

LMAN Lesions Prevent Song Degradation after Deafening without Reducing HVC Neuron Addition

Luisa L. Scott,^{1,2} Ernest J. Nordeen,³ Kathy W. Nordeen³

¹ Neuroscience Program, University of Rochester, Rochester, New York 14642

² Section of Neurobiology, University of Texas at Austin, Austin, Texas 78712

³ Department of Brain and Cognitive Sciences, University of Rochester, Rochester, New York 14627

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ABSTRACT: In some songbirds perturbing auditory feedback can promote changes in song structure well beyond the end of song learning. One factor that may drive vocal change in such deafened birds is the ongoing addition of new vocal-motor neurons into the song system. Without auditory feedback to guide their incorporation, the addition of these new neurons could disrupt the established song pattern. To assess this hypothesis, the authors determined if neuronal recruitment into the vocal motor nucleus HVC is affected by neural signals that influence vocal change in adult deafened birds. Such signals appear to be conveyed via LMAN, a nucleus in the anterior forebrain that is necessary for vocal change after deafening. Here the authors tested whether LMAN lesions might restrict song degradation after deafening by reducing the addition or survival of new HVC neurons that would otherwise corrupt the ongoing song pattern.

Using [³H]thymidine autoradiography to identify neurons generated in adult zebra finches, it was shown here that LMAN lesions do not reduce the number or percent of new HVC neurons surviving for either several weeks or months after [³H]thymidine labeling. However, the authors confirmed previous reports that LMAN lesions restrict vocal change after deafening. These data suggest that neurons incorporated into the adult HVC may form behaviorally adaptive connections without requiring auditory feedback, and that any role such neurons may play in promoting vocal change after adult deafening requires anterior forebrain pathway output. © 2007 Wiley Periodicals, Inc. *Develop Neurobiol* 67: 1407–1418, 2007

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INTRODUCTION

Songbirds require auditory feedback to develop normal song patterns (Konishi, 1965a; Nottebohm, 1968; Price, 1979), but species vary widely in their contin-

ued use of such feedback to maintain and/or reshape song behavior as adults. Canaries normally restructure their song seasonally, and deafening in adulthood leads to rapid and severe disruption of song structure (Nottebohm et al., 1976). In contrast, white-crowned sparrows, a species that maintains a stereotyped song throughout adulthood, retain their song structure for many months after adult deafening (Konishi, 1965b). But in several other species that normally modify songs only during adolescence (e.g., zebra and bengalese finches), altering or preventing auditory feedback in adulthood leads to eventual vocal deterioration (Nordeen and Nordeen, 1992; Okanoya and Yamaguchi, 1997; Woolley and Rubel, 1997; Leonardo and Konishi, 1999).

Correspondence to: L.L. Scott (luisa_scott@mail.utexas.edu).
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The gradual vocal changes that follow perturbations of auditory input in some species could reflect either a gradual corruption of patterned activity in the neural pathways controlling song production, or a more active readjustment of these pathways prompted by the altered feedback. One potential source of variability in the control of song behavior is the ongoing addition of new vocal-motor neurons into the song system (see Fig. 1). Adult generated neurons are incorporated into HVC (acronym used as proper name), a pallial nucleus necessary for song production. This region conveys both premotor and auditory information to downstream vocal motor nuclei and to an anterior forebrain pathway (AFP) that is necessary for vocal plasticity. Across several paradigms, the rate of neuronal incorporation in HVC correlates with vocal plasticity, suggesting that new HVC neurons normally provide a substrate for vocal plasticity. For instance, early developmental, as well as seasonally recurring, periods of song learning coincide with the incorporation of massive numbers of new HVC neurons (Nordeen and Nordeen, 1988a,b; Kirn and DeVogd, 1989; Kirn et al., 1994). In fact, even when the early period of song learning is extended in zebra finches, this prolonged period of vocal plastic-

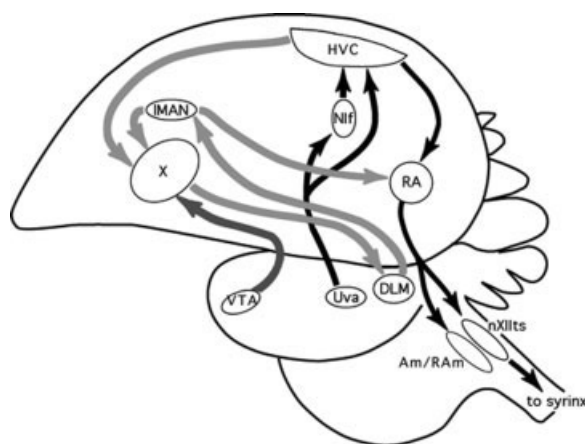


Figure 1 Simplified schematic of the avian song system. The vocal motor pathway (black arrows) includes nucleus uvulaeformis (Uva), nucleus interfacialis (Nif), HVC (acronym used as proper name), robust nucleus of the arcopallium (RA), nucleus ambiguus (AM), nucleus retroambiguus (RAm), and tracheosyringeal portion of the XII cranial nerve nucleus (nXIIts). The anterior forebrain pathway (gray arrows) connects the HVC to the RA via area X (X), the dorsal-lateral nucleus of the medial thalamus (DLM) and the lateral magnocellular nucleus of the anterior nidopallium (LMAN). Projection from ventral tegmental area (VTA) into the AFP is also shown. Major projections in the vocal motor pathway and anterior forebrain pathway are ipsilateral.

ity is accompanied by heightened addition of late generated HVC neurons (Wilbrecht et al., 2006).

In adult birds producing stable song, neuron addition is relatively modest, but some HVC neuron addition continues throughout life in all songbirds studied to date (Goldman and Nottebohm, 1983; Nordeen and Nordeen, 1988b; Alvarez-Buylla et al., 1990; Alvarez-Buylla et al., 1992; Alvarez-Buylla and Kirn, 1997; Tramontin and Brenowitz, 1999; Scott et al., 2000; Absil et al., 2003). While continuing incorporation and turnover of new HVC neurons does not always correlate with adult song remodeling (Tramontin and Brenowitz, 1999), such neurons could provide an important substrate for naturally-occurring or induced vocal plasticity. For instance, HVC receives auditory input, and we have hypothesized that the accumulation of new neurons without the benefit of auditory information could disrupt the production of stable song either by corrupting sensorimotor mapping within this region or introducing noise into the vocal motor pathway (Nordeen and Nordeen, 1992). In support of this idea, species differences in the rate of vocal deterioration correlate with species differences in HVC neuron addition after deafening (Scott et al., 2000). Also, age-related decreases in the rate of behavioral change after deafening correlate with age-related declines in rates of HVC neuron addition (Lombardino and Nottebohm, 2000; Wang et al., 2002). However, there have yet to be any direct tests of the functional relationship between HVC neuron addition and vocal stability after deafening.

The present study assesses the link between adult HVC neuronal incorporation and vocal plasticity by exploiting the observation that disruption of the connection from the AFP to the motor pathway prevents behavioral change after deafening. Several studies suggest that signals generated in the AFP are necessary for the behavioral change observed in adult birds following perturbations of auditory input. The output nucleus of this pathway, the lateral magnocellular nucleus of the anterior nidopallium (LMAN), indirectly connects HVC to the vocal motor nucleus RA (robust nucleus of the anterior nidopallium), and lesions of LMAN prevent the loss, addition, and reorganization of song syllables that normally occur after adult deafening or tracheosyringeal nerve cuts (Williams and Mehta, 1999; Brainard and Doupe, 2000). This suggests that the AFP provides a signal that permits and/or instructs vocal change when mismatches exist between expected auditory feedback and internal representations of song (Bottjer et al., 1984; Troyer et al., 1996; Williams and Mehta, 1999; Brainard and Doupe, 2000; Troyer and Bottjer, 2001; Margoliash,

2002). We reasoned that if the accumulation of “untrained” HVC neurons is directly responsible for vocal change after deafening, then LMAN lesions that prevent this behavioral change must also reduce HVC neuron addition (e.g., by reducing activity and/or trophic support normally conveyed through the LMAN-RA pathway). Indeed, previous work has shown that LMAN lesions prevent the increase in HVC neuronal incorporation seen after unilateral tracheosyringeal nerve cuts (Wilbrecht et al., 2002b). However, our results indicate that LMAN lesions restrict adult vocal plasticity after deafening without curtailing the recruitment or long-term survival of new HVC neurons.

METHODS

Treatment Groups

The songs of adult male zebra finches (4–5 months of age) raised in free-flight aviaries were recorded prior to any manipulations. Birds were then randomly assigned to LMAN lesion or sham lesion treatment groups. Bilateral electrolytic lesions were made in LMAN by injecting current through a tungsten microelectrode (World Precision Instruments, Sarasota, FL) connected to a Model A-365 Stimulus Isolator (World Precision Instruments) at four stereotaxically targeted locations (each 45–50 s at 100 μ A) within the nucleus. Electrolytic lesions were employed to replicate methodology used in previous studies documenting the stabilizing influence of LMAN lesions (Williams and Mehta, 1999; Brainard and Doupe, 2000). Sham-lesioned animals received similar surgery except that the electrode was not lowered into the brain. Following surgery, animals were housed in group-cages (all male) for the remainder of the experiment. After at least a week, animals were again recorded to document that the lesions did not alter syllable morphology or sequencing (Nordeen and Nordeen, 1993). Two weeks after surgery, all animals were deafened bilaterally by cochlear extirpation. All cochlea were extracted with the distal end (lagena) intact. After 2 or 3 days of recovery, all animals began a regime of [3 H]thymidine injections (New England Nuclear, 6.7 Ci/mmol, 1.0 mCi/37 MBq; dose: 2.5 μ Ci/gm body weight) to label dividing cells. Injections were delivered 2 times per day (at \sim 9 AM and 5 PM) for 8 days. Songs were re-recorded 4 months after deafening, and birds then were sacrificed to assess the long-term incorporation of new HVC neurons.

An additional set of animals underwent treatment similar to the above group to test whether LMAN lesions in deafened animals affect short-term neuronal recruitment in HVC. Adult male zebra finches (either 4–5 or 8–9 months of age) received bilateral LMAN lesions or sham lesions (age counterbalanced across groups), and then were deafened 2 weeks later. After 2 to 3 days, all animals were given [3 H]thymidine injections 2 times per day for 10 days.

Animals were sacrificed 1 month after deafening (18 days after the last thymidine injection), allowing sufficient time for new neurons to arrive in HVC (Burd and Nottebohm, 1985; Alvarez-Buylla and Nottebohm, 1988; Burek et al., 1994; Kim et al., 1999). In two lesioned and one sham animal, the retrograde tracer fluorogold was injected into RA 4 days before sacrifice.

Behavioral Analysis

Female-directed songs were recorded, amplified, and digitized using Avisoft Recorder software. Sonograms were produced using Avisoft SASlab Pro with parameters set as follows: fast-fourier transform length = 256 points, frame size = 100%, and FlatTop window with 75% overlap. Song syllables were defined as acoustic units surrounded by intervals of baseline energy lasting at least 10 ms. Comparison of songs recorded prior to LMAN (or sham) lesions and prior to deafening revealed no significant differences in syllable number, structure, or order, confirming that songs were stable at the time of deafening. To verify the effect of LMAN lesions on the songs of deaf birds (Brainard and Doupe, 2000), songs recorded immediately prior to deafening were compared with songs recorded 3.5 months after deafening. We focused on changes in the acoustic structure (phonology) of song syllables because these changes occur slowly, and therefore are most likely to be caused by the gradual accumulation of naïve neurons in vocal motor circuitry. For each bird, the phonological similarity between each syllable in the post-deaf song and the pre-deaf syllable it most resembled in contour, frequency, and duration was scored on a 0–3 scale (0 = no resemblance, 1 = slightly similar, 2 = highly similar, 3 = matched) by an observer blind to experimental group. The proportion of post-deaf syllables unmatched (scored 0) to a pre-deaf syllable measured syllable addition after deafening. Also, an average syllable score was obtained from 12.5 s of post-deaf song (quiet periods were spliced out of this sample). Group differences in syllable addition and average syllable score were assessed using a one-tailed Mann–Whitney U-test. A one-tailed Wilcoxon signed-rank test assessed group differences in the percent of pre-deaf song that was deleted (no syllable in post-deaf song scored 2 or 3) because the control group exhibited no variation in this dependent measure. Group data are presented as average \pm SEM.

Anatomical Analysis

Animals were transcardially perfused with 0.9% saline, followed by 10% formalin in a 1 M phosphate buffer. The brains were removed and post-fixed in formalin before being embedded in paraffin. Coronal forebrain sections (10 μ m) through LMAN were mounted onto slides and stained with thionin. Serial sections (10 μ m) through HVC were mounted onto slides and processed for thymidine autoradiography.

To estimate the extent of the LMAN lesions, cross-sectional areas of left and right LMANs were measured every

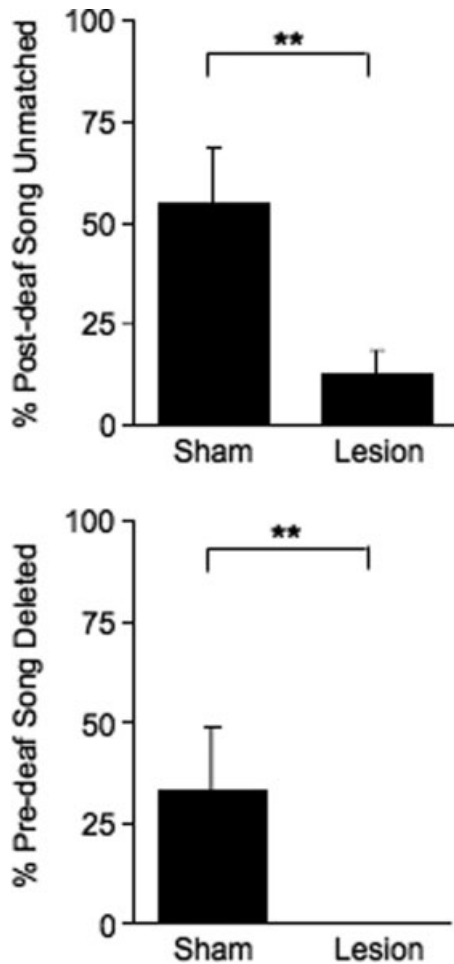


Figure 2 By 3.5 months after deafening, the proportion of post-deaf song syllables that were unmatched (score = 0) to a pre-deaf syllable was significantly greater in sham ($n = 6$) than in LMAN-lesioned ($n = 5$) animals (upper). Also, sham-lesioned birds deleted one third of their pre-deaf song syllables whereas LMAN-lesioned birds did not delete any syllables (lower) ** one-tailed $p < 0.02$. Data shown are mean + SEM.

40–60 μm and used to estimate LMAN volumes in control (sham-lesioned) and LMAN-lesioned birds. Lesion size is expressed as percent of mean control LMAN volume in the corresponding hemisphere. For long survival birds, lesioned animals with reduced LMAN volumes bilaterally (with respect to mean control volumes) showed minimal vocal changes after deafening and were included for analysis of both behavior and neuronal incorporation. All but one of these animals sustained substantial damage to both LMANs such that their volumes fell below the smallest LMAN volume measured in the controls (mean reduction = $73\% \pm 9.3\%$). Although the LMAN volume in the remaining lesioned animal overlapped with smaller control LMAN volumes (see Fig. 6), this and all other lesioned birds sustained damage in the caudal portion of the LMAN where

the RA-projections leave the nucleus (pers. observation; Kittelberger and Mooney, 1999). The inclusion of this latter bird did not change the outcome of any group comparisons. For the short survival group, half of the animals sustained damage that was substantially greater in the right than the left hemisphere, whereas the remaining animals sustained large bilateral LMAN lesions. Therefore, LMAN lesions were considered “unilateral” if LMAN volume on one side was reduced by $<50\%$, while LMAN volume on the other side was reduced by $>50\%$ and extended through the caudal portion of the nucleus. Among these animals, the right hemisphere always sustained the larger lesion. LMAN lesions were considered “bilateral” if LMAN volume in both hemispheres was reduced by $>50\%$ and extended through the caudal portion of each nucleus. Even in the “bilateral” group, LMAN lesions were generally larger on the right than on the left side (mean: $85\% \pm 8\%$ vs $75\% \pm 10\%$ reduction in volume). Therefore, for the short survival birds we restricted our analysis of HVC to the right hemisphere (i.e., side of maximal damage).

For analyses of HVC neuronal incorporation, slides were dipped in emulsion (NTB2, Kodak) and stored at 4°C for 25 days, developed (D19, Kodak), fixed (Kodak fixer), and stained with thionin. We estimated the overall density of HVC neurons and thymidine-labeled HVC neurons using the following procedure. Throughout the rostrocaudal extent of the HVC, counting fields (>21 per bird) were chosen in every fourth section via systematic random selection. Neurons were identified at $100\times$ by their clearly distinct nucleus and densely stained cytoplasm. Background silver grain density was measured over nonsomal portions of the tissue and the number of background silver grains per mean nuclear area was calculated for each autoradiography processing batch. Neurons with at least six exposed silver grains over the nucleus ($>20\times$ background) were counted as thymidine-labeled. The density of HVC neurons, the density of thymidine-labeled HVC neurons, and the percent of HVC neurons that were thymidine-labeled were assessed for each animal. Cross-sectional areas of HVC measured every 40–60 μm were used to estimate HVC volume, the total number of HVC neurons, and the number of thymidine-labeled HVC neurons for each bird. Nuclear profiles of HVC neurons (50 profiles/bird) were measured in 1–2 randomly chosen fields, and labeled HVC nuclear profiles (>15 profiles/bird, mean = 28.3) were measured in 2–3 randomly chosen sections. There was no significant effect of treatment on the average nucleus profile size of HVC neurons or thymidine-labeled HVC neurons ($p > 0.14$) indicating that cell-splitting errors would occur equally for LMAN-lesioned and sham-lesioned birds. Thus, neuron counts were not corrected because our analysis focused on relative group comparisons.

Independent two-tailed t -tests were used to compare HVC anatomical data from sham vs. LMAN-lesioned birds in the long survival groups. In the short survival group, the 4- to 5-month-old and 8- to 9-month-old birds did not differ significantly in measures of HVC neuronal incorporation ($p > 0.19$) and so were combined for analysis. HVC anatomical data from the short-survival groups were compared using

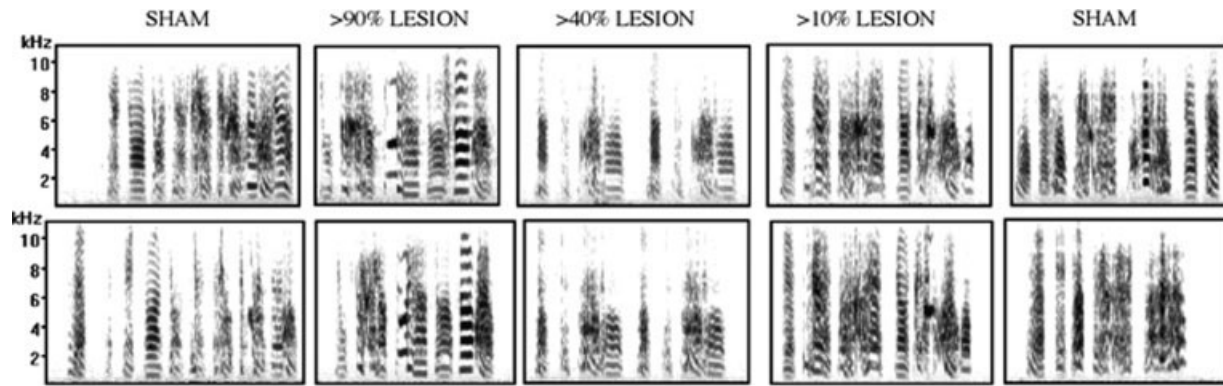


Figure 3 Sonograms illustrating representative songs produced prior to deafening (upper) and 3.5 months after deafening (lower). The songs of sham-lesioned birds changed substantially after deafening (first and last panels). In contrast, LMAN-lesioned birds (see middle panels) typically reproduced the structure of pre-deaf song with only minor alterations. Calibration bar = 250 ms.

single factor ANOVAs (“bilateral” lesions, “unilateral” lesions, and controls). Data presented are mean \pm SEM.

Finally, to evaluate whether a random sampling strategy might have underestimated group differences in neuronal incorporation, we conducted a more complete analysis of thymidine-labeling in a subset of the long survival animals. In three birds with the least behavioral change (LMAN

lesion + deaf), and in three birds with the most behavioral change (deaf only), all thymidine-labeled neurons within the boundaries of HVC were counted in every section through the rostral-caudal extent of this nucleus in the left hemisphere (side of maximal damage). The volume of the HVC analyzed was calculated and used to estimate the density of thymidine-labeled HVC neurons. Total neuron den-

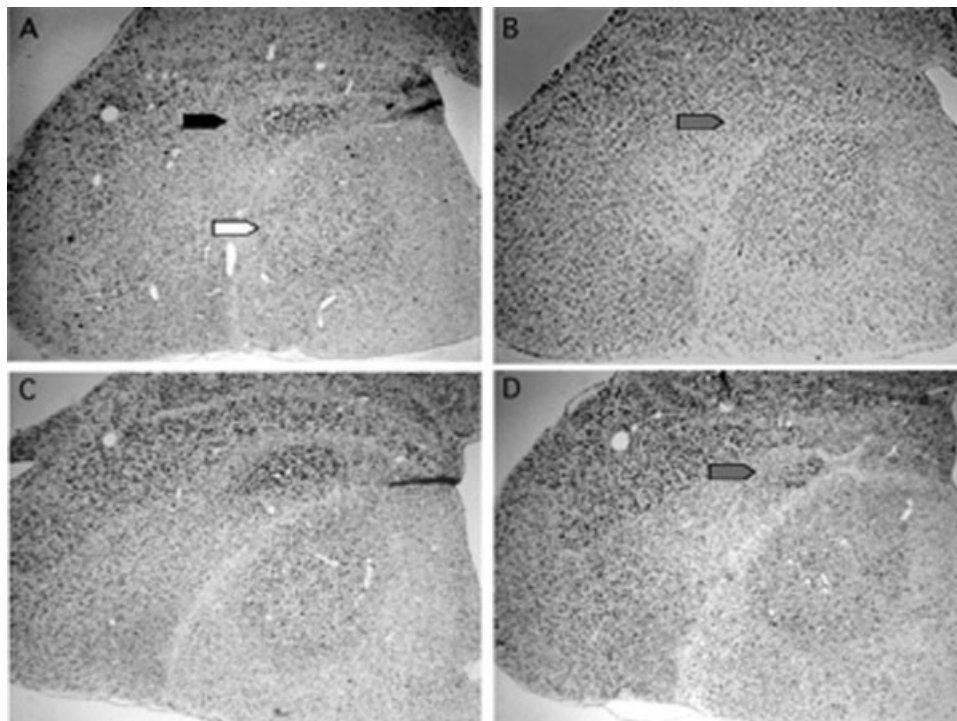


Figure 4 Photomicrographs of sham (A and C) and LMAN (B and D) lesions. LMAN (black arrow) and Area X (white arrow) are indicated in A. The LMAN lesion (gray arrow) shown in B is complete, and the LMAN volume shown in D is 44% smaller than the average LMAN volume in sham-lesioned birds.

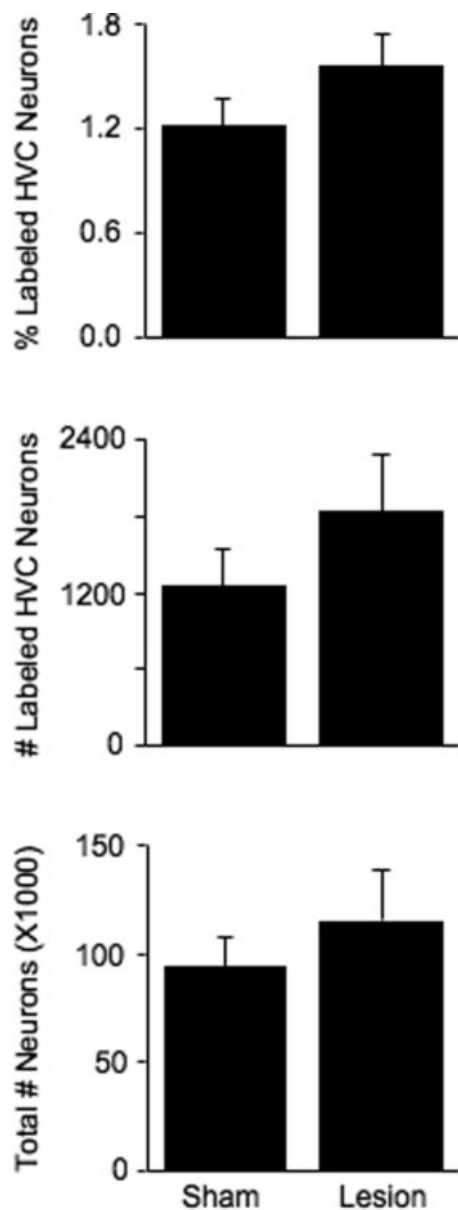


Figure 5 LMAN lesions did not alter the percentage of new neurons present in deafened birds 3.5 months after [^3H]thymidine injections (upper). These LMAN lesions also did not alter the uncorrected number of [^3H]thymidine-labeled HVC neurons (middle) or the total uncorrected number of HVC neurons (lower) 3.5 months after deafening. Data shown (mean \pm SEM) are for the left hemisphere.

sity measurements then were used to calculate the % of thymidine-labeled HVC neurons. Among the six animals comprising this more complete but focused analysis, the incidence of thymidine-labeled HVC neurons (LMAN-lesioned: $1.4\% \pm 0.4\%$; Sham: $1.0\% \pm 0.1\%$) was similar to that obtained in these same animals using the random sampling strategy described above (LMAN-lesioned: $1.7\% \pm 0.2\%$; Sham: $1.1\% \pm 0.5\%$), despite the fact that the two analyses were done by different experimenters. Moreover,

the overall results of this further analysis were similar to those obtained for the entire data set, and thus only the full data set obtained by the random sampling strategy are reported in the Results.

RESULTS

We replicated previous reports that bilateral LMAN lesions restrict song degradation after deafening in adult zebra finches (see Fig. 2). By 3.5 months after deafening (long survival group), sham-lesioned deaf birds sang significantly more song unmatched to pre-deaf syllables than did birds with LMAN-lesions ($55.1\% \pm 13.6\%$ vs. $12.5\% \pm 6.3\%$; one-tailed $p < 0.01$). Also, sham-lesioned animals no longer produced $32.9\% \pm 15.6\%$ of their song syllables whereas LMAN-lesioned birds did not omit any of their pre-deaf song syllables (one-tailed $p < 0.05$). There was little overlap in these measures of vocal change after deafening between sham-lesioned and LMAN-lesioned animals. Comparison of syllables produced 3.5 months after deafening to predeafening song syllables revealed significantly lower similarity scores in sham-lesioned as compared to LMAN-lesioned birds (average similarity = 1.19 ± 0.35 vs. 2.37 ± 0.20 , one-tailed $p < 0.01$). Interestingly, song remained stable after deafening even in those birds where LMAN lesions spared a significant portion of the nucleus (see Fig. 3).

Relative to the average LMAN volume in the sham-control group, lesions in the long survival group reduced LMAN volume by $65\% \pm 17\%$ and $57\% \pm 19\%$ on the left and right side respectively (Fig. 4). However all lesions damaged at least the posterior portion of LMAN where RA projecting axons leave the nucleus. The volume of LMAN in each hemisphere was reduced by $>95\%$ in 2 animals, by 40–60% in 2 animals, and by 10–14% in the remaining animal.

Table 1 The Percentage of Thymidine-Labeled HVC Neurons in Each Hemisphere of the Long Survival Groups

	Left HVC	Right HVC
LMAN-lesioned	1.56 ± 0.19	1.60 ± 0.20
Control	1.21 ± 0.17	1.36 ± 0.20
	($p = 0.16$)	($p = 0.38$)

Although LMAN lesions were somewhat larger on the left than on the right side (% lesioned = 65 ± 17 and 57 ± 19 , respectively), LMAN lesions did not significantly alter the % of thymidine-labeled HVC neurons within either hemisphere. Data shown are mean \pm SEM.

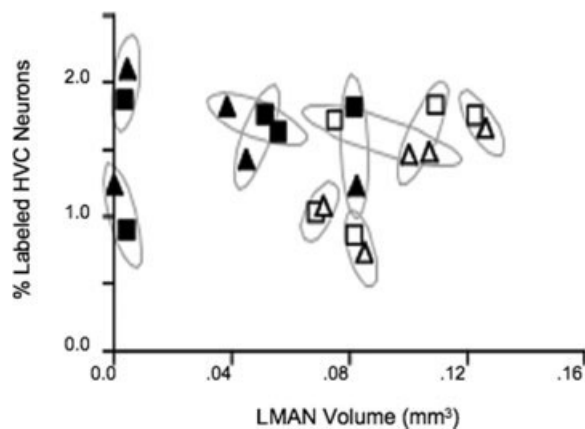


Figure 6 The percent of [^3H]thymidine-labeled neurons in the HVC did not correlate with the volume of LMAN in either LMAN-lesioned (black symbols) or sham-lesioned (open symbols) animals. Data shown are from animals sacrificed 3.5 months after [^3H]thymidine injections. Circles enclose measurements from the left (triangle) and right (square) hemisphere of the same animal.

Although bilateral LMAN lesions restricted vocal change after deafening, lesions did not suppress the long-term survival of new HVC neurons. As shown in Figure 5 for the hemisphere of maximal damage (left side) in the long survival group, LMAN-lesioned and sham-lesioned birds did not differ significantly in the percent of HVC neurons that were thymidine-labeled ($p = 0.16$) or the number of HVC neurons that were thymidine-labeled ($p = 0.26$). Estimates of total HVC neuron number in the left hemisphere also did not differ significantly between LMAN- and sham-lesioned animals ($p = 0.42$). Similarly, in the right hemisphere of these long survival animals, the % thymidine-labeled HVC neurons did not differ significantly between treatment groups (see Table 1). Finally, neither LMAN- nor sham-lesioned birds exhibited a correlation between LMAN volume and the percent of thymidine-labeled HVC neurons surviving for several months in the ipsilateral hemisphere (see Fig. 6).

While the long survival birds revealed no effect of LMAN lesion on the incorporation of new neurons, additional animals were generated to evaluate if LMAN lesions alter short-term neuronal incorporation. Birds surviving 1 month after deafening (18 days after the last thymidine injection) were classified as having bilateral lesions if LMAN volume was reduced by at least 50% in each hemisphere and both lesions extended through the posterior portion of the nucleus. Relative to the average volume of LMAN in controls, LMAN volume was reduced in these birds by $75\% \pm 10\%$ (range: 54%–99%) and

$85\% \pm 8\%$ (range: 62%–100%) on the left and right side respectively. The remaining birds were classified as having right hemisphere lesions (see methods): in

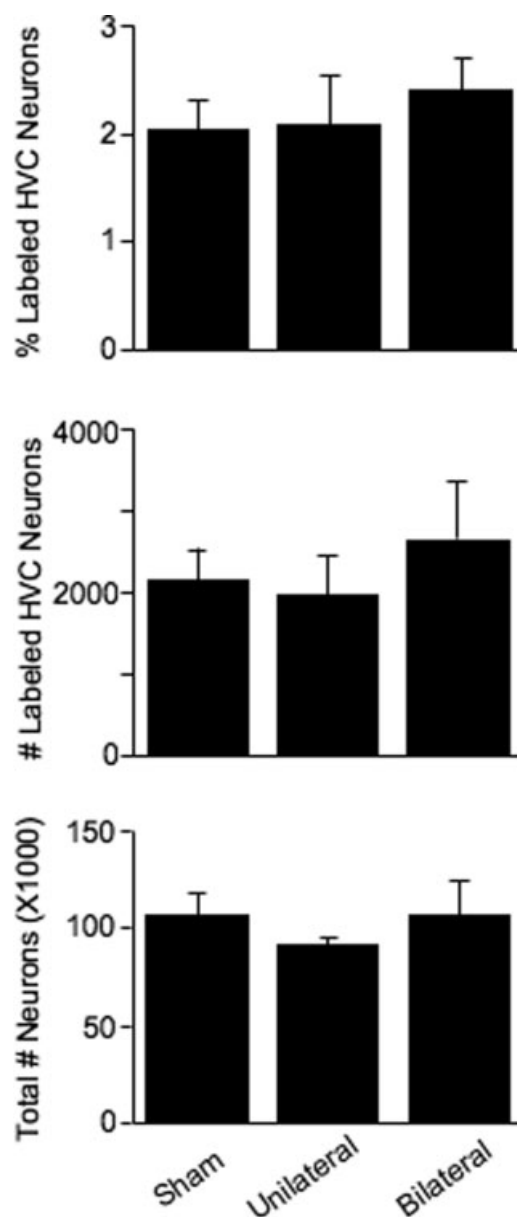


Figure 7 Neither bilateral nor unilateral LMAN lesions altered the percent of new neurons present in birds sacrificed 1 month after deafening and the onset of [^3H]thymidine injections (upper). LMAN lesions also did not alter the uncorrected number of thymidine labeled HVC neurons (middle) or the total uncorrected number of HVC neurons (lower) in this short survival treatment group. Bilateral lesions reduced each LMAN volume by $>50\%$ and extended through the caudal region of each nucleus. Animals with unilateral LMAN lesions met the same criteria for the right LMAN while the left LMAN had $<50\%$ damage. Data shown (mean + SEM) are for the right hemisphere.

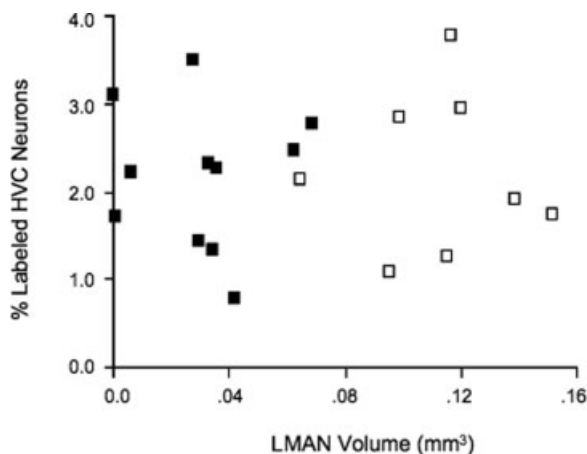


Figure 8 The volume of LMAN did not correlate with the percent of [^3H]thymidine-labeled neurons in the HVC in either LMAN-lesioned (black symbols) or sham-lesioned (open symbols) animals. Data shown are for the right hemisphere of animals sacrificed 1 month after deafening and the onset of [^3H]thymidine injections.

these birds LMAN volume was reduced on the left and right side by $33\% \pm 6\%$ (range: 11–49%) and $84\% \pm 10\%$ (range: 59–100%), respectively.

Neither bilateral ($n = 5$) nor right hemisphere ($n = 5$) LMAN lesions altered the recruitment of new neurons into the right HVC during the first month after deafening. As shown in Figure 7, the percent of HVC neurons that were thymidine-labeled in the short survival birds did not differ between birds with bilateral LMAN lesions, right hemisphere LMAN lesions, or sham lesions ($p = 0.74$). Likewise, the number of thymidine-labeled HVC neurons and the total number of HVC neurons did not differ between these groups ($p > 0.5$). Combining the unilateral and bilateral lesion groups, there was no correlation between right LMAN volume and the percent of thymidine-labeled neurons recruited into the right HVC 18 days after the last thymidine injection (Fig. 8). Also in sham-lesioned birds, there was no correlation between LMAN volume and the percent of thymidine-labeled HVC neurons.

A substantial proportion of new HVC neurons added in adult male zebra finches are RA-projection neurons (Wang et al., 1999). In all three of the short survival animals (two lesioned and one sham) that received fluorogold injections into RA 4 days prior to sacrifice, some of the retrogradely labeled HVC neurons were also thymidine-labeled. Two of these animals had $>97\%$ LMAN lesion ipsilateral to the fluorogold-labeled HVC-RA projections, indicating that new HVC neurons innervate RA even if virtually all of LMAN has been lesioned.

DISCUSSION

The results of this study indicate that the gradual accumulation of new, untrained HVC neurons does not inevitably disrupt the production of stable learned song patterns. Rather, the findings clearly indicate that when AFP function is compromised, adult song structure can withstand neuronal incorporation within the HVC, even when auditory feedback is completely absent. One interpretation of these results is that neurons incorporated into the adult HVC normally form behaviorally adaptive (or at least nondisruptive) connections without relying on instructive signals related to auditory feedback. Alternatively, newly incorporated HVC neurons may indeed be a crucial source for feedback-driven behavioral change, but require normal AFP function to realize that role.

Before discussing these interpretations further, it is important to consider how our results relate to other work that has demonstrated effects of experience on HVC neuron addition. Adult deafening alone has been reported to decrease HVC neuronal recruitment in male zebra finches (Wang et al., 1999), although this observation was not replicated in juvenile birds (Wilbrecht et al., 2002a). Whether or not these conflicting results reflect age-related differences in the effect of deafening has not been tested directly. We cannot speak directly to this point since our study focused exclusively on whether LMAN lesions affect HVC neuronal incorporation in the context of vocal plasticity induced by deafening, and thus all of the animals were deafened. Another manipulation that alters HVC neuronal recruitment is unilateral tracheosyringeal nerve cuts made prior to vocal learning. Unilateral nerve cuts at 26d posthatch produce a transient increase in HVC neuronal incorporation within the contralateral hemisphere of male zebra finches (Wilbrecht et al., 2002b). This effect is evident only among neurons born during the later part of sensorimotor learning (cell birth marker given 61–65d posthatch); it is not apparent among neurons born substantially earlier in song development, or in adults. Importantly, LMAN lesions made at the time of unilateral nerve cuts prevent changes in HVC neuron addition, while such lesions alone do not alter HVC neuronal incorporation. Since bilateral tracheosyringeal nerve cuts do not alter HVC neuron addition (but are more disruptive behaviorally than unilateral cuts), the authors suggest that the observed increase in HVC neuron addition following unilateral nerve cuts results from the contralateral hemisphere's increased participation in sensorimotor learning, and that LMAN lesions reduce the recruitment because such

lesions disrupt learning. Consistent with this view, deafening also blocks the induced unilateral increase in neuronal recruitment. In the current study, adult deafening deprived both hemispheres of the auditory feedback that would allow for HVC's participation in adult song maintenance. Thus, any "use related" increase in HVC neuron addition would not be expected in the paradigm employed in our study.

Because the incorporation of new HVC neurons in adult zebra finches is modest (Nordeen and Nordeen, 1988b; Alvarez-Buylla et al., 1990) and deafening may further reduce the rate of HVC neuronal incorporation and turnover (Wang et al., 1999), it is possible that adult-generated HVC neurons never comprise a large enough portion of circuitry to disrupt ongoing behavior, even in the absence of auditory feedback. However, while deafening reduces the initial incorporation of new HVC neurons, it also reduces the loss of those new neurons that are successfully incorporated (Wang et al., 1999). Since LMAN lesions can preserve stable song patterns for at least 1 year after deafening (Brainard and Doupe, 2000), it seems likely that even the low rate of neuronal incorporation evident in adult deafened birds would translate into an appreciable accumulation of new neurons within this time period. This supposition assumes that in adult deafened and/or LMAN-lesioned birds, new HVC neurons actually contribute functionally to song production. It is not known whether adult born HVC neurons actually convey premotor commands during song or participate in the robust ZENK induction observed among HVC neurons during song production. Another important question that deserves further attention in this context is whether HVC neuronal turnover involves all members of the RA-projecting and inter-neuronal populations, or whether the turnover is confined to only a subset of these neuron types.

It is possible that HVC neurons added in adult birds can insert appropriately into existing circuitry by molecular cues alone. For instance, new neurons could form roughly appropriate connections through cues provided by the dying neurons that they replace. In mouse cortex, targeted apoptosis leads to increased expression of brain derived neurotrophic factor (BDNF) expression in local GABAergic interneurons adjacent to degenerating neurons (Wang et al., 1998), and new neurons are attracted to apoptosing projection neurons and form connections with the targets of these neurons (Arlotta et al., 2003; Magavi et al., 2005). Within HVC, photolytic partial ablation of the HVC-RA pathway stimulates a robust increase in HVC neuronal incorporation that leads to replacement of the lost HVC-RA neurons and is accompa-

nied by a variable degree of song recovery (Scharff et al., 2000). Also, a singing-related increase in BDNF expression is associated with enhanced new neuron survival (Li et al., 2000). Chemotrophic cues may aid in roughing out the connections of adult generated HVC neurons and molecular signaling from RA could guide new HVC-RA neurons to form connections organized by topography, or firing order during song production. Indeed, even early deafening does not appear to prevent the development of topography in several of the song control pathways (Iyengar and Bottjer, 2002).

While our results indicate that the incorporation of new HVC neurons is not sufficient to drive behavioral change after deafening, plasticity afforded by these new cells could be necessary for, or accelerate, such change. For instance, the ongoing recruitment of new neurons, expressing immature molecular phenotypes that favor synaptic change, may provide a critical substrate for the active adjustment mechanisms mediated by the AFP. In adult hippocampus, plasticity is enhanced among newly generated granule cells, perhaps owing to their paucity of fast inhibitory inputs and larger Ca^{2+} spikes (Wang et al., 2000; Snyder et al., 2001; Schmidt-Hieber et al., 2004). In the adult rodent visual system, complete visual deprivation promotes a reemergence of juvenile patterns of NMDA receptor expression and GABAA/AMPA receptor ratios that are hypothesized to enable the reemergence of ocular dominance plasticity (He et al., 2006). Although adult deafening has not been associated with any overall changes in the expression pattern of NMDAR subunits within the song system (Scott et al., 2004), young HVC-RA projection neurons could express phenotypes that favor synaptic change, and be a preferred target for AFP-derived molecular signals that promote plasticity when feedback fails to meet expectations. One such signal could be BDNF. The recruitment of new HVC neurons is fostered by this tropic factor (Li et al., 2000), it is transported anterogradely from LMAN to RA (Johnson et al., 1997), and can provoke changes in song behavior when infused directly into the RA (Kittelberger and Mooney, 2005). It is possible that BDNF could be especially effective at promoting synaptic changes at the nascent connections being formed by new HVC-RA neurons. Adult deafening also results in a transient rise in protein kinase C expression within RA (Watanabe et al., 2002), a biochemical change implicated in various forms of plasticity (Akers et al., 1986; Kano et al., 1995; Neill et al., 2001). Interestingly, this molecular response also is blocked by LMAN lesions (Watanabe et al., 2006).

Finally, preliminary work suggests that adult deafening also increases the expression of the immediate early gene *Arc* within RA (Horita et al., 2005), another genetic signaling event that may foster synaptic remodeling (Steward and Worley, 2002). It will be important to determine if these molecular changes are restricted to, or exaggerated within the recently recruited HVC-RA projection neurons.

Another possibility is that new HVC neurons added in the absence of auditory feedback actually cause the AFP to send the error correction signal that permits or instructs change. For example, one working hypothesis is that during song development, a sensory-motor map is established within HVC that links specific patterns of premotor activity in the HVC-RA neurons to corresponding auditory feedback received by HVC circuitry (Troyer et al., 1996; Troyer and Doupe, 2000; Troyer and Bottjer, 2001). When song is produced, a corollary discharge of the premotor neural activity is conveyed to the AFP through the HVC-Area X projection, and this information constitutes an “expected auditory feedback” signal. A comparison between this signal and the stored template determines whether the AFP should produce a corrective or plasticity-permissive signal that would promote plasticity within the vocal motor pathway. When auditory feedback is absent or altered, the accumulation of naïve HVC neurons could drive alterations in the sensory-motor map within HVC, in turn altering the corollary discharge signal sent to the AFP. Normally this would provoke behavioral change, but LMN lesions would restrict such change by preventing AFP-generated error correction signals from reaching the vocal-motor pathway. A complete understanding of how neuronal recruitment in adulthood contributes to natural or induced vocal plasticity will require a means of attenuating and/or augmenting HVC neuronal turnover and measuring parallel changes in the rate of behavioral change.

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