simultaneous measurements of the level and variability of activity of identified neurons in multiple nuclei in the song system are necessary to elucidate the origins and extent of context-dependent differences in variability as well as their consequences for song production and plasticity.

In addition, combining manipulations of one part of the circuit with simultaneous recordings in another part of the circuit would help to clarify how neural responses are transformed in the AFP. For example, neurons in Area X receive afferent input from both HVC and from LMAN. Combining recordings of single neurons in Area X with reversible inactivation of LMAN, either during singing or during playback of song stimuli, would reveal whether the feedback projection from LMAN alters the firing properties of neurons in Area X. Similar experiments altering the activity in HVC, the primary source of afferent input to the AFP, while recording activity in the AFP could help to distinguish between properties of AFP neurons that are merely driven by HVC neurons from those that emerge in the AFP.

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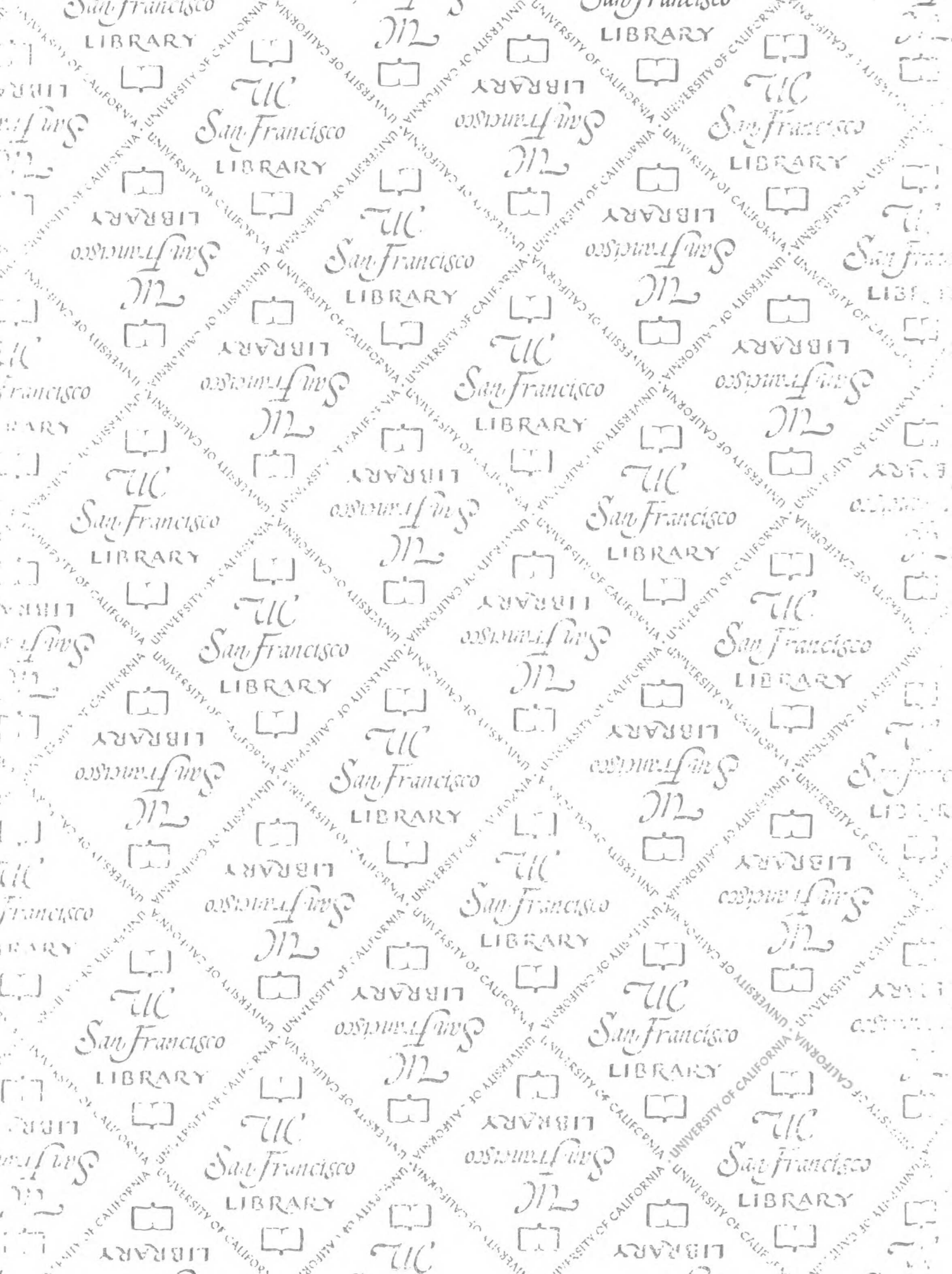
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