

Sensorimotor Nucleus Nif Is Necessary for Auditory Processing But Not Vocal Motor Output in the Avian Song System

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Cardin, Jessica A., Jonathan N. Raksin, and Marc F. Schmidt. Sensorimotor nucleus Nif is necessary for auditory processing but not vocal motor output in the avian song system. *J Neurophysiol* 93: 2157–2166, 2005. First published December 8, 2004; doi:10.1152/jn.01001.2004. Sensorimotor integration in the avian song system is crucial for both learning and maintenance of song, a vocal motor behavior. Although a number of song system areas demonstrate both sensory and motor characteristics, their exact roles in auditory and premotor processing are unclear. In particular, it is unknown whether input from the forebrain nucleus interface of the nidopallium (Nif), which exhibits both sensory and premotor activity, is necessary for both auditory and premotor processing in its target, HVC. Here we show that bilateral Nif lesions result in long-term loss of HVC auditory activity but do not impair song production. Nif is thus a major source of auditory input to HVC, but an intact Nif is not necessary for motor output in adult zebra finches.

INTRODUCTION

Bird song is a learned behavior governed by a series of discrete, interconnected brain areas collectively known as the song system. As in other systems (duLac and Knudsen 1990; Moss and Sinha 2003; Oestreich and Zakon 2002), sensory responses to specific stimuli play a role in fine-tuning motor representations and output. The premotor nuclei of the song system generate stereotyped, precisely timed activity patterns underlying song production (Hahnloser et al. 2002; Schmidt 2003; Yu and Margoliash 1996) and many of these nuclei exhibit robust auditory responses. Auditory feedback to the song system is necessary for both song learning and maintenance (Brainard and Doupe 2000; Konishi 1965; Leonardo and Konishi 1999; Lombardino and Nottebohm 2000; Nordeen and Nordeen 1992). Because of these characteristics, the song system is a useful model in which to study the complex interactions between premotor pattern generation and sensory feedback. However, neither the generation of motor patterns underlying song nor the ascending flow of sensory feedback is well understood.

The song system includes several major pathways that are involved in song learning, perception, and production (Fig. 1). The forebrain nucleus interface of the nidopallium (Nif) provides a major input to nucleus HVC (used as a proper name) (Cardin and Schmidt 2004; Coleman and Mooney 2004; Janata and Margoliash 1999), which projects to both the descending motor and anterior forebrain pathways. Nif and HVC both exhibit strong premotor activity that is time-locked to the

production of song elements (Cardin and Schmidt 2004; Hahnloser and Fee 2003; McCasland 1987; Yu and Margoliash 1996) and bilateral HVC lesions cause severe, lasting song deficits (Nottebohm et al. 1976). In addition, Nif and HVC both demonstrate auditory responses that covary with the animal's behavioral state (Cardin and Schmidt 2003, 2004). Acute administration of muscimol or γ -aminobutyric acid (GABA) into Nif completely eliminates spontaneous bursting activity and both sub- and suprathreshold auditory responses in the ipsilateral HVC (Cardin and Schmidt 2004; Coleman and Mooney 2004), suggesting that Nif provides a major auditory input to HVC. Nif is thus well positioned to participate in both premotor and auditory processing. In Bengalese finches, whose songs exhibit a higher level of temporal organization than those of zebra finches, Nif lesions have been suggested to decrease song complexity (Hosino and Okanoya 2000). However, the role of Nif in motor production of song in zebra finches has not been fully investigated.

To address these issues, we explored the effects of bilateral, excitotoxic Nif lesions on song behavior and auditory activity in HVC. After Nif lesion, there was a profound loss of spontaneous and auditory activity in HVC during both wakefulness and sedation. Despite these effects, Nif lesions did not impair motor production of song. Thus although Nif appears to be the major source of auditory input to HVC, it is not necessary for motor production of song in adult zebra finches.

METHODS

Acoustic and neural recordings

Nine adult male zebra finches (*Taeniopygia guttata*), ranging from 200 to 400 days of age, were obtained from our breeding colony or from a local supplier. Three birds were unlesioned controls and 6 birds received Nif lesions. All procedures described here were approved by an institutional animal care and use committee at the University of Pennsylvania.

Each bird's vocalizations were recorded in a sound isolation chamber. Birds were continuously monitored by a video camera. All data presented here are directed songs elicited by a female visible through a Plexiglas divider. After an initial 3-day recording period, each bird was sedated with 0.08 ml of 1.5 mg/ml diazepam (Abbott Laboratories, North Chicago, IL) and sharp glass electrodes were lowered into both HVC and Nif as described in detail by Cardin and Schmidt (2003, 2004). Each Nif was thoroughly mapped before lesion by recording spontaneous and auditory activity at evenly spaced sites that spanned the nucleus and surrounding forebrain areas in a 3-dimen-

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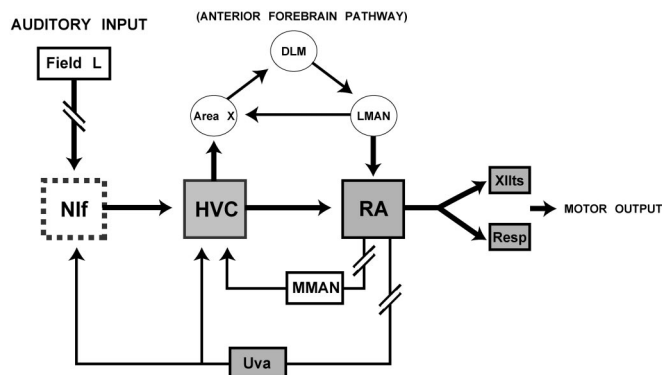


FIG. 1. Song system schematic. HVC (used as a proper name) receives auditory input indirectly from Field L by way of nucleus interfascicularis (Nif) and projects to both the motor and anterior forebrain pathways. Interrupted arrows denote one or more intermediate structures. Motor structures are shown in gray. Area X, song-related region of the basal ganglia; DLM, medial portion of the dorsal lateral nucleus of the anterior thalamus; Nif, nucleus interface of the nidopallium; LMAN, lateral magnocellular nucleus of the anterior nidopallium; MMAN, medial magnocellular nucleus of the anterior nidopallium; RA, robust nucleus of the arcopallium; Resp, respiratory areas of the brain stem; Uva, nucleus uvaformis of the thalamus; Xlts, tracheosyringeal portion of the hypoglossal nucleus.

sional grid. Nif was easily identified by its coincident bursting with HVC and its intermediate degree of song selectivity (Cardin and Schmidt 2004). Baseline HVC auditory responses were assessed by recording the multiunit neural responses to presentations of the bird's own song. Stimuli used to assess auditory responsiveness consisted of 2 complete motifs of the bird's own song (BOS) digitized at 40 kHz (Goldwave, St. Johns, NF, Canada). Four to 6 injections of 50–75 nl of 7 mg/ml ibotenic acid (Biosearch Technologies, Novato, CA) were then made in each Nif by pressure injection through a glass pipette connected by polyethylene tubing to a Hamilton syringe. Each injection was made over the course of 3 min and the pipette was slowly withdrawn to minimize spread up the pipette track. Birds recovered in the sound-recording chamber so that all postlesion vocalizations could be recorded.

Vocal output from all 6 Nif-lesioned birds was recorded continuously for 3 days prelesion and 30 days postlesion using acquisition software written in Labview (National Instruments, Austin, TX) by A. Leonardo. The vocal output of the 3 unlesioned control birds was recorded continuously for the same duration. Two birds, ZF282 (control) and ZF38 (100%+ bilateral lesion), were also recorded on day 60.

Postlesion auditory responses were tested in all 6 Nif lesion birds. In 2 cases (ZF130 and ZF290), birds were implanted with one nichrome wire electrode (0.5–1.2 M Ω) in each HVC during the initial lesion surgery, as described in detail by Cardin and Schmidt (2003, 2004). HVC auditory responses in these 2 birds were assessed several times during wakefulness and diazepam sedation each day for the first 5 days after Nif lesion. In addition, HVC activity was recorded during song. One additional bird (ZF38) was implanted with HVC electrodes 31 days postlesion to assess HVC activity during song. In 4 cases (ZF120, ZF127, ZF38, and ZF139), birds were sedated 30 days after Nif lesion and acute recordings were used to assess spontaneous and auditory-evoked activity in at least 2 recording sites in HVC. Because HVC activity was generally very low after Nif lesion, 100 nl of 10% BDA was injected at the end of each experiment to confirm recording location. See Table 1 for a summary of experimental manipulations.

Histology

Birds were deeply anesthetized with 0.1 ml of 50 mg/ml Nembutal (Abbott Laboratories) and transcardially perfused with 0.9% saline

and 4% paraformaldehyde. Brains were cryoprotected in 30% sucrose and sectioned at 50 μ m on a freezing microtome. Lesion size and chronic electrode placement were confirmed using cresyl violet staining. Lesions were easily identified because of gliosis and loss of neurons (see Fig. 2, A and B). In birds ZF120, ZF127, ZF130, and ZF290, portions of Nif were spared (see Fig. 2A). In these cases, we used the public domain program ImageJ (Wayne Rasband; <http://rsb.info.nih.gov/ij/>) to trace the outlines of the remaining portions of Nif on digital images of each successive 50- μ m section of tissue (Brenowitz and Lent 2001; Scharff and Nottebohm 1991). The areas from all sections were first converted to volume by accounting for the 50- μ m thickness of the tissue sections and then summed to give the total remaining Nif volume. Using the same measurement technique, we calculated mean prelesion Nif volume by measuring Nif volume in each hemisphere of the 3 unlesioned control birds. The proportion of Nif remaining after the lesion was then calculated for each lesioned bird by dividing the remaining volume of Nif by the mean control Nif volume. This number was then subtracted from 1 and multiplied by 100 to give the percentage of Nif lesioned. In birds ZF38 and ZF139, the lesions were extensive and clearly included all of Nif and some of the surrounding tissue (see Fig. 2B). In these birds, the lesion of Nif was designated as 100%+. For identification of postlesion acute recording sites in HVC, several sections containing HVC were also processed with an avidin–biotin–horseradish peroxidase complex kit followed by a reaction with a peroxidase substrate kit (Vector Laboratories, Burlingame, CA). All data presented here are from confirmed HVC recording sites.

Data analysis

Data were analyzed as described in detail by Cardin and Schmidt (2003, 2004) using Matlab (The MathWorks, Natick, MA) routines written by J. A. Cardin and M. F. Schmidt. Spike events in the multiunit data from acute and chronic experiments were measured by using a peak-detection algorithm. For each data set, the threshold was

TABLE 1. Birds and lesion sizes

	Bird	Lesion Size	Postlesion Auditory Response
Chronic recordings	290 ^m	L 59%	No
		R 67%	No
	130 ^m	L 0%	Yes
		R 100%+	No
Acute recordings	38 ^m	L 100%+	No
		R 100%+	No
	139	L 100%+	No
		R 100%+	No
	120	L 86%	No
		R 29%	Yes
	127	L 62%	No
		R 90%	No
Unlesioned controls	282	L 0%	N/A
		R 0%	N/A
	420	L 0%	N/A
		R 0%	N/A
	489	L 0%	N/A
		R 0%	N/A

Six birds received Nif lesions and 3 were used as unlesioned controls. Two birds (ZF130 and ZF290) were implanted with chronic electrodes in HVC during the initial lesion surgery and their HVC premotor activity and auditory responses were recorded over the next 3–5 days during wakefulness and sedation. Four birds (ZF38, ZF120, ZF127, and ZF139) were sedated 30 days postlesion and HVC auditory responsiveness was assessed by acute recordings. Three birds (ZF282, ZF420, and ZF489) did not receive Nif lesions and were used as controls for assessments of song production over time. One bird (ZF38) was implanted 31 days postlesion to assess premotor activity in HVC. Birds with premotor recordings are denoted by “m”.

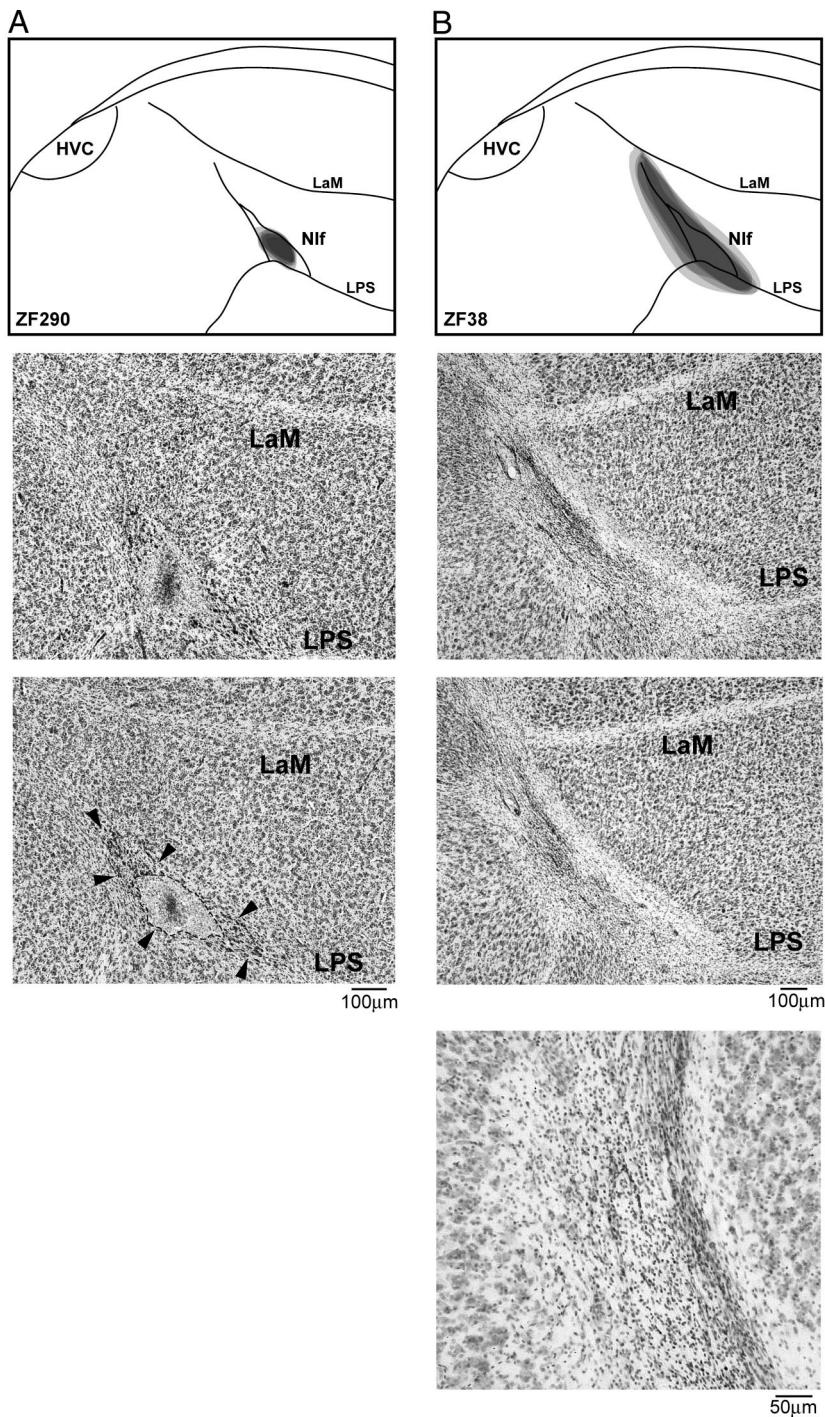


FIG. 2. Nif lesion histology. *A, top panel:* a standardized schematic of a parasagittal section through the middle of Nif. Lesioned portions of each successive section of tissue are represented as overlaid gray areas. This example is of a 59% lesion of Nif in the left hemisphere of bird ZF290. *Bottom panels:* images of 2 successive sections containing Nif and the lesion. In the *bottom panel*, the border of Nif is denoted by arrowheads and the lesion is outlined by a dashed line. *B, top panel:* a schematic representation of the left hemisphere of ZF38. This lesion included both Nif and some surrounding tissue (100%+). *Middle panels:* pictures of 2 successive tissue sections showing the extensive lesion. *Bottom panel:* a higher magnification view of the lesion from an adjacent tissue section, showing gliosis and loss of neurons. LaM: lamina mesopallialis; LPS: lamina pallio-subpallialis.

visually positioned at a point clearly above background noise but low enough to detect all observed spike events.

We first determined the presence of significant auditory responses by using both response strength and variance measurements taken from peristimulus time histograms (PSTHs; bin size = 10 ms) as described in detail in Cardin and Schmidt (2003, 2004). For each PSTH we performed 2 calculations: 1) an unpaired *t*-test comparing the set of bin totals representing the auditory stimulus period to the set of bin totals representing a baseline period of equivalent duration, and 2) an F-test to compare the variance of the sets of PSTH bin totals representing the summed neural activity during the same stimulus and baseline periods. The recorded response was defined as a significant auditory response if $P < 0.05$ for either of the above tests.

Next, we quantified auditory responsiveness by measuring response strength (RS) for each set of auditory trials. Response strength is a measure of the change in mean firing rate during the auditory stimulus of each trial

$$RS = FR_{STIM}/FR_{SPONT}$$

FR_{STIM} is the mean firing rate during the auditory stimulus and FR_{SPONT} is the mean spontaneous firing rate during the 2 s before the onset of the auditory stimulus. An RS of 1 thus indicates no change in firing in response to the stimulus, whereas a more positive RS indicates an increase in firing in response to the stimulus and an $RS < 1$ indicates a decrease in firing in response

to the stimulus. All RS measurements are presented as means $\pm$ SE.

Three birds were used to assess premotor activity in HVC after Nif lesion. For each bird, we measured the mean HVC firing rate during five 1-s epochs of spontaneous activity during nonsinging wakefulness and five 1-s epochs of premotor activity taken from 5 song bouts. Mean spontaneous firing rate was compared with mean premotor firing rate by a paired *t*-test. Firing rates are shown as means $\pm$ SE.

Song analysis

Vocalizations were sampled at 22.05 kHz, high-pass filtered at 400 Hz, and analyzed with the Sound Analysis Pro software package written by D. Swigger and O. Tchernichovski (Tchernichovski and Mitra 2002; Tchernichovski et al. 2001). A total of 30 randomly selected complete song motifs were analyzed from each of 3 time points: 1 day prelesion (PRE-1), 1 day postlesion (POST-1), and 30 days postlesion (POST-30). Each motif used for analysis was the first motif of a song bout. Derivative power and Wiener entropy thresholds were manually adjusted until the automated identification of individual song syllables was consistent across all motifs. Spectral derivatives represent changes in the power at a given frequency over time (Tchernichovski et al. 2000). Overall motif duration was defined as the period from the onset of the first syllable to the offset of the final syllable of the motif. For each syllable, we measured mean duration, mean pitch, and mean frequency modulation (FM) and compared them across the 3 time points. We also compared motif duration at the 3 time points.

Within-bird comparisons across time points were made by one-way ANOVA analysis with Dunnett's post hoc test. Because syllables could not be reliably identified on the first postlesion day in the two 100%+ lesion birds, songs from PRE-1, POST-2, and POST-30 were compared by ANOVA in these birds. Because of the large number of statistical comparisons, the threshold for significance was set at $P < 0.01$. All measurements of individual birds' songs are shown as means $\pm$ SD.

We also compared the degree of change in syllable duration, motif duration, pitch, and FM over time between the control and lesion groups. We first calculated the mean values for syllable duration, motif duration, pitch, and frequency for the control and lesion groups and compared them by unpaired *t*-test to confirm that there were no significant differences between the 2 groups on day PRE-1. We next normalized all measurements of each syllable on the PRE-1, POST-1, and POST-30 days to the mean PRE-1 value for that syllable. The normalized values were averaged across syllables to give mean normalized syllable duration, pitch, FM, and motif duration for each bird. Similarly, all individual measurements of motif duration were normalized to the mean PRE-1 motif duration for that bird. The normalized values from all birds in each group were then averaged to give means at each time point. These calculations allowed us to compare the magnitude of the changes in song characteristics of the control and lesion groups on days POST-1 and POST-30 by unpaired *t*-test. In addition, we also compared the variances of the distributions of the groups at each time point by an F-test. All normalized values are shown as means $\pm$ SD.

RESULTS

As shown in Table 1, histology revealed a combination of bilateral lesion sizes in each of the 6 lesioned birds. Four birds (ZF120, ZF127, ZF130, and ZF290) had lesions predominantly restricted within the borders of Nif and little damage to the surrounding neural structures. Two birds (ZF38 and ZF139) had complete bilateral Nif lesions that also included some damage to nearby areas such as Field L. Examples of restricted

and extensive lesions are shown in Fig. 2, A and B, respectively. Three additional birds (ZF282, ZF420, and ZF489) did not receive lesions.

Nif lesions eliminate HVC auditory responses

Large Nif lesions led to a lasting impairment in HVC auditory responsiveness. Two birds with chronic implants were tested for auditory responses during wakefulness and sedation on the first 5 postlesion days. Four birds were tested for auditory responses during acute recordings 30 days postlesion. Figure 3A shows representative examples of acute recordings from the right HVC of bird ZF38 (100%+ right and left Nif lesioned). Before the lesion, there was a song-selective HVC auditory response to the bird's own song (*left panel*). Thirty days after bilateral Nif lesion, HVC auditory responsiveness was completely eliminated (*right panel*). Spontaneous HVC activity was also reduced (see sample traces, Fig. 3A).

In past experiments, we observed that HVC auditory responses, especially during wakefulness, are not always detected by analyses of mean firing rate (Cardin and Schmidt 2003, 2004). Each set of awake and sedated auditory trials was therefore tested for the presence of a significant auditory response by a more sensitive combination of criteria based on both mean and variance measures (see METHODS). Using these criteria, we found no significant postlesion HVC auditory responses to the BOS stimulus during sedation in any of the hemispheres with $\geq 50\%$ Nif lesion ($n = 10$ lesioned hemispheres in 6 birds) (see Table 1). Similarly, after Nif lesions there were no significant HVC auditory responses during wakefulness in the chronically implanted birds ($n = 3$ lesioned hemispheres in 2 birds). In contrast, when only 29% (right hemisphere, bird ZF120) or 0% (left hemisphere, bird ZF130) of Nif was lesioned, HVC continued to show significant auditory responsiveness ($P < 0.05$; see METHODS).

Because the above analyses determine the presence of a significant auditory response without quantifying the response magnitude, we also measured the response strength (RS) of HVC activity in response to the BOS stimulus. Figure 3B shows the relationship between lesion size and HVC response strength. As shown in Fig. 3C, mean prelesion sedated HVC RS was 3.23 ± 0.16 , indicating robust prelesion auditory responses ($n = 12$ hemispheres in 6 birds). Lesions of $< 50\%$ Nif ($n = 2$ hemispheres in 2 birds) did not significantly affect HVC auditory responsiveness (mean RS 3.01 ± 0.18) ($P > 0.05$; one-way ANOVA with Dunnett's post hoc test), but lesions of $> 50\%$ Nif ($n = 10$ hemispheres in 6 birds) resulted in a mean HVC RS of 0.99 ± 0.07 , indicating complete loss of auditory responsiveness ($P < 0.01$). Mean sedated spontaneous firing rates in HVC were also significantly reduced from 19.7 ± 6.3 to 3.1 ± 1.7 spike events/s after $> 50\%$ Nif lesion ($n = 10$ hemispheres in 6 birds) ($P < 0.01$; unpaired *t*-test). Together, these results support the role of Nif in providing the major source of auditory and spontaneous input to HVC. In addition, because the lack of auditory responsiveness persists 30 days after lesion, other structures that project to HVC, such as MMAN (medial magnocellular nucleus of the anterior nidopallium) and Uva (nucleus uvaeformis of the thalamus), do not appear to compensate for the loss of auditory input from Nif.

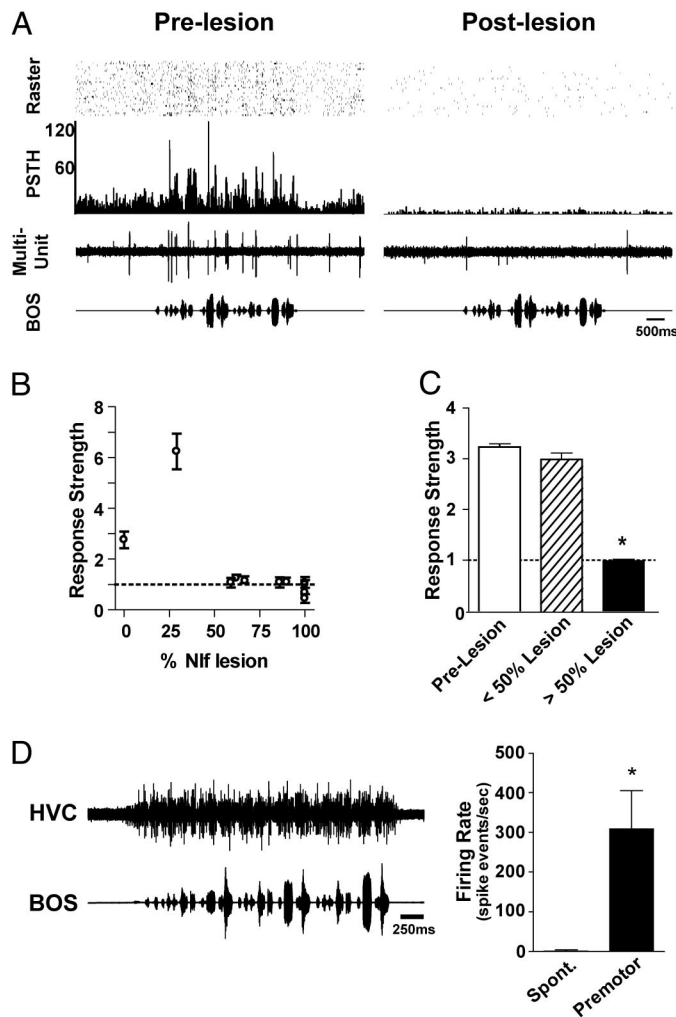


FIG. 3. Nif lesions eliminate auditory activity in the ipsilateral HVC. *A*: multiunit recordings from the right HVC of bird ZF38 (bilateral 100%+ Nif lesion). During prelesion acute recordings (*left panel*), there was an HVC auditory response to the bird's own song. In contrast, 30 days after Nif lesion (*right panel*), there was no auditory response and very little spontaneous activity in the same HVC. In each case, the stimulus was presented 30 times. For each panel in *A*: *Top section*: raster plot of the auditory trials. *Middle section*: peristimulus time histogram (PSTH) resulting from the sum of the raster plots in units of spike events per bin (bin size = 10 ms). *Trace* is an example of multiunit activity during one auditory trial. *Bottom section*: amplitude waveform of the auditory stimulus. *B*: plot of the Nif lesion size in each hemisphere vs. the postlesion response strength of the ipsilateral HVC during 30 presentations of the bird's own song. *C*: HVC auditory responses were robust before lesion (open bar) and were not significantly affected by <50% Nif lesion (hatched bar). However, >50% Nif lesion completely eliminated all HVC auditory responsiveness (black bar) ($P < 0.01$; one-way ANOVA with Dunnett's post hoc test). *D*: premotor activity in HVC after complete Nif lesion. HVC demonstrated robust premotor activity during song even in the absence of Nif (*left panel*) (left hemisphere of ZF38; 100%+ Nif lesion). Mean premotor firing rate in HVC during song was significantly higher than spontaneous activity during awake, nonvocalizing periods (*right panel*) ($n = 3$ birds). Asterisks denote $P < 0.01$. Error bars indicate SE.

In addition to assessing auditory responses, we also observed premotor activity in HVC during song. An example of premotor HVC activity in a Nif-lesioned bird (left hemisphere of ZF38, 100%+ Nif lesion) is shown in the *left panel* of Fig. 3*D*. Although mean spontaneous activity during wakefulness in the chronically implanted birds was only 0.8 ± 0.9 spike events/s, the HVC firing rate increased to 308.4 ± 166.3 spike

events/s during song ($n = 5$ hemispheres in 3 birds) ($P < 0.001$; paired *t*-test). These results demonstrate premotor activity in HVC, despite the absence of Nif input.

Song output is spared by Nif lesions

To quantitatively assess the effects of Nif lesions on song output, we used a rigorous comparison of each bird's song at 3 time points: 1 day prelesion (PRE-1), 1 day postlesion (POST-1), and 30 days postlesion (POST-30). Mean duration, pitch, and FM of each syllable and mean motif duration were measured at each of the time points. Although Nif-lesioned birds demonstrated some small long-term changes in song over 30 days, these changes were not different from those observed in unlesioned control birds. Similarly, we did not observe any changes in syllable order in either the control or Nif lesioned birds.

The unlesioned control birds (ZF282, ZF420, and ZF489) demonstrated stable song over short periods and small but significant changes over 30 days. Representative examples from bird ZF282 are shown in Fig. 4*A* and the mean values of each measurement are shown in Table 2. Between PRE-1 (Fig. 4*A*, *first panel*) and POST-1 (Fig. 4*A*, *second panel*), ZF282 demonstrated a small but significant increase in the FM of syllable *b* ($P < 0.0001$; one-way ANOVA with Dunnett's post hoc test). All other measurements were unchanged between PRE-1 and POST-1 ($P > 0.01$). However, by POST-30 a number of characteristics of ZF282's song had changed significantly (Fig. 4*A*, *third panel*). Syllables *a*, *b*, and *d* demonstrated significant increases in FM between PRE-1 and POST-30 ($P < 0.01$). Syllable *c* showed a significant increase in duration ($P < 0.0001$) and a significant decrease in FM ($P < 0.01$). No changes were observed in syllable *e*. The song of bird ZF282 was also recorded on day POST-60 (data not shown). Significant changes in the duration of syllables *a*, *c*, and *d* and the FM of syllables *a*, *b*, and *c* were observed between POST-30 and POST-60 ($P < 0.01$). Results from the other 2 control birds, ZF420 and ZF489, showed a similar degree of short-term stability and significant long-term changes at both the syllable and motif levels. These results suggest that normal zebra finches demonstrate a small but consistent degree of change in song characteristics over time.

The songs of the 4 birds whose lesions were restricted within Nif (ZF120, ZF127, ZF130, and ZF290) did not demonstrate any significant changes in song characteristics between PRE-1 and POST-1, suggesting no immediate impact of Nif lesion on song production. Over 30 days, all 4 birds showed small but significant long-term changes similar to those observed in the control birds. Representative examples from bird ZF127 are shown in Fig. 4*B* and mean values for each measurement are shown in Table 2. There were no significant changes in any of the individual syllable measurements or in overall motif duration between PRE-1 (Fig. 4*B*, *first panel*) and POST-1 (Fig. 4*B*, *second panel*). Syllable *b* demonstrated significant decreases in duration and FM between PRE-1 and POST-30 ($P < 0.01$) (Fig. 4*B*, *third panel*). Syllable *c* showed a significant decrease in duration over the same period ($P < 0.01$). In addition, motif duration increased significantly ($P < 0.01$). There were no significant changes in syllables *a*, *d*, or *e* between PRE-1 and POST-30. Interestingly, because all observed changes in syllable duration were decreases, the increased motif duration in

this bird appears to arise from longer silent periods such as that between syllables *c* and *d* (Fig. 4*B*, *third panel*). Analysis of birds ZF120, ZF130, and ZF290 showed a similar pattern of short-term stability and small long-term changes (data not shown).

The 2 birds with 100%+ Nif lesions (ZF38 and ZF139) demonstrated transient song degradation on POST-1, followed by rapid recovery of a normal song. Representative examples from ZF139 are shown in Fig. 4*C* and mean values for each

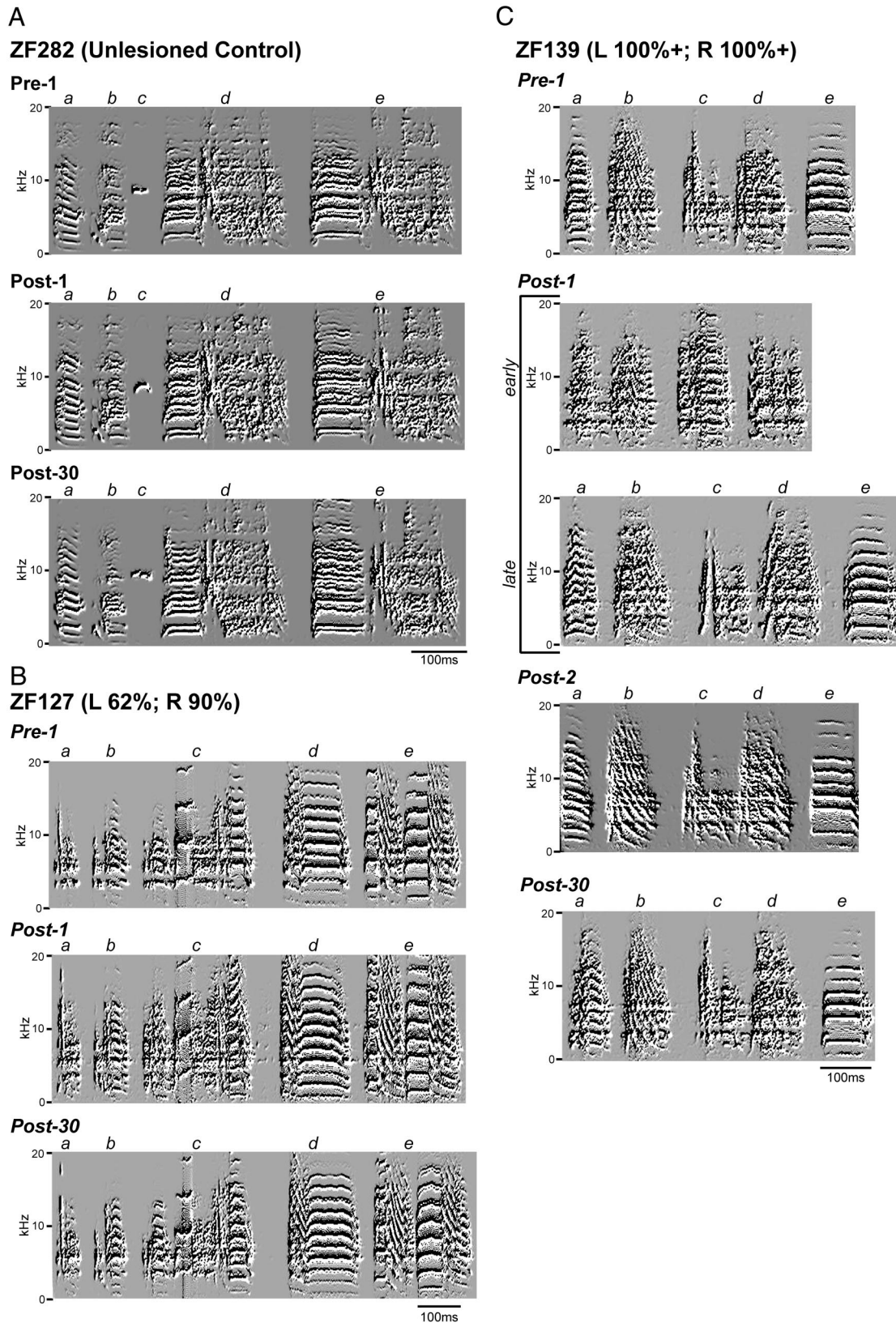


TABLE 2. Song analysis of control and Nif-lesioned birds

		Control Bird			Nif Lesion Birds					
		ZF282 (L 0%, R 0%)			ZF127 (L 62%, R 90%)			ZF139 (L 100%, R 100%)		
		PRE-1	POST-1	POST-30	PRE-1	POST-1	POST-30	PRE-1	POST-2	POST-30
Syllable <i>a</i>	Duration	52.41 ± 1.01	53.04 ± 0.6	50.4 ± 0.79	59.62 ± 1.09	56.8 ± 1.01	55.67 ± 0.83	60.17 ± 2.51	59.00 ± 2.24	70.42 ± 1.53
	Pitch	630.3 ± 11.7	647.1 ± 14.23	699.9 ± 20.88	1719 ± 38.81	1726 ± 31.83	1798 ± 60.39	1018 ± 44.85	1006 ± 43.23	1115 ± 32.77
		*								
Syllable <i>b</i>	FM	31.4 ± 0.84	30.67 ± 0.68	35.44 ± 0.54	53.33 ± 3.26	52.75 ± 3.28	51.76 ± 3.43	34.03 ± 0.99	32.71 ± 1.10	34.98 ± 0.68
	Duration	60.43 ± 0.72	62.20 ± 0.59	60.57 ± 0.90	82.76 ± 0.82	81.35 ± 0.78	78.38 ± 0.84	85.9 ± 0.86	85.40 ± 0.87	85.94 ± 0.42
Syllable <i>c</i>	Pitch	851.0 ± 17.17	796.4 ± 17.07	885.8 ± 19.92	1369 ± 22.80	1394 ± 26.92	1291 ± 36.52	1596 ± 42.59	1623 ± 48.31	1610 ± 29.2
	FM	37.63 ± 0.62	40.85 ± 0.71	42.39 ± 0.59	44.52 ± 3.50	43.55 ± 2.95	41.48 ± 2.60	50.97 ± 0.62	50.97 ± 0.63	50.62 ± 0.51
Syllable <i>d</i>	Duration	30.01 ± 0.72	31.37 ± 0.67	40.96 ± 0.52	251.6 ± 0.87	250.7 ± 0.67	247.2 ± 1.19	84.34 ± 1.18	84.46 ± 0.65	79.98 ± 0.56
	Pitch	2861 ± 99.25	2899 ± 103.2	2845 ± 123.1	1830 ± 20.98	1832 ± 28.34	1880 ± 40.39	1746 ± 34.19	1629 ± 24.64	1718 ± 31.3
Syllable <i>e</i>	FM	39.9 ± 1.3	37.52 ± 1.32	34.15 ± 1.21	40.19 ± 1.75	39.35 ± 2.54	39.75 ± 1.52	48.79 ± 0.85	49.23 ± 0.58	49.96 ± 0.47
	Duration	220.9 ± 0.77	222.3 ± 0.67	221.3 ± 1.01	158.5 ± 1.0	158.4 ± 0.80	155.7 ± 4.63	91.59 ± 0.52	91.87 ± 0.52	95.76 ± 0.53
Syllable <i>f</i>	Pitch	1400 ± 19.92	1320 ± 23.21	1453 ± 26.95	1013 ± 24.62	963.1 ± 26.0	973.7 ± 18.94	1456 ± 30.8	1515 ± 34.19	1514 ± 28.10
	FM	40.83 ± 0.33	39.61 ± 0.43	42.49 ± 0.37	25.05 ± 1.56	25.8 ± 1.94	24.6 ± 1.85	41.52 ± 0.55	41.96 ± 0.47	42.68 ± 0.51
Syllable <i>g</i>	Duration	255.60 ± 1.53	257 ± 1.68	256.3 ± 0.88	220.3 ± 1.0	222.2 ± 1.70	222.2 ± 1.70	85.34 ± 0.66	83.29 ± 1.22	87.62 ± 0.66
	Pitch	1463 ± 23.41	1360 ± 22.70	1419 ± 24.88	1231 ± 21.81	1202 ± 30.29	1222 ± 30.89	1023 ± 16.86	977.8 ± 18.02	948.4 ± 11.56
Motif duration	FM	38.27 ± 0.44	37.13 ± 0.31	37.95 ± 0.39	39.57 ± 1.70	40.1 ± 1.12	38.6 ± 4.08	18.07 ± 0.28	17.0 ± 0.36	17.78 ± 0.18
		705 ± 1.4	706.9 ± 1.2	707.1 ± 1.24	916.7 ± 1.71	915.2 ± 2.90	932.2 ± 2.94	415.1 ± 0.91	417.8 ± 4.31	441.2 ± 1.32

Values are means ± SD. Individual measurements of song characteristics for the 3 birds shown in Fig. 4 (ZF282, ZF127, and ZF139). The duration (ms), pitch (Hz), and frequency modulation (degrees) of each syllable and motif duration were sampled from 30 song motifs on PRE-1, POST-1, and POST-30. Because POST-1 song was degraded in ZF139, POST-2 song was used for statistical comparison with PRE-1. * $P < 0.01$; ** $P < 0.001$.

measurement are given in Table 2. Early song bouts on POST-1 (Fig. 4C, second panel) were so degraded that it was not possible to identify individual syllables using rigorous criteria (see METHODS). The temporal and harmonic structures of all syllables were severely disrupted. In addition, no vocalizations similar to the original syllable *e* were produced early on POST-1. However, by the end of the first postlesion day, the bird had recovered his initial song structure (Fig. 4C, third panel), although overall motif duration was prolonged relative to that of PRE-1. The bird's song remained stable thereafter, and there were no significant differences between PRE-1 and POST-2 song (Fig. 4C, fourth panel). On POST-30 (Fig. 4C, fifth panel), the bird's song was normal and showed only the small degree of long-term change that was observed in the control and restricted lesion birds. Syllable *a* demonstrated a significant increase in duration between PRE-1 and POST-30 ($P < 0.001$). No significant changes were observed in syllable *b* ($P > 0.01$). Over the same period, syllable *c* demonstrated a significant decrease in duration ($P < 0.001$). The duration of syllable *d* increased significantly between PRE-1 and POST-30

($P < 0.0001$). Syllable *e* demonstrated a significant decrease in pitch ($P < 0.001$). The second 100%+ lesion bird, ZF38, was recorded for 60 days postlesion. Like ZF139, ZF38 also showed transient song degradation early on POST-1 and recovery by the end of the first postlesion day (data not shown). ZF38 showed no significant changes between PRE-1 and POST-2 and small but significant changes in 5 of 7 syllables between PRE-1 and POST-30. A similar number of small changes were observed between POST-30 and POST-60. No long-term song degradation like that seen in deafened birds was observed in either 100%+ lesion bird during the 30- or 60-day observation period. In summary, we observed that 3/3 control birds and 6/6 Nif lesion birds demonstrated significant changes in syllable duration, pitch, and FM over 30 days. Similarly, 2/3 control birds and 4/6 Nif lesion birds demonstrated significant changes in motif duration over 30 days.

We next compared the magnitude of these changes in song over time in the control and lesion groups. We first compared the prelesion song characteristics of the 2 groups. There were no significant differences in the mean prelesion syllable dura-

FIG. 4. Nif lesions do not impair song production. Songs of each bird were compared at 1 day prelesion (PRE-1), 1 day postlesion (POST-1), and 30 days postlesion (POST-30). Each panel is an example of one song motif plotted as a spectral derivative. Spectral derivatives represent changes in the power at a given frequency over time. All birds showed some variability in the fine structure of song elements both within and between days. A: examples of song from the unlesioned control bird ZF282. This bird showed a significant change in syllable *b* between PRE-1 and POST-1. Thirty days later there were small but highly significant changes in syllables *a*, *b*, *c*, and *d* ($P < 0.01$; one-way ANOVA with Dunnett's post hoc test). B: song production was not affected by Nif lesions between 50 and 100%. Bird ZF127 (62% left Nif lesion, 90% right Nif lesion) showed no changes in song between PRE-1 and POST-1. By POST-30, as observed in the control birds, there were small but significant changes in syllables *b* and *c* and motif duration ($P < 0.01$). C: lesions that included all of Nif and some of the surrounding tissue caused brief song degradation, followed by rapid recovery. Bird ZF139 (bilateral 100%+ Nif lesion) sang a degraded song early on POST-1. However, by the end of POST-1, the song had completely recovered normal syllable structure. There were no significant changes between PRE-1 song and POST-2 song in this bird. By POST-30, as in the control and restricted-lesion birds, there were small but significant changes in syllables *a*, *c*, *d*, and *e* ($P < 0.01$).

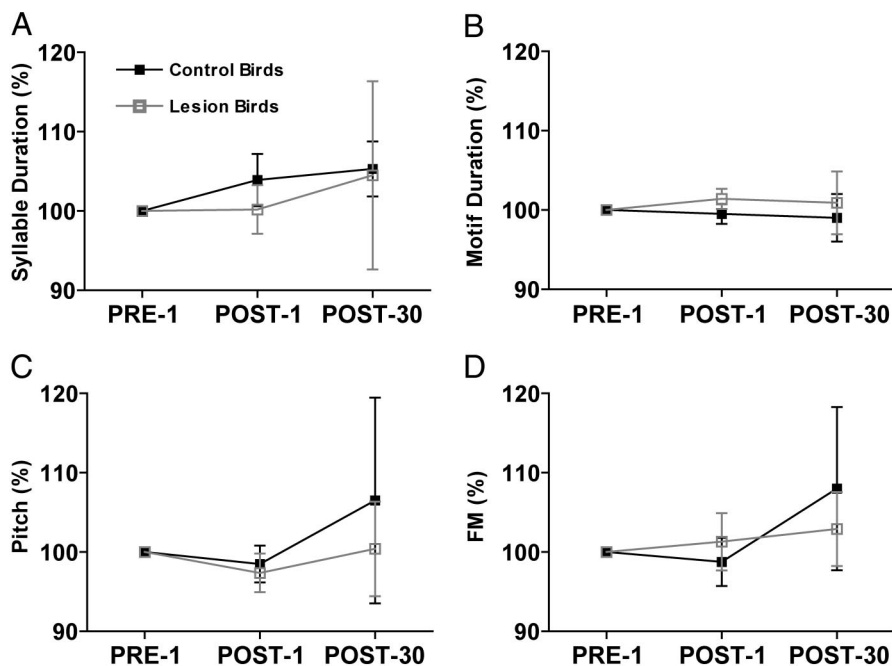


FIG. 5. Comparison of song characteristics in control and Nif lesioned birds. Each plot shows mean normalized values for the control and lesion groups, expressed as percentages of the mean prelesion values. Means and variances of the 2 distributions were compared by unpaired *t*-test and F-test, respectively. *A*, syllable duration: there were no significant differences in mean normalized syllable duration between the control and lesion birds on POST-1 or POST-30. *B*, motif duration: similarly, there were no significant differences in mean normalized motif duration between the groups on POST-1 or POST-30. *C*, pitch: likewise, there were no significant differences in mean normalized pitch between the control and lesion groups on POST-1 or POST-30. *D*: FM (frequency modulation): no significant differences in mean normalized FM were observed between the 2 groups on POST-1 or POST-30. Control birds are represented in black and Nif lesioned birds are represented in gray. Error bars denote mean $\pm$ SD.

tion (control 154.2 ± 98.8 ms; lesion 129.5 ± 85.5 ms), pitch (control 1341.1 ± 883.5 Hz; lesion 1202.6 ± 385.6 Hz), FM (control 36.6 ± 10.5 deg; lesion 37.1 ± 15.2 deg), or motif duration (control 746.2 ± 82.0 ms; lesion 793.53 ± 219.1 ms) measurements of the 2 groups ($n = 3$ control and 6 lesion birds; $P > 0.05$, unpaired *t*-test).

To compare the 2 groups at POST-1 and POST-30, all syllable measurements at each time point were normalized to the mean PRE-1 values for that syllable. We then averaged these values across syllables to give mean normalized syllable duration, pitch, and FM values for each bird. Similarly, all motif duration measurements were normalized to the mean PRE-1 value. Figure 5 depicts the mean normalized values for both groups as percentages of the mean PRE-1 values ($n = 3$ control and 6 lesion birds). As noted above, no measurements of POST-1 song were possible for either 100%+ lesion bird (ZF139 and ZF38).

There were no striking differences in the trajectories of the control and lesioned birds' song characteristics over time. There were no significant differences in either the mean or the variance of the syllable duration measurements between 2 groups at either POST-1 (control $103.9 \pm 3.3\%$; lesion $100.2 \pm 3.1\%$; $P > 0.05$) or POST-30 (control $105.2 \pm 3.5\%$; lesion $104.5 \pm 11.9\%$; $P > 0.05$) (unpaired *t*-test, F-test) (Fig. 5A). Similarly, there were no significant differences in the mean or the variance of the motif duration values on day POST-1 (control $99.5 \pm 1.2\%$; lesion $101.4 \pm 1.3\%$; $P > 0.05$) or POST-30 (control $99.0 \pm 3.0\%$; lesion $100.9 \pm 4.0\%$; $P > 0.05$) (Fig. 5B). Likewise, there were no significant differences in either mean or variance of the pitch measurements on POST-1 (control $98.5 \pm 2.3\%$; lesion $97.4 \pm 2.4\%$; $P > 0.05$) or POST-30 (control $106.5 \pm 13.0\%$; lesion $100.4 \pm 6.0\%$; $P > 0.05$) (Fig. 5C). No significant differences were observed in the mean or the variance of the FM values on POST-1 (control $98.8 \pm 3.0\%$; lesion $101.3 \pm 3.6\%$; $P > 0.05$) or POST-30 (control $108.0 \pm 10.3\%$; lesion $102.9 \pm 4.7\%$; $P > 0.05$) (Fig. 5D). Thus the magnitude of the observed changes in

song over time did not differ between the control and lesion groups. Together with the above observations, these data suggest that Nif lesions neither eliminate nor induce abnormal variability in song production of adult zebra finches within the time period studied here.

DISCUSSION

We used bilateral excitotoxic lesions to examine the role of nucleus Nif in auditory and premotor processing in the avian song system. Nif lesions led to a loss of auditory responsiveness in HVC but did not impair song production. These results suggest that, although Nif is a crucial component of the ascending auditory input to the song system, an intact Nif is not necessary for the generation of the premotor activity patterns underlying song.

Previously, we found that inactivation of Nif by muscimol injection leads to a loss of most spontaneous and all auditory activity in HVC (Cardin and Schmidt 2004). In the present study, lesions that encompassed more than 50% of Nif resulted in a profound loss of both spontaneous activity and auditory responsiveness in the ipsilateral HVC. The reduction in spontaneous and evoked HVC activity after Nif lesion was equivalent in magnitude to that observed after muscimol injection into Nif (Cardin and Schmidt 2004). The lack of spontaneous or stimulus-evoked HVC activity after Nif lesion suggests that Nif provides both the major auditory input and significant spontaneous drive to HVC. This is consistent with recent findings that GABA injection into Nif abolishes the subthreshold synaptic responses evoked in HVC by auditory stimuli (Coleman and Mooney 2004). Reduced HVC activity was observed in hemispheres where lesion sizes ranged from 50 to 100%+ of Nif ($n = 10$), but not in the hemisphere where only 29% of Nif was lesioned. Although we cannot rule out the possibility of some residual Nif input spared by the lesions, we consistently observed no auditory activity and extremely little spontaneous activity in HVC after Nif lesion.

The above results support the role of NIf as the main auditory input to HVC. Although NIf inputs may simply provide a depolarizing offset that allows another auditory input to drive HVC, it is unlikely that another structure provides substantial auditory input to HVC. Field L, the primary auditory forebrain structure, predominantly projects to the shelf region around HVC and makes very few contacts within HVC itself (Fortune and Margoliash 1995; Kelley and Nottebohm 1979). The auditory response latencies of MMAN, another forebrain structure that projects to HVC, are longer than those of HVC neurons (Vates et al. 1997). Finally, the thalamic structure Uva projects directly to both NIf and HVC but has not been shown to demonstrate auditory responses (Wild 1994).

If NIf is indeed the main auditory input to HVC, bilateral NIf lesions should eliminate auditory feedback to the song system. Because song maintenance in adult zebra finches is dependent on auditory feedback, bilateral NIf lesions might be expected to lead to a long-term degradation of song similar to that observed after deafening (Brainard and Doupe 2001; Lombardino and Nottebohm 2000; Nordeen and Nordeen 1992) or other disruption of auditory feedback (Leonardo and Konishi 1999). In Bengalese finches, preliminary evidence suggests that NIf lesions may cause some immediate decrease in song syntax complexity, but do not cause long-term (up to 3 wk) song degradation (Hosino and Okanoya 2000). In contrast, deafening in Bengalese finches causes a rapid decrease in song stereotypy and syllable phonology in the first week, followed by long-term song degradation over a period of months (Okanoya and Yamaguchi 1997; Woolley and Rubel 1997). In the current study, we did not observe any long-term degradation of song after NIf lesion in zebra finches, even in the one 100%+ lesion bird evaluated 60 days postlesion. However, several studies have found that the effects of deafening on song are age-dependent. Whereas young-adult zebra finches have been shown to demonstrate rapid loss of song structure after deafening, these effects may take 2 to 3 months to develop in birds over 200 days of age (Brainard and Doupe 2001; Lombardino and Nottebohm 2000). Because all birds used in the current study were a minimum of 200 days old, the lack of song degradation at 30 and 60 days may not be surprising. Future long-term examination of young- and old-adult birds with bilateral NIf lesions will be necessary to determine whether loss of NIf input has an impact similar to that of deafening.

In contrast to the effects of NIf lesions on HVC auditory processing, loss of NIf did not impair song output. Previous studies using mostly qualitative measurements of song have observed a high degree of stereotypy of adult zebra finch song (Immelmann 1969; Lombardino and Nottebohm 2000; Price 1979; Scharff and Nottebohm 1991). In the current study, we used a quantitative approach (Tchernichovski and Mitra 2002; Tchernichovski et al. 2001) to compare song characteristics over time both within individual birds and between the control and lesion groups. We observed that unlesioned control birds demonstrated stable song over short periods but showed significant changes over 30 days. Like the control birds, birds with restricted NIf lesions showed short-term stability and some long-term changes in song. Overall, we found no significant difference in motif duration, syllable duration, pitch, or FM between control and lesion birds at any time point. These

results suggest that an intact NIf is not necessary for motor production of song in adult zebra finches.

The 2 birds whose lesions included all of NIf and some of the surrounding tissue demonstrated transient song degradation. During the beginning of the first postlesion day, both birds showed disruption in the temporal and harmonic structure of the songs they produced and we were unable to quantitatively identify individual syllables. Remarkably, by the end of the first postlesion day both birds had recovered normal song. Thirty days after lesion, they showed the same degree of long-term change as that of the control and restricted-lesion birds. These results agree well with preliminary observations by Vu et al. (1995), who observed that relatively large lesions centered on NIf caused transient changes in song. Because the lesions in our 100%+ birds included some tissue surrounding NIf, we cannot distinguish whether the transient degradation observed on the first postlesion day was the result of a total NIf lesion, damage to other areas such as Field L, or a compound effect of both. However, as both birds rapidly recovered their songs, our results suggest that the maximum immediate effect of NIf lesion on song production is a short-term disruption. These results are consistent with some previous findings in Bengalese finches, where NIf lesions in birds with simple songs do not appear to disrupt song phrase structure within a 3-wk period (Hosino and Okanoya 2000).

Previous studies have observed that both HVC and NIf exhibit premotor activity time-locked to the production of song (Cardin and Schmidt 2004; Hahnloser and Fee 2003; McCasland 1987; Yu and Margoliash 1996). HVC lesions lead to a dramatic, immediate loss of normal song production (Nottebohm et al. 1976), suggesting that HVC plays a pivotal role in the generation of the premotor activity underlying song. In contrast, all birds with restricted NIf lesions sang a normal song immediately after lesion and even the 2 birds with 100%+ lesions sang a normal song by one day postlesion. Although we cannot rule out the possibility of premotor contribution from residual NIf inputs spared by the lesion, these results suggest that an intact NIf is not necessary for generation of the precisely timed activity patterns necessary for song production in zebra finches. Indeed, we observed HVC premotor activity in our chronically implanted NIf-lesioned birds. The above results raise the possibility that the robust activity in NIf during song represents a corollary premotor input from Uva, which demonstrates premotor activity (Williams and Vicario 1993) and may be necessary for song production (Coleman and Vu 2000; Vu and Coleman 2001). An alternative possibility is that activity in NIf during song may be primarily auditory in nature. Although our data suggest that NIf does not act as a pattern generator for song, it is possible that NIf may exert a subtle effect on the patterns of HVC activity.

These results, together with those of our earlier study, suggest that ascending auditory input reaches HVC by way of NIf. Generation of the premotor patterns underlying zebra finch song production depends on HVC, but does not require an intact NIf. Sensory and premotor representations of song thus converge in HVC, which may be a primary site of sensorimotor integration in the song system.

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