Acknowledgements S.S. and I.A.A. were supported by the Genome Canada Foundation.

Competing interests statement The authors declare that they have no competing financial interests.

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

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Cortical–basal ganglia circuits have a critical role in motor control and motor learning[1]. 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[2–5]. Here, we investigate functional contributions of this circuit to the control of song, a complex, learned motor skill. We test the hypothesis that neural activity in the AFP of adult birds can direct moment-by-moment changes in the primary motor areas responsible for generating song. We show that song-triggered microstimulation in the output nucleus of the AFP induces acute and specific changes in learned parameters of song[6,7]. 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. 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.

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 (Fig. 1a): (1) a motor pathway that is required throughout life for normal song production[8]; and (2) a basal ganglia–forebrain circuit[9] (the AFP) that is necessary for song learning and plasticity but not for the production of adult song[2–5]. 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[2–5], 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[10,11]. Such a modulatory influence might serve to introduce into song the stochastic variability that is necessary for reinforcement learning[11], or to systematically bias song towards desired targets in a manner that eventually becomes encoded in the song motor pathway[10]. Consistent with this hypothesis, the AFP exhibits patterned neural activity in singing birds[12,13]. However, previous studies have failed to demonstrate any direct contribution of the AFP to the production of adult birdsong. In adult zebra finches, lesions of LMAN (lateral magnocellular nucleus of the anterior nidopallium), the output nucleus of the AFP (Fig. 1a), have not been reported to affect ongoing song. Moreover, whereas stimulation in the motor pathway of quiescent birds can elicit vocalizations[14,15], comparable microstimulation in LMAN does not. Finally, a previous study did not report any gross effects of LMAN microstimulation on song[14]. 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.

Here, we 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 a song (Fig. 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; ref. 6), as well as increases and decreases in sound amplitude (a parameter of song that is precisely controlled; refs 7, 16). Figure 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 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 1 and 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 ms, but it could be as short as 35 ms (see Supplementary Fig. 1). In addition, the effects of stimulation typically terminated within 60–70 ms after the end of the stimulus train (Fig. 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 ms[14,17]. 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 (note the standard deviation (s.d.) of the histograms in Fig. 2b), increased the variability in 30% of cases (Fig. 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 shift systematically 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 2). Figure 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 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[18].

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 400–1,000 μm dorsal to LMAN (Fig. 2c). At higher current intensities, stimulation outside of LMAN rarely caused significant changes in syllable structure (Fig. 2c). In contrast, we found that stimulation with higher current intensities both within LMAN and at control sites dorsal to LMAN could induce the suspension of ongoing motifs, as seen previously with stimulation of the premotor nucleus HVC[14]. 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 may instead result from antidromic activation of nucleus HVC (Fig. 1a), which sends projections through the anterior forebrain[14,19].

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)[12,20] (Fig. 3a–c, e). We found that the mean fundamental frequency of syllables did not differ systematically between these two conditions, but the variability in the fundamental frequency was significantly greater during undirected song (Fig. 3d, f). Moreover, across birds, there was a significant corre-

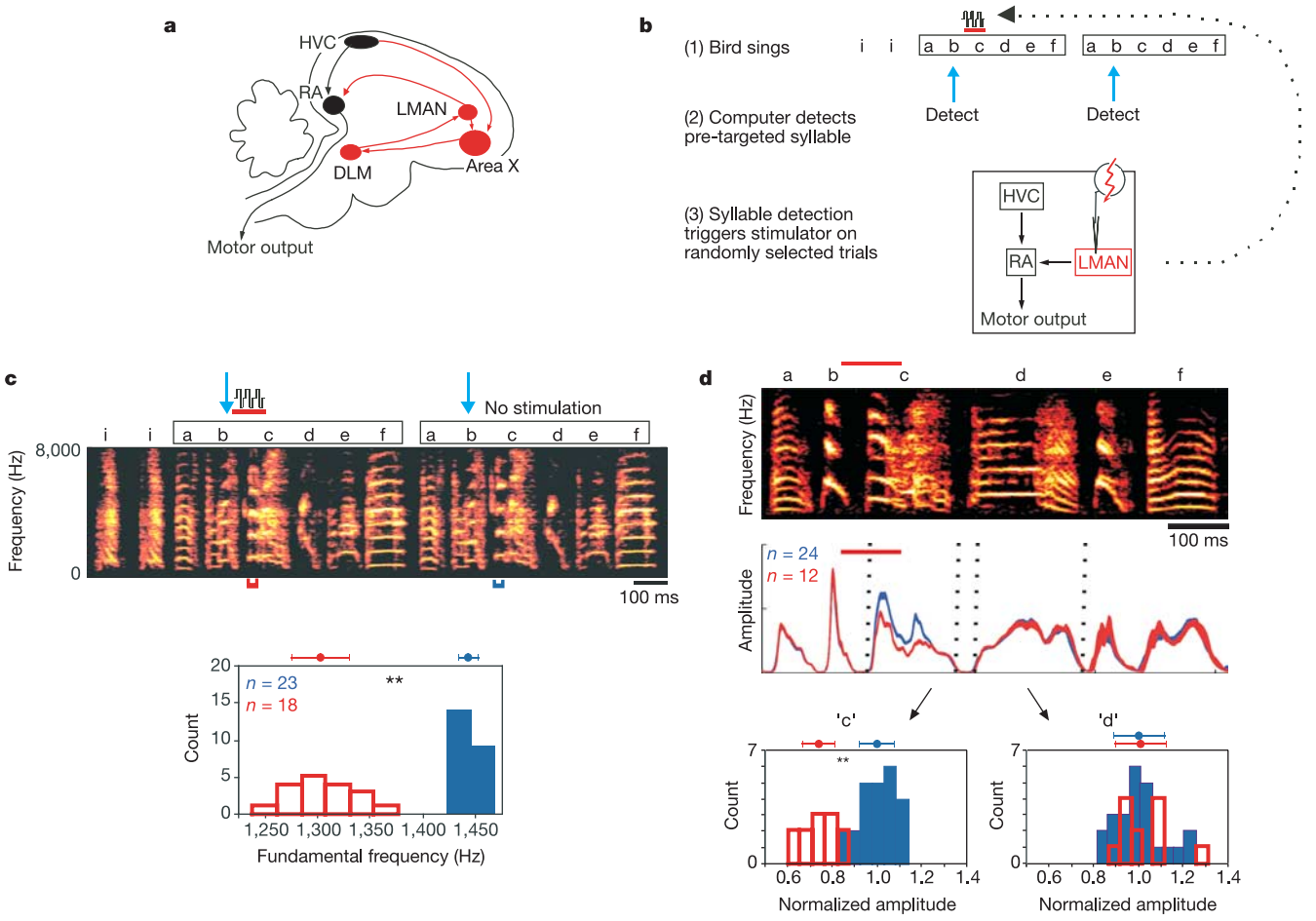

Figure 1 Song-triggered microstimulation in LMAN elicits acute changes in learned parameters of syllable structure. **a**, Song system. The motor pathway includes HVC and RA. The AFP consists of area X, the medial portion of the dorsolateral thalamus (DLM) and LMAN. **b**, Experimental design. 'i' indicates 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 syllable '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 trials (red). Bottom: stimulation (40 μA) significantly reduced the amplitude of syllable 'c' (~27%; $P < 0.0001$), but not that of the next syllable, 'd' ($P = 0.74$).

lation between the magnitude of changes in LMAN variability and the magnitude of changes in song variability ($R^2 = 0.83$; Supplementary Fig. 2). The observed correspondence between variability in LMAN activity and variability in syllable structure (Fig. 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 (for example, Figs 1c and 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 (Fig. 4a, b; Supplementary Table 3). Thus, the artificial introduction of variability in LMAN activity was sufficient to recapitulate one natural difference between directed and undirected song (compare Fig. 3f with Fig. 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 (Fig. 4c; 2–5 syllables per bird). Immediately after the lesions, this context-dependent difference in song variability was eliminated (1–3 days after lesion; Fig. 4c). Thus, LMAN is necessary for modulating naturally occurring differences in song variability.

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[4]. One possibility is that LMAN provides an instructive signal to RA that systematically biases the motor pathway towards a particular goal[3,4,10]. 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[21,22].

A second possibility is that neural activity in LMAN contributes to plasticity by generating variability in the motor pathway and subsequent song output[3,11]. 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[11,23]. 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 (for example, altered muscle tone or innervation) and for continued motor exploration 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 (for example, during directed song), our results suggest that it

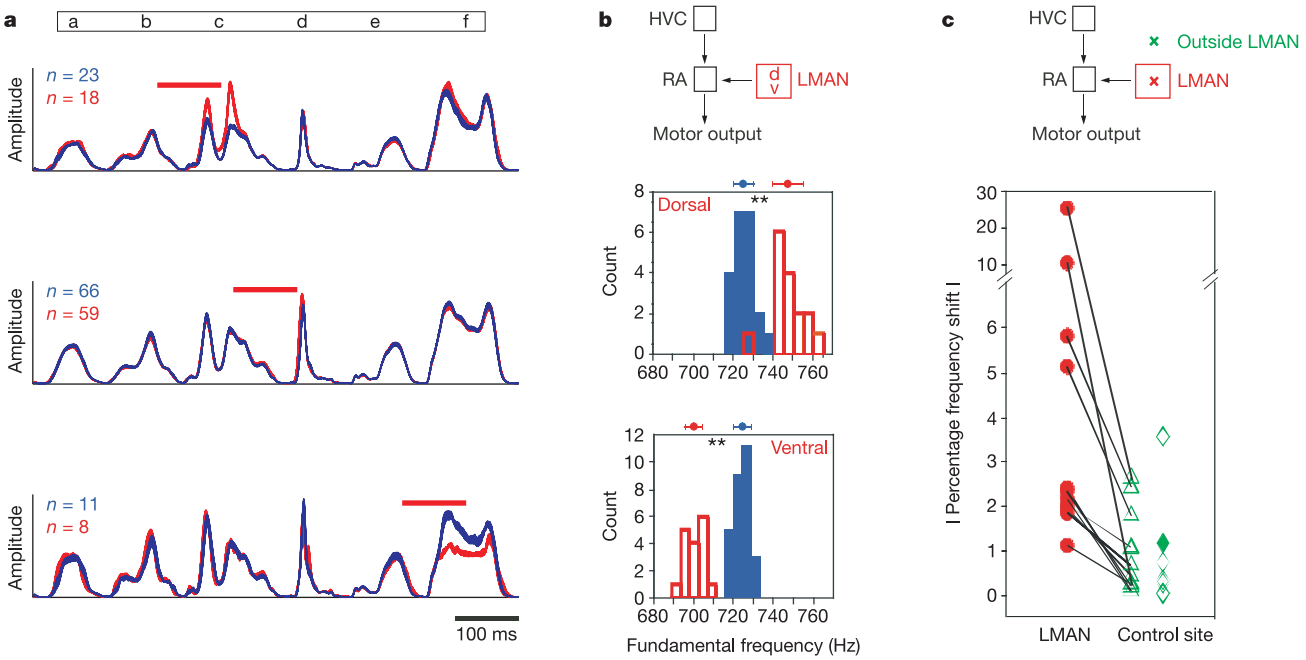

Figure 2 Specificity of stimulation. **a**, Stimulation at one site could elicit qualitatively different effects on different syllables. A fixed pattern of stimulation (30 μA; red bar) increased the amplitude of syllable 'c', decreased the amplitude of syllable 'f', and had little effect on other syllables (for example, '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 the 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.

should systematically bias vocal production. In contrast, when AFP activity is variable across motif renditions (for example, 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[4,5].

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[24]. 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[25]. Moreover, LMAN input to RA is unusual in that it is mediated predominantly by NMDAR (*N*-methyl-D-aspartate receptors)[24,26], 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[27,28], 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 (for example, Huntington's disease) or in too little movement (for example, 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[29,30]. We suggest that in songbirds, the AFP can modulate ongoing motor activity and drive specific changes in motor output on a moment-

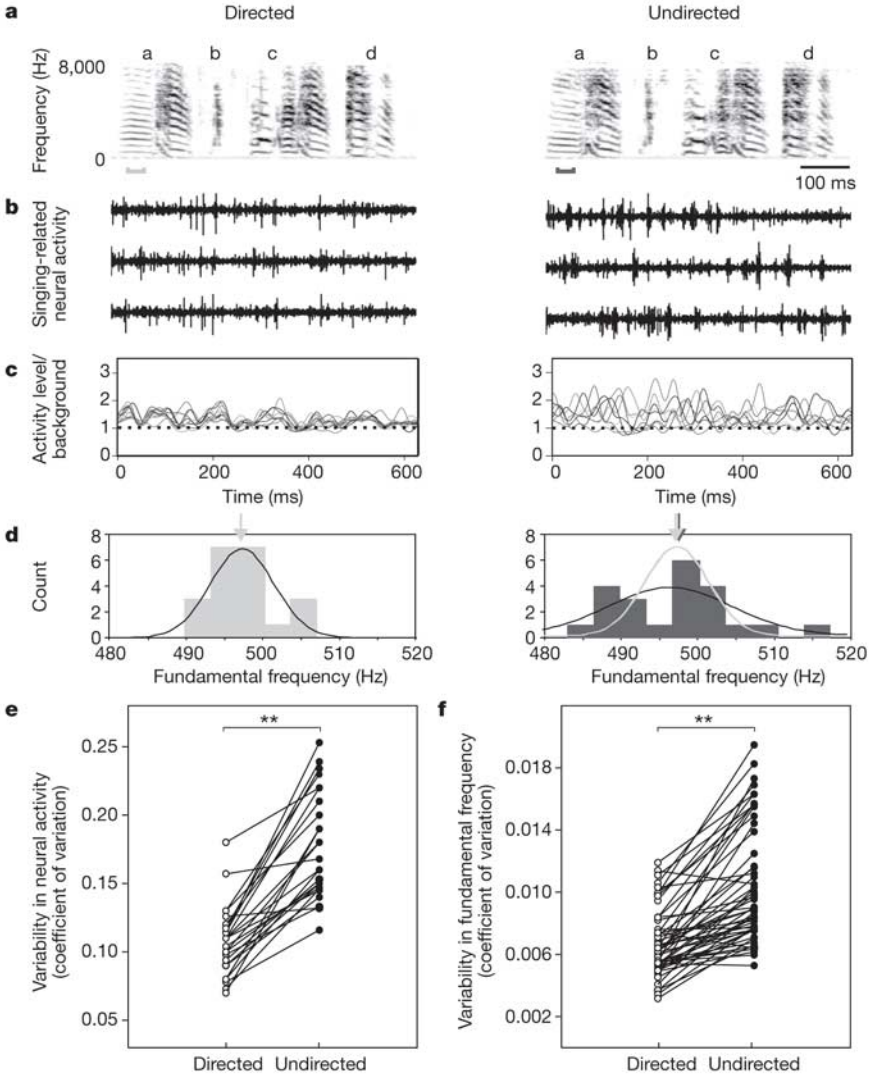

Figure 3 Context-dependent changes in variability. **a**, Spectrograms of the motif 'abcd' during directed (left) and undirected (right) singing. **b**, LMAN multi-unit activity during three renditions of the motif. **c**, LMAN activity waveforms for ten renditions of the motif. Note that the singing-related 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 and 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 grey 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; $P < 0.0001$).

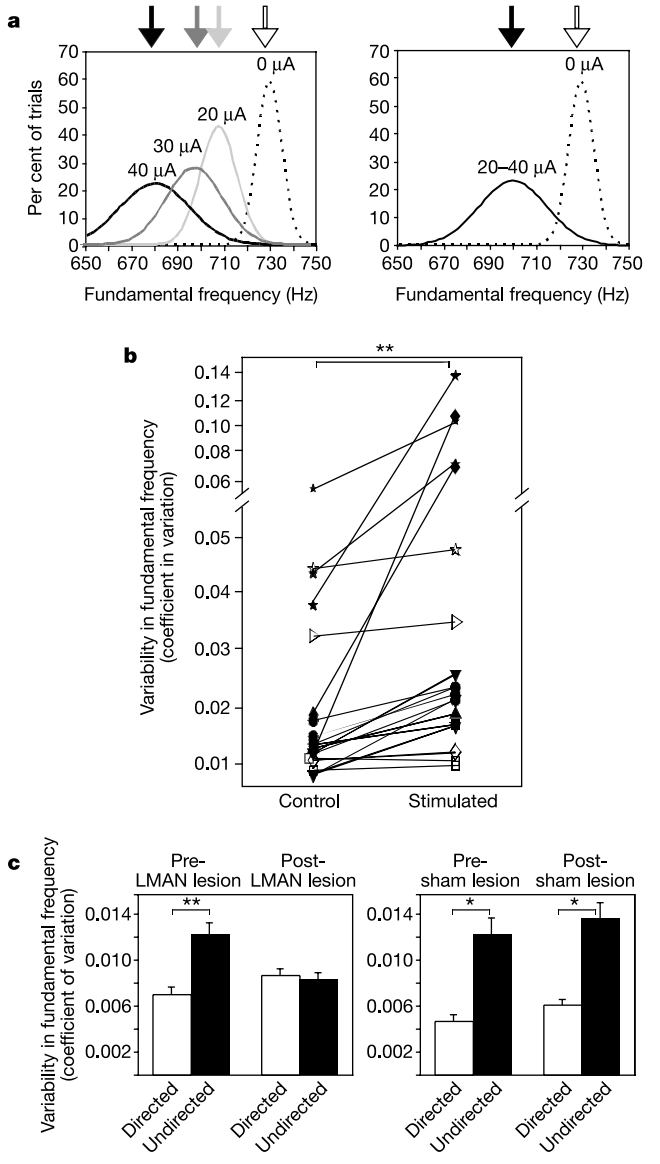

Figure 4 Contributions of the AFP to real-time song modulation. **a**, Left: gaussian fits of fundamental frequency for one syllable for control (dotted) and stimulated trials of different current intensities. Right: varying stimulation across trials increased the variability in fundamental frequency relative to controls ($P = 0.022$). **b**, Variability in fundamental frequency for control and stimulated 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 the 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, $P = 0.03$).

by-moment basis, perhaps by adjusting the gain of active synapses in the motor pathway. In addition, we show that introduction of variability in the activity of LMAN (the output nucleus of the AFP) can drive variability in motor performance. Finally, we find 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. □

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[13]. 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[13]. 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 9,000 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 400–1,000 μm dorsal to LMAN ($n = 3$ birds) or at sites in LMAN selected on the basis of their characteristic singing-related activity[13] ($n = 5$ birds). At sites at least 400 μm dorsal to LMAN, there was no conspicuous change in multi-unit firing during singing when compared with the spontaneous neural activity during non-singing periods. At the conclusion of experiments, site locations were confirmed in 40-μm Nissl-stained brain sections by their positions relative to the depth of marker lesions.

Song-triggered microstimulation

To deliver electrical stimuli reproducibly during specific parts of a 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 ms trains of biphasic current pulses at 400 Hz (0.4 ms per phase; 2.5 ms between phases)[14]. 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[4]. 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 were presented in a separate cage. Each female presentation lasted ≤2 min, 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[12,13]. 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 of activity across motif renditions was 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. To improve the resolution of the estimates, we performed a parabolic interpolation of the autocorrelation function 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 8,000 Hz, rectified, and smoothed with a 2-ms 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

non-parametric Mann–Whitney *U*-test (for within-syllable changes) and paired sign test (for group changes). Comparisons of variability of 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 $P < 0.05$.

Received 3 August; accepted 21 October 2004; doi:10.1038/nature03127.

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Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements We thank N. Hessler for a portion of the data in Fig. 3e. We thank A. Basbaum, S. Lisberger, J. Sakata and B. Wright for helpful comments on this manuscript and A. Arteseros and K. McManaway for technical assistance. This work was supported by an HHMI Predoctoral Fellowship (M.H.K.), the MacArthur Foundation, the Steven and Michele Kirsch Foundation, NARSAD and NIH (A.J.D.), and the HHMI Biomedical Research Support Program grant, the McKnight Foundation, the Klingenstein Fund, a Searle Scholars Award and NIH (M.S.B.).

Competing interests statement The authors declare that they have no competing financial interests.

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Ultrabithorax is required for membranous wing identity in the beetle *Tribolium castaneum*

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The two pairs of wings that are characteristic of ancestral pterygotes (winged insects) have often undergone evolutionary modification. In the fruitfly, *Drosophila melanogaster*, differences between the membranous forewings and the modified hindwings (halteres) depend on the Hox gene *Ultrabithorax* (*Ubx*). The *Drosophila* forewings develop without Hox input, while *Ubx* represses genes that are important for wing development, promoting haltere identity[1,2]. However, the idea that Hox input is important to the morphologically specialized wing derivatives such as halteres, and not the more ancestral wings, requires examination in other insect orders. In beetles, such as *Tribolium castaneum*, it is the forewings that are modified (to form elytra), while the hindwings retain a morphologically more ancestral identity. Here we show that in this beetle *Ubx* 'de-specializes' the hindwings, which are transformed to elytra when the gene is knocked down. We also show evidence that elytra result from a Hox-free state, despite their diverged morphology. *Ubx* function in the hindwing seems necessary for a change in the expression of *spalt*, *iroquois* and *achaete-scute* homologues from elytron-like to more typical wing-like patterns. This counteracting effect of *Ubx* in beetle hindwings represents a previously unknown mode of wing diversification in insects.

Many modern insects have wings on their second (T2) and third (T3) thoracic segments. Wing morphology often differs greatly between species, and sometimes between forewing and hindwing in the same species. In *Drosophila*, the forewing is used for flight, while the hindwing (haltere) is highly reduced and used only for balance (Fig. 1a). *Ubx* promotes haltere identity by repressing expression of some wing genes, including those of the *spalt* (*sal*) complex[2] (Fig. 1a), but not others such as *optomotor blind* (*omb*; *bifid*, *bi* in Flybase)[2]. Removing *Ubx* function causes the transformation of haltere to forewing[1] (typically referred to simply as 'wing'). In contrast, the forewing is thought to be a Hox-free state, because inactivating or overexpressing *Antennapedia* (*Antp*), the only Hox gene expressed in the forewing, has almost no effect on wing morphology[3,4]. No wings develop on T1 or the abdominal segments, because *Sex combs reduced* (*Scr*), *Ubx* and *abdominal-A* (*abd-A*) repress wing development in these segments[3] (Fig. 1a). Despite the divergence of hindwing morphology between dipterans (flies) and lepidopterans (butterflies and moths), *Ubx* also regulates hindwing identity in the butterfly *Precis coenia*, albeit by regulating a different set of target genes than those in *Drosophila*[5,6]. Weatherbee *et al.*[5] proposed that diversification of wing morphology among insects was achieved both by modification of a basic wing-gene network that controls both fore- and hindwing development in a species-specific and *Ubx*-independent manner, and by the divergence of *Ubx*-regulated target genes (rather than by changes in *Ubx* expression) in the hindwing.

Applying these models to beetle wing development is confusing, as the situation in beetles is the opposite to that in *Drosophila*: the T2 segment bears sclerotized elytra (wing covers) (Fig. 1b, c), whereas