Hemispheric Coordination Is Necessary for Song Production in Adult Birds: Implications for a Dual Role for Forebrain Nuclei in Vocal Motor Control

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Ashmore RC, Bourjaily M, Schmidt MF. Hemispheric coordination is necessary for song production in adult birds: implications for a dual role for forebrain nuclei in vocal motor control. J Neurophysiol 99: 373–385, 2008. First published October 31, 2007; doi:10.1152/jn.00830.2007. Precise coordination across hemispheres is a critical feature of many complex motor circuits. In the avian song system the robust nucleus of the arcopallium (RA) plays a key role in such coordination. It is simultaneously the major output structure for the descending vocal motor pathway, and it also sends inputs to structures in the brain stem and thalamus that project bilaterally back to the forebrain. Because all birds lack a corpus callosum and the anterior commissure does not interconnect any of the song control nuclei directly, these bottom-up connections form the only pathway that can coordinate activity across hemispheres. In this study, we show that unilateral lesions of RA in adult male zebra finches (Taeniopigia guttata) completely and permanently disrupt the bird’s stereotyped song. In contrast, lesions of RA in juvenile birds do not prevent the acquisition of normal song as adults. These results highlight the importance of hemispheric interdependence once the circuit is established but show that one hemisphere is sufficient for complex vocal behavior if this interdependence is prevented during a critical period of development. The ability of birds to sing with a single RA provides the opportunity to test the effect of targeted microlesions in RA without confound of functional compensation from the contralateral RA. We show that microlesions cause significant changes in song temporal structure and implicate RA as playing a major part in the generation of song temporal patterns. These findings implicate a dual role for RA, first as part of the program generator for song and second as part of the circuit that mediates interhemispheric coordination.

INTRODUCTION

Voluntary behaviors in vertebrates are controlled, or modulated, by motor control regions in the telencephalon (Girard and Berthoz 2005; Hoshi and Tanji 2007; Jürgens 2002; Suthers and Margoliash 2002). Although some behaviors, such as language, might be controlled primarily by a dominant specialized hemisphere (Foundas 2001; Geschwind 1970), others often require fine-tuned coordination between hemispheres (Carson 2005; Kelso et al. 1979; Schmidt and Ashmore 2007; Suthers 1997). Although cortico-cortical projections are likely to play an important role in interhemispheric coordination (Brinkman and Kuypers 1973; Donchin et al. 1998), other mechanisms involving a bottom-up coordinating role from thalamus or brain stem might be equally important. Evidence for these alternative coordination mechanisms has been described in the avian song system (Schmidt and Ashmore 2007) and also in the mammalian eye saccade system, where interhemispheric communication in split-brain monkeys can occur in the complete absence of forebrain commissures (Berman et al. 2005).

The presence of hemispheric coordination in a neural system implies that motor control areas that are involved in such coordination, even if they do not contain direct contralateral projections, play a dual role in controlling motor output. At one level, output signals instruct downstream motor structures, whereas at another level, these structures transmit signals that serve to coordinate both hemispheres. Characterizing and differentiating between these different signals is difficult in mammalian brains because coordination can occur through either ascending bilateral brain stem systems or direct cortico-cortical influences. Manipulations, such as lesions or transections, that target only one of these coordination pathways, as has been performed in the eye saccade system (Berman et al. 2005), may therefore yield ambiguous results because the unaffected pathway might compensate for any induced impairment.

Song production in birds is an example of a behavior that requires finely tuned coordination between hemispheres (Schmidt 2003; Schmidt et al. 2004; Williams 1985) and might be ideally suited for investigating the neural mechanisms of hemispheric coordination. Specifically, forebrain, thalamic, and brain stem nuclei responsible for song production, known collectively as the song system, are duplicated in both halves of the brain (Nottebohm and Arnold 1976; Striedter and Vu 1998; Wild 1997; Wild et al. 2000), but the telencephalic nuclei are not connected across the midline even by the anterior commissure, which is the only inter-hemispheric commissure present in birds. Projections influencing song system nuclei in the opposite hemisphere originate from either the bilateral projections of the dorsomedial posterior nucleus of the thalamus (DMP) (Vates et al. 1997) or the thalamic nucleus uvaformis (Uva), to which certain brain stem vocal-respiratory nuclei project bilaterally (Reinke and Wild 1998; Striedter and Vu 1998). Based on their physiological and anatomical properties, these bilateral projections are well placed for mediating the coordination and synchronization observed during song production (Ashmore et al. 2005; Schmidt et al. 2004; Wild et al. 2000). The song system therefore provides a powerful model system for investigating the mechanisms of interhemispheric coordination that are not mediated by direct commissural projections at the forebrain level. By extension, it also provides a model for characterizing the different types of signals that might be sent by nuclei with projections to both peripheral...
motor nuclei and forebrain targets of recurrent bilateral signals from the brain stem.

One forebrain nucleus that may perform such a dual role is the robust nucleus of the arcopallium (RA) (Vicario 1991; Wild 1993, 1997). RA forms part of the descending motor pathway in each hemisphere, receives inputs from HVC (used as a proper name) and the lateral magnocellular nucleus of the anterior nidopallium (LMAN), and sends independent output projections to nuclei controlling the muscles of the vocal organ (nXIIIs) and brain stem nuclei that play an important role in vocal-respiratory control (nucleus retroambigualus, RAm; nucleus paraambigualus, PAm; the dorsomedial nucleus of the intercollicular complex, DM; see Fig. 1). These brain stem structures are connected, directly or indirectly, across the midline, and two of these nuclei (PAm and DM) project back to the forebrain vocal control nucleus HVC, via Uva. RA activity from each hemisphere is therefore likely integrated at the level of these brain stem vocal-respiratory nuclei before being processed and relayed back to the forebrain (Schmidt and Ashmore 2007; Vu et al. 1994). In addition, RA also projects to the thalamic nucleus DMP, which projects bilaterally back to HVC via the forebrain nucleus MMAN (Foster and Bottjer 1993; Vates and Nottebohm 1995).

In this study, we investigated whether RA contributes to song production by participating in both interhemispheric coordination and ipsilateral motor program generation. To do this, we made lesions of RA in adults and juveniles and assessed the effects of these lesions on song. We found that complete unilateral lesions of RA in adult birds completely disrupted song structure and reduced but did not completely eliminate song attempts. This suggests that the remaining hemisphere is incapable of producing normal song in the absence of input from the contralateral RA. In contrast to adult birds, similar lesions performed in juvenile birds before they learn to sing did not prevent the later acquisition of normal song vocalizations. These results suggest interdependence between the two hemispheres in normal adults, that this interdependence is mediated in part by activity originating in RA, and that the interdependence is established during the critical developmental period for song acquisition. To test the contribution of RA to ipsilateral motor production in a manner that could be isolated from its role in interhemispheric coordination, we performed targeted microlesions in the remaining RA of adult birds that had received complete unilateral RA lesions as juveniles. Our findings suggest that RA, in addition to mediating interhemispheric coordination, also plays a significant ipsilateral role in establishing the song temporal structure, rather than simply following motor commands about temporal sequence from HVC.

**FIG. 1.** Unilateral lesions of the robust nucleus of the arcopallium (RA) in songbirds. A: diagram of the avian song system, showing the functional effect of a complete unilateral lesion of RA. Such a lesion completely eliminates all activity from the ipsilateral HVC or the anterior forebrain pathway from reaching brain stem vocal-motor structures (nucleus retroambigualus (RAm), nucleus paraambigualus (PAm), dorsomedial nucleus of the intercollicular complex (DM), and nuclei controlling the muscles of the vocal organ (nXIIIs) and thereby functionally eliminates any contribution that hemisphere might have on song production. The anatomical connections shown in this diagram represent the major projections in the song system and have been compiled from a number of different sources (Nottebohm et al. 1982; Reinke and Wild 1998; Stokes et al. 1974; Striedter and Vu 1998; Sturdy et al. 2003; Vates et al. 1997; Wild 2004). Weak projections have been left out. DLM, dorsal lateral nucleus of the medial thalamus; DMP, dorsomedial posterior nucleus of the thalamus; LMAN, lateral magnocellular nucleus of the anterior nidopallium; MMAN, medial magnocellular nucleus of the anterior nidopallium; Area X, Area X of the medial striatum; NIf, nucleus interfacialis of the nidopallium. B: cresyl violet stained parasagittal section from an adult zebra finch that received an RA lesion as a juvenile. Left: intact RA; right: results of ibotenic acid lesions of RA. Scale bar = 1 mm.
Methods

Animals

Adult male zebra finches (Taeniopygia guttata) ranging from 120 to 500 days of age were obtained from our breeding colony and from a local supplier. Juvenile male zebra finches ranging from 45 to 60 days of age were obtained from our breeding colony. Birds were housed under constant 12:12 light:dark conditions and given food and water ad libitum. All procedures described here were approved by an institutional animal care and use committee at the University of Pennsylvania.

Unilateral RA lesions

We performed unilateral RA lesions in two sets of birds. One set consisted of adults the song of which had been previously recorded (n = 6). The other set consisted of juvenile birds around the onset of the sensorimotor period of song learning (n = 23). This developmental time period (posthatch day 45–60) follows a point where HVC is known to already innervate RA (Mooney and Rao 1994), resulting in the presence of identifiable neural activity in RA, which precedes extensive practice of song (Immelmann 1969; Konishi and Akutagawa 1985). Under ketamine/xylazine anesthesia, each bird received unilateral injections of ibotenic acid directed at either the left or right RA. Injection sites were identified using extracellular recordings to confirm characteristic neural activity in RA. Three or four injections of ibotenic acid were made to maximize the extent of the lesion. Injections were made using a Hamilton syringe (Hamilton, Reno, NV), and the total amount of ibotenic acid injected ranged from 1.0 to 2.0 µl of a 6.6-mg/ml solution, pH 7.0–8.0. Lesions were verified by frozen section histology and cresyl violet staining. The lesions ranged in extent from those that were very specific to the RA region, to those encompassing much of the caudal portion of the targeted hemisphere.

Electrical microstimulation

Methods for chronic implantation of electrodes and stimulation during song are described in detail in Ashmore et al. (2005). In the present study, we implanted electrodes bilaterally in HVC of six adult birds that had previously received unilateral lesions of RA as juveniles. All birds were between 120 and 200 days posthatch at the time of implant. Electrode locations were chosen based on intrinsic activity in HVC evident under anesthesia. Despite the absence of one RA, due to unilateral lesion, typical bursting was seen in HVC in both hemispheres. To determine the significance of the number of song- and syllable-level effects seen with stimulation, the proportion of effects in songs that received stimulation was compared with the proportion of effects scored in control songs (0 µA, no stimulation data). The comparison was performed using a heterogeneity G-test (Sokal and Rohlf 2001).

Targeted RA microlesions

In 15 adult birds that had received RA lesions as juveniles, we recorded song for a period ranging from 7 to 15 days. Birds were then anesthetized with a mixture of ketamine and xylazine (see preceding text). Lesions were targeted to either the dorsal (n = 8) or ventral (n = 7) region of either the left or right RA. Lesions were made by passing 10 or 15 µA of DC through the recording electrode for periods ranging from 30 to 60 s. Birds with ventral and dorsal lesions received a similar distribution of these lesion parameters. Lesion locations and extent were verified by frozen section histology. Lesions ranged in size from ~50 to 250 µm in diameter, comprising 0.2–4% of the estimated volume of each RA.

Analysis of song before and after complete RA lesions

To test the effect of complete unilateral RA lesions in adult birds, we compared songs before and after lesions. Because postlesion songs consisted primarily of introductory notes or other vocal units that did not match prelesion syllables, we were unable to perform meaningful comparisons of individual syllables or stereotypy. Instead, we quantified the difference in syllable structure by determining the number of postlesion vocal units (introductory notes, syllables, and calls) during singing attempts that could be matched to prelesion syllables. To do this, we employed a classifier based on the Mahalanobis distance of vocal units from sample sets of syllables, where each vocal unit was defined by four acoustic parameters: pitch, acoustic entropy, FM, and duration. These parameters were calculated using the Sound Analysis software package (Tchernichovski et al. 2000). For each bird, the sample sets were computed for canonical prelesion syllables (excluding introductory notes and calls). A classification threshold for each syllable was determined by first calculating the mean distance of each sample syllable from its own sample set (each instance of syllable ‘A’ compared with all ‘A’s for 1 bird). The mean distance plus 2 SD was chosen as the threshold. The classifier was applied to all vocal units (including introductory notes and calls) in a set of prelesion songs and then to all vocal units in a set of postlesion songs. The postlesion vocalizations were taken from the first singing attempts within 10 days of the lesion that had sufficient amplitude to allow calculation of acoustic parameters.

To verify that songs were temporally, and not just acoustically, degraded after complete adult RA lesions, we also examined vocal unit durations in more detail. One possibility was that overall syllable timing remained intact after lesions, which would preserve vocal unit durations, but that vocal units failed identification or classification due to acoustic feature degradation. Therefore we compared the distributions of vocal unit lengths before and after lesions. Because the duration distributions were not normally distributed, we performed comparisons using a ranked sum test (Mann-Whitney). In one case, we also compared the ratio of introductory notes to all vocal units per singing attempt, and tested for significance using a nonpaired Student’s t-test.

To test the effect on adult song of RA lesions performed in juvenile birds, adult songs of 12 birds with juvenile RA lesions were compared with songs of six randomly selected songs from normal adult males. Acoustic features were analyzed using the Sound Analysis software package (Tchernichovski et al. 2000) and included duration, acoustic entropy, minimum pitch, maximum pitch, and FM. Stereotypy was calculated as previous described (Scharff and Nottebohm 1991). To quantify the production of introductory notes, we computed the ratio of the number of introductory notes appearing in songs to the total number of vocal units (introductory notes, syllables, and calls) for each bird. These were compiled into mean proportions and compared via Student’s t-test.

Analysis of song before and after targeted RA microlesions

For targeted RA microlesions, songs were analyzed on specific days (baseline prelesion, 1 day prior to lesion, 1st day of song production after the lesion, and 7 days postlesion). For each day analyzed, 50 songs were selected at random throughout the day. If <50 songs were produced, all songs for a given day were used for analysis. For a given set of songs, we calculated the number of introductory notes distributed throughout each song. As with complete juvenile lesions (see preceding text), we computed the ratio of introductory notes to all vocal units produced during song (syllables, introductory notes, and calls).

For each day, we also calculated the probability of a song bout ending with a given vocal unit. These vocal units could include syllables, introductory notes or calls. Thus for each day we generated a profile of ending “vocal unit” probability distributions. To compare
across days, we calculated the Euclidean distance between these distributions in $n$-dimensional space, where $n$ represented the total number of possible ending vocal units. Because we wanted to maintain the same scale in each dimension, we chose to use simple Euclidean distance rather than a scale-invariant measure (such as the Mahalanobis distance). We analyzed these distributions before and after lesions in each bird by performing a chi-square test between the baseline day and each subsequent day (df = number of vocal units – 1).

**Histology**

Birds were deeply anesthetized with 0.1 ml of 50 mg/ml pentobarbital sodium (Nembutal; Abott Laboratories, Abbott Park, IL) and perfused with 0.9% saline and 4% paraformaldehyde. Brains were cryoprotected in 30% sucrose and sectioned at 40 μm on a freezing microtome. Sections were cut parasagitally for verification of forebrain electrode placement and for lesions of RA.

**RESULTS**

**Complete unilateral lesions of RA in adults eliminate song production**

The motor circuit for song production consists of two parallel descending pathways, one in each hemisphere, that connect the forebrain (HVC and RA) to brain stem nuclei that control respiration (RAm, PAm, and DM) and syringeal muscles (nXIIts). We assessed the interdependence of these pathways in song production by unilaterally lesioning RA and examining the effects on song production. We performed complete unilateral ibotenic acid lesions of RA in either hemisphere ($n = 3$ right and 3 left) of six adult male zebra finches (Fig. 1). RA lesions in adult birds caused dramatic impairment of song production in all birds (6/6). In all cases, birds that sang normal songs before the lesions were unable to produce stereotyped renditions of these songs after the lesions (Fig. 2, Table 1). For two birds (with right RA lesions, *birds 1* and 2 in Table 1), no singing attempts were recorded for ≤10 days postlesion. The third bird with a right RA lesion made numerous singing attempts, but these attempts consisted primarily of long strings of introductory notes, occasionally ending with one poorly formed syllable (Fig. 2A, *bird 6* in Table 1). The three birds with left RA lesions all sang within 10 days postlesion, but these songs consisted of introductory notes and syllable-like vocal units that could not readily be matched to prelesion syllables (Fig. 2B).

To quantify the effects of RA lesion on song, we determined the number of prelesion and postlesion vocal units (including introductory notes and intrasingo calls) that could be matched to prelesion syllables (excluding introductory notes and calls) using a classifier based on acoustic features (pitch, acoustic entropy, FM, and duration; see METHODS for details). We found that 59.7 ± 14.4% of all vocal units of songs compared prior to the RA lesion could be matched to a canonical set of syllables ($n = 810$ of 1,401 vocal units in 6 birds). This contrasts to the same analysis performed after the lesion where only 5.7 ± 6.4% of vocal units could be classified ($n = 121$ of 1254 vocal units in 6 birds, $P < 0.005$, paired t-test).

All postlesion songs appeared degraded in their temporal structure but the inability to identify individual syllables prevented any rigorous comparison of song stereotypy before and after lesion. We therefore decided to assess potential degradation of temporal structure by quantifying changes in vocal unit duration without consideration for vocal unit identity. The reasoning for such analysis was that songs that are temporally intact but acoustically degraded (i.e., the bird sang the same syllables but with acoustic distortion) would show a similar distribution of vocal unit duration before and after RA lesions. For three of the four birds that sang in the 10-day postlesion period, the difference in the distribution of vocal unit duration was highly significant, indicating that different vocal units were produced following the lesion ($n = 1,013$ vocal units in 3 birds, $P < 0.0005$, Mann-Whitney). For one bird (*bird 6* in Table 1, Fig. 2A), the distributions were not different ($n = 282$ vocal units, $P = 0.16$, Mann-Whitney) because the song consisted almost entirely of introductory notes with occasional renditions of a vocal unit that matched a prelesion syllable in length. To verify that song sequence structure had nonetheless been degraded for this bird, we calculated the ratio of introductory notes to vocal units produced in a set of prelesion songs and compared this to postlesion song ratios and found that they were significantly different ($P < 0.0005$, nonpaired t-test). We also recorded singing attempts from this bird at later time points. At 63 days postlesion, we found no recovery of song structure. In fact the bird no longer produced the vocal unit that matched the prelesion syllable and songs consisted primarily of introductory notes intermixed with occasional calls. For this time point, the distribution of vocal unit durations was significantly different from the prelesion distribution ($n = 102$ vocal units, $P < 0.0005$, Mann-Whitney). By 334 days postlesion, the bird was still attempting to sing but had not yet recovered his normal song structure (Fig. 2A).

We also recorded singing attempts for one of the birds that did not initially sing during the first 10 days (*bird 2* in Table 1). At 60 days postlesion for this bird, songs consisted primarily of introductory notes and occasional calls, with no evidence of any prelesion song syllables. At 220 days postlesion, some temporal structure had begun to develop in the form of more consistent temporal spacing between introductory notes. However, no complete motif structure was evident in these vocalizations. Thus for both birds, song recovery was not seen even after many months.

**Complete unilateral lesions of RA in juveniles do not prevent acquisition of normal song**

The effect of RA lesions in adult birds suggests that left and right descending motor pathways are interdependent. To test whether this dependence is established during juvenile song maturation, we performed complete unilateral ibotenic acid lesions of RA in juvenile birds during the sensorimotor period of song development (45–60 days posthatch). All of the lesions ($n = 23$) were extensive, and always included all of RA. Lesions typically spread beyond RA and included sections of the surrounding arcopallium. In a subset of these ($n = 2$), lesions also impacted the ventral nidopallium, although in these birds the nucleus HVC was still visibly intact. Despite the extensive damage done by the injections, however, and in contrast to adult lesions, all birds receiving juvenile RA lesions eventually acquired adult songs that were generally indistinguishable from those produced by intact adult control birds (Fig. 3).
To compare songs of adult birds having received RA lesions as juveniles to normal adult songs, we quantified several song features in a subset of juvenile lesioned birds (n/H11005 429 songs in 12 birds) and compared them to a randomly selected pool of intact adult control songs (n/H11005 124 songs in 6 birds). Motifs of adult zebra finches that had received RA lesions as juveniles contained the same overall number of unique syllables (4.6 /H11006 1.2) as normal adult birds (5.0 /H11006 1.1, NS, t-test). Many of the syllables consisted of complex conjunctions of multiple notes typically observed in normal zebra finches. The proportion of introductory notes in a given song (Fig. 3B) was not different from intact controls (RA-lesioned: 22 /H11006 8%, intact: 18 /H11006 6%, NS; 2-tailed unpaired t-test) and estimates of singing rate showed a variable but species-typical rate of song production (32.7 /H11006 29.3 song/hr during the day for RA-lesioned birds vs. 22.0 /H11006 15 song/hr for controls, NS, 2-tailed unpaired t-test).

To assess the possible effects of juvenile RA lesions on syllable acoustic structure, we also compared several key acoustic features between both groups. On average, the syllables of RA lesioned birds did not differ significantly from intact birds in duration, entropy, FM, and peak frequency.
measures (see Methods). Syllables of lesioned birds differed slightly but significantly from controls, however, in their pitch or fundamental frequency. Song syllables of lesioned birds tended to have a lower maximum pitch ($P < 0.005$, 2-tailed unpaired $t$-test) and higher minimum pitch ($P < 0.01$, 2-tailed unpaired $t$-test), indicating that these birds were not able to achieve the same dynamic range as their control counterparts (Fig. 3D).

Song production under the control of a single hemisphere

Having established that adult birds with juvenile unilateral RA lesions sang normal songs, we wanted to verify that throughput from HVC to the periphery on the lesioned side was functionally eliminated. To do this, we chronically implanted stimulating electrodes in each HVC of adult birds having received unilateral RA lesions as juveniles. One set of electrodes was placed contralateral to the lesion (intact side) and one ipsilateral to the lesion (lesioned side). We chose HVC because it lies upstream of RA, and previous studies have shown that HVC stimulation during singing will interrupt song production. In this analysis, we measured latency only for stimulation in RA ipsilateral to the RA lesion had no effect.

Consistent with previous results, low-intensity ($15–60 \mu A$) stimulation during singing in HVC on the intact side disrupted both the production of individual syllables and the structure of the whole song. Syllable-level effects included acoustic distortion of the ongoing syllable or premature truncation of the syllable. Song-level effects included stopping the song completely after stimulation (stop) or a premature stopping of the song sequence followed by a restart from the beginning of the motif (restart). These effects from intact-side HVC stimulation were similar to those observed after HVC stimulation in normal birds (Fig. 4). By contrast, stimulation $\leq 100 \mu A$ delivered in HVC ipsilateral to the RA lesion had no effect. Because this level of stimulation will always result in song disruption (in an intact hemisphere or in birds without forebrain song system lesions), this result is consistent with a complete elimination of motor output originating in HVC in that hemisphere.

Compiled results are shown in Fig. 4 for a total of 4,255 songs in six birds. Stimulation of $15–60 \mu A$ in the HVC contralateral to the RA lesion (on the intact side) caused syllable-level effects in 45.3% of the songs and song-level effects in 55.1% ($n = 1,926$ and 2,345 of 4,255 songs in 6 birds). The proportion of these songs showing syllable-level effects was significant at stimulation intensities of 30, 45, and $60 \mu A$ ($P < 0.001$; $G$-test), when compared with a control set of songs with no stimulation. For instance, $45 \mu A$ stimulation caused syllable distortion in only 5.6% of songs (74 of 1,333 songs in 6 birds) but caused 40.3% of songs to truncate (573 of 1,333 songs; Fig. 4B). This level of stimulation also caused restarting in 48.2% of songs (642 of 1,333 songs), and stopping in 17.9% (239 of 1,333 songs; Fig. 4C).

Although these stimulation results were qualitatively similar to those seen for HVC stimulation in normal birds (Ashmore et al. 2005), HVC stimulation in RA-lesioned birds produced three notable differences. First, the ratio of truncations to distortions was higher for the RA-lesioned group. Lesioned birds had nearly three times as many truncations as distortions (ratio $= 2.75$; $n = 531$ truncation/193 distortions of 1,275 songs in 6 birds), whereas normal birds had more distortions than truncations (ratio $= 0.59$; $n = 171$ truncation/289 distortions of 684 songs in 4 birds). The same trend was also seen for 30- and 60-$\mu A$ stimulation.

Second, both groups showed a similar percentage of song-level effects (Fig. 4C), but the distribution of these effects between restarting the motif and stopping it altogether was different. Stimulation in the RA-lesioned group caused motif restarts to occur almost three times as often as stops (ratio $= 2.69$; $n = 622$ restart/231 stops of 853 songs in 6 birds), whereas for the normal group this distribution was more even (ratio $= 1.21$; $n = 279$ restart/230 stops of 509 songs in 4 birds). Results for 30 and 60-$\mu A$ were similar.

Finally, we found that the latency to produce an effect was shorter for RA-lesioned birds. Latency to syllable truncation was used as an indicator of how rapidly stimulation affected song production. In this analysis, we measured latency only for truncations that preceded a restart of the motif. The mean latency for the RA-lesioned birds was $53.9 \pm 7.0$ ms ($n = 338$ songs in 6 birds), whereas the previously reported mean latency for the intact birds was $78.9 \pm 15.4$ ms ($n = 73$ songs in 4 birds). This latency was significantly shorter ($P < 0.01$, NANOVA) and less variable ($P < 0.001$, $F$ test) for the

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**TABLE 1. Summary of adult RA lesions**

<table>
<thead>
<tr>
<th>Lesion Site</th>
<th>Sang Before 10 d</th>
<th>Classified Units, %</th>
<th>Mean Duration, ms</th>
<th>Intro Note Quantity</th>
<th>Other Recorded Times, days</th>
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<tbody>
<tr>
<td>Bird 1</td>
<td>Right</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bird 2</td>
<td>Right</td>
<td>No</td>
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<td>pre 43</td>
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<tr>
<td></td>
<td></td>
<td>post 15</td>
<td>76±44</td>
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<td>Yes</td>
<td>pre 53</td>
<td>135±65</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>post 13</td>
<td>113±5</td>
<td>$P &lt; 0.0005$</td>
<td></td>
</tr>
<tr>
<td>Bird 5</td>
<td>Left</td>
<td>Yes</td>
<td>pre 55</td>
<td>153±97</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>post 2</td>
<td>91±34</td>
<td>$P &lt; 0.0005$</td>
<td></td>
</tr>
<tr>
<td>Bird 6</td>
<td>Right</td>
<td>Yes</td>
<td>pre 52</td>
<td>115±85</td>
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<tr>
<td></td>
<td></td>
<td>post 5</td>
<td>91±34</td>
<td>[$P = 0.16$, 27±13%]</td>
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<td>[$P = 0.0005$, 80±15%]</td>
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</table>

Results from unilateral lesions of RA in 6 adult birds. Site: left or right side lesion. Sang before 10 d: whether or not the bird made song attempts within 10 days after the lesion. Classified units: the percent of vocal units that could be matched to a canonical set of syllables based on acoustic features. Mean duration: the mean length in milliseconds of all vocal units, ± SD, followed by a column indicating the significance level of the pre- versus post-lesion comparison of vocal unit duration distributions (Mann-Whitney). Intro note quantity: the mean ± SD of the percentage of introductory notes comprising all vocal units in each singing attempt, followed by the significance of the comparison (unpaired $t$-test). The last column indicates later post-lesion days when singing attempts were recorded for two of the birds.
Targeted microlesions in RA of unihemispheric birds reveal the nature of RA motor commands

Birds with only one intact descending motor pathway (after juvenile RA lesions) provided us with a unique system to probe RA function using targeted microlesions. With the contralateral RA removed, the results of small lesions in the isolated intact RA could be evaluated without any confound of compensation from the contralateral side and in isolation from RA’s role in interhemispheric coordination. We performed targeted single electrolytic microlesions to either the dorsal or ventral region of RA (Fig. 5) in a total of 15 birds. Subsequent histology revealed that these lesions ranged in size from ~50 to 250 μm in diameter. In all cases, lesions of the ventral or dorsal RA disrupted song by degrading the temporal structure and production of motifs (Fig. 6). Birds with the most pronounced behavioral deficits (n = 6/15) did not produce any song for one or more days after the lesion before eventually making song attempts within a 7-day period. In other cases (n = 2/15), birds sang the day after the lesion but with a complete disruption of song sequence. In the remaining birds (n = 7/15), song was at least partly intact, with only minor disruptions. Despite the range of lesion sizes, no significant correlation was seen between lesion extent and metrics of song deficit. All disrupted songs were typified by the inclusion of additional introductory notes, sometimes to the exclusion of normal song syllables (Fig. 6A). These introductory notes were sometimes punctuated by calls or by other syllables that could not be matched to the bird’s repertoire of syllables prior to lesion. Some birds also produced songs that ended on syllables that were atypical for ending song bouts prior to lesion.

To quantify song deficits after targeted microlesions in RA, we first examined the number of introductory notes produced throughout singing attempts and computed ratios of the number of introductory notes relative to the number of overall vocal units (syllables, introductory notes, and calls) for a given day. We collected these values for a baseline day (baseline), for the day immediately prior to the lesion, for the first day of song production following the lesion, and for the seventh day after the lesion (postlesion 7; Fig. 5B). These ratios are shown in Fig. 6B, normalized to the proportions for the baseline day. For lesions targeted to either the dorsal or ventral RA, no significant difference was seen between lesion extent and metrics of song deficit. All disrupted songs were typified by the inclusion of additional introductory notes, sometimes to the exclusion of normal song syllables (Fig. 6A). These introductory notes were sometimes punctuated by calls or by other syllables that could not be matched to the bird’s repertoire of syllables prior to lesion. Some birds also produced songs that ended on syllables that were atypical for ending song bouts prior to lesion.

RA-lesioned birds also impaired the ability to maintain song

RA-lesioned birds than the normal birds (syllables from 30- and 45-μA stimulation combined, Fig. 4D). This longer truncation latency for normal birds may reflect a delay for stimulation to affect the unstimulated hemisphere, allowing this side to continue driving song production longer than in the RA-lesioned set.

FIG. 3. Normal song acquisition after unilateral lesions of RA in juvenile birds. A: sample song of an adult bird (>100 days posthatch) that received a unilateral ibotenic acid lesion of RA as a juvenile (~ posthatch day 50). Songs from all birds receiving such treatment were qualitatively identical to nonlesioned control birds. B: proportion of introductory notes during song bouts. The amount of introductory notes appearing in songs is shown as a percentage of all vocal units produced during singing bouts for 12 birds with juvenile RA lesions and 6 intact control birds. No significant difference is seen between the 2 groups. C: song stereotypy. Quantification of syllable sequence similarity (Scharff and Nottebohm 1991) across songs for any given individual having received a unilateral RA lesion as a juvenile did not show any difference in the level of song stereotypy when compared with the intact control group. D: RA lesioned birds show a slight decrease in the dynamic range of syllable acoustic features. Minimum acoustic pitch (the mean of the minimum pitch across all syllables for each bird) is plotted against maximum acoustic pitch for juvenile lesioned birds (•) and the set of intact control birds (●). Both measures were significantly different between groups, as reflected by the tendency to cluster separately in this plot.
once it was started. Two striking examples of such an effect are shown in Fig. 7A. Prior to RA microlesions, most songs ended on one of two syllables, but lesions completely changed this distribution with songs much more likely to end on syllables that were low probability ending syllables prior to the lesion. To quantify this effect, we calculated the percentage of times that songs produced within a given day ended on different syllables, calls, or introductory notes. We compared this “ending syllable distribution” of songs recorded on postlesion days with songs much more likely to end on one of two syllables, but lesions completely changed this.

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with dorsal lesions were significant for both postlesion days ($n = 8$, $P < 0.05$ for both, 1-tailed paired $t$-test), but only the first postlesion day was significant for the ventral lesion group ($n = 7$, $P < 0.05$, 1-tailed paired $t$-test). As with the distribution of introductory notes, the postlesion day 7 values for dorsal and ventral lesions showed a trend toward prelesion levels, although the dorsal group remained significantly higher.

To determine which individual subjects contributed to these results, we compared the distribution of ending syllables for each time point for each bird. No birds showed a significant change in distribution between baseline and the day prior to lesion. For the dorsal lesion group, seven of eight (88%) showed significant changes on one or both postlesion days compared with baseline ($0.0005 < P < 0.05$, $\chi^2$). For the ventral lesion group, only four of seven (57%) showed significant changes on postlesion days ($P < 0.05$, chi-square). Taken together, these results suggest that dorsal RA lesions were more likely to disrupt the ending syllable distribution than lesions in ventral RA.

FIG. 6. Effects on song of targeted microlesions of RA in adult birds with complete lesions of the contralateral RA as juveniles. A: examples of song attempts from 2 birds before and after targeted microlesions in the dorsal (top) and ventral (bottom) regions of the intact RA. i, introductory notes; x, call-like syllables (harmonic stacks) that could not be matched to any syllables in the prelesion song. B: proportion of introductory notes comprising songs for birds with dorsal ($n = 8$) and ventral ($n = 7$) lesions. Data are shown for 4 time points: baseline (7 days prior to lesion), the day prior to lesion, the 1st day of song production after the lesion, and 7 days postlesion. Values at each time point were normalized for each bird to their baseline value, then compiled into the means shown here. Error bars represent the SD. *, significance by 1-tailed paired $t$-test.

FIG. 7. Effect of targeted RA microlesions on the distribution of ending syllables. A: distributions of ending syllables are shown for 2 birds, where the different areas of the circle represent the proportion of songs ending with a given syllable. These examples are taken from 7 days prior to the respective lesions (left) and 7 days after the lesions (right). B, left: distributions prior to lesions; right: distributions after lesions. Data were compiled for 4 time points: baseline (7 days prior to lesion), the day prior to lesion, the 1st day of song production after the lesion, and 7 days postlesion. For each bird at each time point, a distribution of proportions was compiled for each vocal unit (syllables, introductory notes, or calls), representing the frequency with which songs ended on that unit. The values plotted indicate the mean (across birds) of the linear distance between the distributions (treated as vectors) at the baseline day and each subsequent day. Error bars represent SE. *, significance by 1-tailed paired $t$-test.
DISCUSSION

Unilateral lesions of RA in adult zebra finches disrupt normal song production

The avian song system contains two parallel descending pathways, one in each hemisphere. Unilateral lesions of the forebrain song nucleus HVC in either the left or right half of the brain have been reported to cause varying effects on song production (Halle et al. 2003a,b; Nottebohm et al. 1976; Williams et al. 1992). These effects appear to depend somewhat on species as well as the hemisphere where the lesion is made. Work in Waterschlag canaries, for example, suggests that HVC lesions to the right hemisphere have little effect on song production, whereas identical lesions on the left side cause a near complete deterioration of song (Nottebohm et al. 1976). Similar lesions in adult zebra finches have more subtle reported effects on song with right HVC lesions causing slightly stronger song deficits than left HVC lesions (Williams et al. 1992). Although these studies suggest that the two hemispheres contribute differentially to song, they also imply that song can be produced with a single HVC. One potential problem with this interpretation is that HVC, known for its capacity for neuronal turnover (Alvarez-Buylla et al. 1988), might be capable of some degree of neuronal regeneration if lesions are not complete; a problem that would be confounded if songs are assessed, as they were in these studies, many days or weeks after the lesions.

In the current study, we circumvented these issues by lesioning RA, a downstream target of HVC, where neurogenesis is absent (Alvarez-Buylla et al. 1988; Scharff et al. 2000). We also examined every vocalization the bird produced on each day of the first week and then at many time points thereafter. We found that complete unilateral lesions of RA in adult birds completely and permanently disrupted the temporal structure of song. Some birds produced abnormally long strings of introductory notes with occasional calls or single syllables, whereas others reverted to a more juvenile-like state with less stereotypy of temporal or acoustic structure. In all cases, little evidence of the prelesion song remained. Similar deficits were also observed in a previous unpublished study after unilateral inactivation of RA in awake, singing adult zebra finches by injections of lidocaine or APV (Lombardino 2000). In these birds, such manipulations caused them to produce impoverished songs, in some cases consisting of only introductory notes and calls. Further support that disruption of a single hemisphere is sufficient to prevent normal song comes from studies lesioning two structures that mediate information flow from RA back to the forebrain (Coleman and Vu 2005). In this study, unilateral lesions of Uva and MMAN caused complete and permanent disruptions of song that were similar to those observed in the present study.

The ability of unilateral RA lesions to disrupt song contradicts models of song production that suggest that song temporal structure can be generated independently by forebrain song control nuclei in each hemisphere (Solis and Perkel 2005; Williams et al. 1992; Yu and Margoliash 1996). If the two hemispheres could generate song motor programs autonomously, then an adult RA lesion should affect one descending pathway without affecting the other, leaving the intact hemisphere to produce song attempts unimpeded. Under this scenario, one RA may be insufficient to drive song output, but if that was the case, no song would be produced. Alternatively, the syringeal and respiratory modulations may be weakened, resulting in a temporally faithful but acoustically impaired song. However, our current results support neither of these scenarios. Singing attempts after unilateral adult RA lesions sometimes showed a reduction in acoustic amplitude but more importantly, they always demonstrated an impaired ability to generate a syllable sequence.

Our finding that unilateral RA lesions cause the impaired ability to generate a normal syllable sequence strongly suggests that integration of RA-output activity from each hemisphere is of fundamental importance to the generation of song. The surprising lack of compensation and adaptive plasticity after this lesion, even when song is assessed a year after the lesion, further reinforces this notion.

Normal song acquisition after unilateral lesions of RA: song production using a single hemisphere

In contrast to adult zebra finches, unilateral lesions in juveniles aged 45–60 days post hatch did not prevent the later acquisition of normal song. With the exception of having slight differences in maximum and minimum pitch, songs showed the same degree of stereotypy as control birds and syllables were indistinguishable in their overall duration and acoustic characteristics. The similarity in acoustic structure was particularly surprising considering that both halves of the syrinx contribute to the acoustic features of zebra finch song (Goller and Cooper 2004). Furthermore, because each syringeal half is largely under ipsilateral neural control, the production of acoustically normal complex syllables would not be expected after a complete unilateral lesion of RA. Some reorganization of the descending projections from intact RA might therefore have taken place to allow for a single RA to control both syringeal halves. The capacity to compensate for the loss of one RA during a critical period of post hatch development is in agreement with the observation in one zebra finch that the absence of an entire hemisphere, due a developmental defect, can nonetheless result in a bird producing a normal song as an adult (Lombardino 2000). These findings suggest that the song system can develop with only one hemisphere but that once the critical period for development has passed, interdependence between the two hemispheres prevents either side from being functional in isolation.

In adult birds that had received unilateral RA lesions as juveniles, we confirmed the functional elimination of the descending motor pathway on the lesioned side by stimulating HVC on the lesioned side during singing. Consistent with the hypothesis that this side was functionally eliminated, stimulation at abnormally high current levels (100 μA) did not cause any disruption in song acoustic or temporal patterns. Stimulation in HVC at much lower current levels (30–60 μA) on the intact contralateral side, however, caused short-latency syllable truncation and song-level temporal distortions similar to those observed in normal intact birds (Ashmore et al. 2005). This finding suggests that the remaining intact side is solely responsible for the generation of the song motor pattern. Interestingly, syllable truncation latency after HVC stimulation in “unihemi-
spheric” birds (~50 ms) was much shorter than after identical HVC stimulation in normal intact birds (~80 ms). This difference in timing is likely caused by the direct interruption of brain stem syringeal and respiratory motor commands after stimulation in unihemispheric birds. In normal birds, in contrast, stimulation must first interrupt premotor activity in the contralateral hemisphere before all premotor drive to the periphery is interrupted. The observed difference in timing agrees with the observation that interruption of contralateral premotor activity in HVC occurs ~20–30 ms after HVC stimulation (Vu et al. 1998).

Our findings are consistent with the notion that forebrain lesions in adult vertebrates have more permanent and severe effects than lesions that occur during development (Bates et al. 2001; Mosch et al. 2005). It has been proposed for other systems that the potential impact of unilateral lesions in forebrain motor areas during development is reduced due to compensation from alternate pathways that converge on the same output (Bernis 1930). In some cases, partial compensation might even be caused by neuronal replacement (Nottebohm 2002) or compensatory input along parallel pathways (Biernaskie et al. 2005; Colby et al. 2005; Uryu et al. 2001). One intriguing generalization that has arisen out of studies of human stroke patients and from mammalian studies is that when recovery does occur after adult injury, the brain often reverts to a more plastic state that is reminiscent of juvenile development, effectively recapitulating processes seen during ontogeny (Cramer and Chopp 2000). To some extent, we observe a similar process after RA lesions in adult birds. Attempted songs take on characteristics common to juvenile sub-song, such as inconsistent temporal structure, and the production of few stereotyped syllables (Deregnaucourt et al. 2005; Scharff and Nottebohm 1991). In light of this observation, recovery in the song system may provide insight into a potentially universal mechanism of functional compensation following injury across vertebrate species.

**Microlesions in RA and their effects on song temporal structure**

Complete lesions of RA in adults and juveniles allowed us to assess the contribution of RA activity to interhemispheric coordination. The ability of birds with a single RA (after juvenile lesions) to sing normal song as adults, on the other hand, offered us a unique opportunity to study the direct role that RA plays in song motor program generation. Structure/function relationships have been studied extensively in many systems by targeting microlesions to specific areas and assessing behavioral effects (Harding and McGinnis 2005; Hernadi et al. 1997; Kriegsfeld et al. 2004). In the avian song system, much has been learned from lesions of entire structures (Braillard and Doupe 2000; Brenowitz 1991; Nottebohm et al. 1976, 1982), but surprisingly few studies have been performed targeting single structures with microlesions for more precise characterization of the structure’s function (Margoliash et al. 1994; Thompson and Johnson 2007). This is probably because the effect of small lesions is likely masked by compensation from the lesioned structure’s contralateral counterpart. In addition, as we have argued in the last section, larger lesions, even unilateral ones, have the ability to affect motor production in both hemispheres, and thus it is generally difficult to determine whether behavioral effects after lesions are due to the targeted structure’s contribution to motor pattern generation or its contribution to interhemispheric communication or both.

In the present study, we avoided these confounds by performing microlesions in birds that use only one hemisphere for song production. By eliminating one of the hemispheres with a juvenile RA lesion, we argue that the behavioral effects after manipulation of the intact RA are not confounded either by RA’s potential role in interhemispheric coordination or by compensation from the contralateral forebrain motor pathway (including the contralateral RA). Under these conditions, targeted lesions of the intact RA disrupted motif structure, resulting in an increase in introductory notes throughout song and termination at atypical vocal units (syllables, calls, or intro notes).

This suggests that the lesions impacted the ability of the system to initiate syllable sequences or to maintain stereotyped sequencing once song started. In general, the temporal structure of song was degraded, in some cases severely, suggesting that RA does not simply mediate moment-to-moment control of the syrinx and respiration but rather that it is part of the network that determines overall temporal pattern and sequencing of song motifs. These findings are consistent with our previous studies showing that stimulation in RA as well as in brain stem nuclei such as PAm that receive RA input and project back to HVC cause profound disruptions in song temporal structure (Ashmore et al. 2005). A role for RA in influencing the temporal pattern and syllable sequencing is further supported by studies in juvenile birds where temporal rearrangements in syllable order are observed after injection of lidocaine or muscimol in LMAN, which together with HVC provides the only other known song system input to RA (Olveczky et al. 2005).

The exact contribution that RA makes to song temporal structure is an issue requiring further investigation. On the one hand, RA lesions might disrupt the generation of motor sequencing signals generated within RA or interrupt the flow of motor sequencing commands originating in HVC, and in juveniles, possibly even from LMAN. On the other hand, because of the recurrent nature of the vocal control system (Schmidt and Ashmore 2007), RA lesions might disrupt sequence generation by preventing RA output from reaching premotor areas such as HVC in the same hemisphere. This output may be sent along any of three pathways. The first pathway is a sparse but direct recurrent projection from the dorsocaudal RA back to HVC (Wild 2004). The second pathway travels through DMP, which in addition to its contralateral projections, also projects to the ipsilateral HVC via the ipsilateral MMAN. The third pathway is via the vocal-respiratory nuclei PAm and DM, which project to the ipsilateral HVC and NIf via the ipsilateral Uva (Fig. 1). A possible way to distinguish between these possibilities might be to perform more subtle manipulations of ventral RA, the topographic projections to nXIIIs of which are purely descending (and presumably responsible exclusively for syringeal control) (Vicaro 1991), and dorsal RA, the projections of which lead to the recurrent pathways, and evaluate differences between the two.

We observed some significant but subtle differences between lesions in these two divisions of RA but caution should be applied when interpreting these results. Although dorsal le-
sions produced a more pronounced effect on introductory notes and ending syllable distribution, measures for ventral lesions also showed trends toward song disruption and showed significant effects on ending syllable distribution at the time point immediately following the lesion. The lack of major differences in lesion effects between two areas in RA that project to seemingly functionally very different target structures is similar to the types of observed effects after microstimulation in dorsal and ventral RA (Ashmore et al. 2005). Microstimulation in dorsal RA and ventral RA during singing (in otherwise intact birds) both resulted in song interruption even though microstimulation in nXIIIs, the target structure of vRA, failed to show any such song interruptions and only caused acoustic distortions. One possible explanation for these observations is that strong interconnections between dRA and vRA, mediated by interneurons within RA (Spiro et al. 1999), may create a nucleus that is less segregated in its functional division than its outputs.

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