Two separate areas of the brain differentially guide the development of a song control nucleus in the zebra finch

(song system/lesion)

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ABSTRACT A brain nucleus that is important for the generation of song in the adult male zebra finch (Poephila guttata), the robust nucleus of the archistriatum (RA), receives dual inputs from two other telencephalic song nuclei: the hyperstriatum ventrale pars caudale (HVc) and the lateral magnocellular nucleus of the anterior neostriatum (L-MAN). We lesioned each of these afferent inputs to the RA early in development, either by themselves or both at the same time in the same side of the brain, to determine what influences each of these nuclei exerts on the normal development of the RA into adulthood. We found that lesioning the HVc in a 20-day-old male zebra finch prevents the later increase in RA volume and soma size that would normally occur around 35 days posthatching. MAN lesions at this same early age, on the other hand, had a large effect on reducing the volume and cell number of RA neurons, without affecting soma size. Lesioning both inputs early in development induced considerable RA neuronal cell death and atrophy of the nucleus. This study shows that the development of the RA is affected differently by each of its two input nuclei.

Songbirds have specialized areas in the brain that are necessary for the generation of song (1). These anatomically different areas are interconnected and are collectively referred to as the song system (Fig. 1). In zebra finches (Poephila guttata) the song system is composed of two distinct pathways that mediate either song acquisition or song production (2, 3). Song acquisition occurs first in the early posthatching period up to around 35–40 days, when the juvenile bird is hearing and learning its father's song but not yet vocalizing it. A discrete neural circuit in the anterior telencephalon makes up one part of the song system that is thought to be important for such early song acquisition. Lesions in any of these anterior song nuclei during early development severely disrupt normal song, whereas lesions in those same areas in adult birds do not (4–6). This part of the song system is initially innervated by the hyperstriatum ventrale pars caudale (HVc). The HVc makes synaptic connections to area X, which is located in the ventral portion of the anterior telencephalon. Area X connects to the medial nucleus of the dorsolateral thalamus, which projects back up to the anterior telencephalon again to make synaptic connections to the lateral magnocellular nucleus of the anterior neostriatum (L-MAN). The L-MAN makes afferent inputs to the robust nucleus of the archistriatum (RA), which is a nucleus involved in song production. The second song system circuit, located mainly in the posterior part of the telencephalon, is important for the motor production of song. After the initial song acquisition phase, juvenile males begin to practice their vocalizations to match them to what they have learned earlier. After the correct matching has occurred, the song crystallizes and remains fairly stable from then on (7). This second song system pathway is again initially innervated by the HVc, which sends its axons down to the RA. The RA goes on to connect to the hypoglossal (nX11s) nucleus, which in turn innervates the syringeal muscles used in singing.

The RA thus occupies an important position within the song system architecture, in which both of the functionally distinct circuits converge. Achievement of correct song is thus critically dependent on the normal development of the RA. In adult zebra finches, the song system is also sexually dimorphic (8). The RA, as is true of most of the other song nuclei, is large in both volume and soma size in males but is almost absent in females. In the early posthatching days of development, the L-MAN already makes afferent inputs to the RA in both sexes (9, 10), but HVc terminals lie just outside of and dorsal to the RA until around 35 days posthatch, when in males they suddenly innervate their target cells. In females, this HVc to RA connection is never made during this time and subsequently the RA undergoes rapid atrophy accompanied by massive cell death (11). The nature and timing of these events are highly suggestive of afferent regulation of neural development. We therefore investigated,

Abbreviations: HVc, hyperstriatum ventrale pars caudale; L-MAN, lateral magnocellular nucleus of the anterior neostriatum; RA, archistriatum; BDA, biotinylated dextran amine.
by selective ibotenic acid lesioning, what developmental influences the MAN and HVc have on the fate of the RA. Our results indicate that lesioning the HVc alone, or the MAN alone, in a 20-day-old male had drastically different effects on the final outcome of the cytoarchitecture of the adult RA. While HVc lesions had an attenuating effect on the later development of both RA volume and soma size, MAN lesions had a large effect on reducing the volume and neuronal cell number in the RA, while not affecting cell size. Unilateral lesions of both HVc and MAN at this same early age, however, led to massive RA atrophy and cell death, such that the eventual adult ipsilateral RA in such a preparation looked similar to the collapsed RA of an adult female.

MATERIALS AND METHODS
A breeding colony in our animal facilities provided us with both adult zebra finches (>90 days old) and chicks of known ages. Twenty-day-old male birds were deeply anesthetized by intramuscular injections of Equithesin and placed in a stereotaxic headholder. Ibotenic acid (Siris Labs, San Rafael, CA; 6.6 mg/ml in phosphate buffer, pH 7.4) was injected unilaterally into either HVc alone (n = 5), MAN alone (n = 6), or both nuclei in the same hemisphere (n = 5). Adult males (n = 4) were also lesioned in both HVc and MAN to test the effects of such dual lesions on the age of the bird. At 90 days of age, all birds were killed by deep anesthesia and transcardially perfused with 0.9% saline followed by 4% buffered paraformaldehyde (pH 7.4). The brains were postfixed for 2–3 days and then cryoprotected overnight in a 30% solution of sucrose in phosphate buffer. Sagittal or coronal sections were cut 30 μm thick on a freezing microtome, collected in cold phosphate buffer, mounted onto gelatin-coated glass slides, and stained with 0.5% cresyl violet. Adult males used as controls in this study were killed and processed in a similar way after a 2-week survival period postlesioning.

RA volume and soma size were determined for each bird on camera lucida drawings of the perimeter of stained cell bodies of RA neurons and the Nissl-defined borders of the whole RA nucleus; then the area of the tracings was calculated using a digitizing tablet. Neurons were discriminated from glia cells by the presence of a pale nucleus containing one or two distinct nucleoli and a well-stained cytoplasm. For neuron density, the number of neurons in the field of view of a 100× (oil immersion) objective was randomly sampled 20 times over the entire nucleus for each bird. The average number of neurons was then divided by the volume of the 30-μm field of view slice to get the neuron density. Neuron number was calculated by multiplying the cell density by the volume of the RA. A more detailed explanation of such measurement protocols has been published (4, 12).

Biotinylated dextran amine (BDA) (Molecular Probes) was used as an anterograde tracer to study the adult connections between HVc and RA in early MAN-lesioned males as well as to verify that 20-day-old females already have this connection (13). A 10% (wt/vol) solution of BDA in 0.2 M KCl was iontophoresed through a finely pulled capillary glass electrode (15-μm tip diameter) for 10 min at 10 μA positive current. After a 2-day survival period, the birds were perfused with a fixative consisting of 4% paraformaldehyde, 0.1 M lysine, and 0.01 M sodium periodate in 25 mM phosphate buffer (pH 7.4). After postfixing the brain for 1 day (4°C), the brains were cryoprotected overnight in a 30% sucrose solution (wt/vol) in 25 mM phosphate buffer and cut at 30 μm on a freezing microtome. The sections were allowed to react with a Vector elite kit (Vector Laboratories) to visualize the transported BDA.

RESULTS
HVc Lesions. Examination of RA volumes in adult birds that had HVc lesions when they were 20 days old reveals that the ipsilateral side was smaller by about 39% than its contralateral counterpart (Fig. 2A; see also Fig. 4A). The mean volume of the ipsilateral RA was 0.150 ± 0.022 mm³ (means ± SEM), and the contralateral volume was 0.246 ± 0.010 mm³. Neuron size within the ipsilateral RA was also smaller by about 32% than the neurons in the contralateral RA (Fig. 3A; see also Fig. 5A). Measurements of the area of the Nissl-stained cell bodies show that the mean ipsilateral RA neuron size was 145 ± 29.9 μm², whereas the contralateral RA neurons were larger at 214 ± 40 μm². Also readily apparent (Figs. 2A and 3A) is the observation that the neurons within the ipsilateral RA were more densely packed in comparison to the unlesioned side (see Fig. 5A). In fact, the mean ipsilateral RA cell density of 6.88 ± 0.654 × 10⁴ cells per mm³ was roughly 1.75 times greater than the contralateral RA density of 3.93 ± 0.469 × 10⁴ cells per mm³. The RA neuron number between the two sides of the brain, however, was roughly the same and differed by <6% (Fig. 4A). The smaller ipsilateral RA volume combined with its higher cell density resulted in the mean ipsilateral RA neuron number calculation to be 10,200 ± 878 cells, while the mean contralateral side cell number was 9651 ± 1183 cells.

In comparison to its ipsilateral RA, the contralateral RA of early HVc-lesioned birds was larger in both volume and neuron size. While the number of RA neurons between the hemispheres was roughly equal, the spacing between those

FIG. 2. Cytoarchitectonic effects on the development of the RA following ibotenic acid lesions. (A) HVc lesions on a 20-day-old male. (B) MAN lesions on a 20-day-old male. (C) Both HVc and MAN lesions on a 20-day-old male. (D) Lesions of both HVc and MAN done on an adult male. IPSI, ipsilateral; CONTRA, contralateral. All young-lesioned birds were examined as adults (>90 days), and the adult-lesioned birds were examined after a 2-week survival period. (Bar = 200 μm.)
neurons was higher on the contralateral side than in the ipsilateral side.

**MAN Lesions.** In contrast to HVC lesions in young males, MAN lesions had a much different effect on the fate of RA development. In 20-day-old males, the RA already receives afferents from MAN but has not yet received synaptic input from the HVC. Ablating MAN during this early period of development resulted in a 72% drop in volume size of the ipsilateral RA when compared to the contralateral side (Figs. 2B and 4B). The mean volume of the ipsilateral RA was 0.063 ± 0.021 mm$^3$ and the contralateral mean RA volume was 0.232 ± 0.015 mm$^3$. Neuron size between the two sides, however, was roughly equal (Figs. 3B and 5B). On the ipsilateral side, the mean neuron size was 195.8 ± 34.4 μm$^2$, while the contralateral RA neuron mean size was very similar at 196.8 ± 31.5 μm$^2$, reflecting a difference of <1%. The density of those neurons within the RA was lower on the lesioned side as compared to the nonlesioned side of the brain but was much lower than the density of cells in RA resulting from early HVC-lesioned birds (Figs. 3A and B and 5A and B). The mean RA density in the MAN-lesioned side of the brain was 3.37 ± 0.460 × 10$^4$ cells per mm$^3$, while the mean RA cell density on the contralateral side was 4.73 ± 0.441 × 10$^4$ cells per mm$^3$. The spacing between RA neurons in such early MAN-lesioned birds was thus about 28% higher on the ipsilateral side as compared to the contralateral side. In comparison to the RA cell density of early HVC-lesioned males (Fig. 5A), the MAN-lesioned birds had over twice as much space between their RA neurons. The largest effect of early MAN lesions, however, was on cell number. The RA in the MAN-lesioned side of the brain had a mean cell number of 2110 ± 583 cells, while the unlesioned side had a mean cell number of 10,981 ± 591. This represents a >80% drop in the number of RA neurons that survived into adulthood.

Lesions of the MAN early in development greatly reduced the volume and cell number of its ipsilateral RA. In comparison to the unlesioned side, RA cell size was roughly the same but the interneuronal spacing was higher on the lesioned side of the brain. Despite the large effect that lesions had on the development of RA, BDA iontophoresed into the adult HVC shows that these two nuclei are synthetically connected.

**HVC and MAN Lesions.** Lesioning both HVC and MAN inputs to the RA in 20-day-old males led to massive RA atrophy accompanied by considerable cell death. One of the most obvious effects of such early dual lesions was the precipitous drop in RA volume that occurred in the ipsilateral side as compared to the contralateral, or unlesioned, side of the brain (Figs. 2C and 4C). The ipsilateral RA mean volume was 0.015 ± 0.010 mm$^3$ while the contralateral side was much larger at 0.218 ± 0.023 mm$^3$. This represents a >93% drop in

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**Fig. 3.** Effect on RA soma size following ibotenic acid lesions. (A) HVC lesions on a 20-day-old male. (B) MAN lesions on a 20-day-old male. (C) Both HVC and MAN lesions on a 20-day-old male. (D) Lesions of both HVC and MAN done on an adult male. All young-lesioned birds were examined as adults (>90 days), and the adult-lesioned birds were examined after a 2-week survival period. (Bar = 20 μm.)

**Fig. 4.** Bar graphs quantifying the effects of ibotenic acid lesions on RA volume and neuronal cell number. (A) HVC lesions on 20-day-old males. (B) MAN lesions on 20-day-old males. (C) HVC and MAN lesions on 20-day-old males. (D) Lesions of both HVC and MAN done on adult males. All young-lesioned birds were examined as adults (>90 days), and the adult-lesioned birds were examined after a 2-week survival period. Data shown are means ± SEM.
HVC and MAN Lesions in Adult Males. To determine whether or not such early lesions of both HVC and MAN were developmentally significant, similar unilateral lesions were done in adult males (>90 days old). After a 2-week survival period, histological examination showed that the volume of the ipsilateral RA shrank by about 38% when compared to the contralateral RA (Figs. 2D and 4D). The mean volume of the ipsilateral RA was 0.142 ± 0.010 mm³, while the contralateral RA was 0.228 ± 0.015 mm³. Neuronal size in the RA following adult lesions of both HVC and MAN was reduced by <6% on the ipsilateral side as compared to the unlesioned side of the brain (Figs. 3D and 5D). The ipsilateral mean cell size in RA was 165 ± 33.8 μm², while the contralateral mean cell size was 175 ± 23.9 μm². Along with the reduction of RA volume following the adult lesions of HVC and MAN, the cell density within the RA also increased on the ipsilateral side to 6.61 ± 0.569 × 10⁴ cells per mm³, as compared to the unlesioned side of 4.13 ± 0.469 × 10⁴ cells per mm³ (Figs. 3D and 5D). Cell number in the RA, however, was nearly identical between the ipsilateral RA at 9369 ± 856 cells and the contralateral RA at 9414 ± 989 cells (Fig. 4D). This reflects a <1% difference of cell number between the lesioned and unlesioned side of the brain.

Ablating both afferent inputs to the adult RA, while reducing the ipsilateral volume and increasing its cellular density, had little effect on the RA’s neuronal size and number.

**DISCUSSION**

The results presented in this paper show that the normal development of the RA is differentially influenced by each of its two input nuclei, HVC or L-MAN. While Johnson and Bottjer (14) recently published a similar paper dealing with early afferent influences on the development of the RA, our study is significantly different in several ways. First, instead of electrolytic lesions, we used ibotenic acid since it is purported not to damage fibers of passage. This property, which may not be as important in MAN lesions, is crucially important in HVC lesions, since MAN axons traverse very close to the HVC in their pathway to the RA (17). Second, we examined the lesion results in adult birds, as opposed to only 2–6 days postlesion in young birds, which have not finished developing. Finally, but most important, we also lesioned HVC, either by itself or in combination with the ipsilateral MAN, to get a complete picture of the contributions each of these nuclei have on the development of the RA.

The results of our early MAN lesions are in general agreement with earlier findings that eliminating early MAN afferent input to the RA greatly reduces the RA’s volume and cell number (14). We further demonstrate that the later increase in RA neuronal size and spacing that normally occurs around 35 days posthatching is not affected by such early MAN lesions and that the surviving neurons are successfully innervated by the HVC in the adult. Survival of the RA during development, however, is not only a matter of afferent connectivity. The L-MAN in a 20-day-old female also makes synaptic contact with the RA (20, 10), but apparently this is not enough in itself, since the RA later atrophies and many of its neurons die nonetheless. Rather, the connectivity between the MAN and RA may serve as an important conduit for the transport of anterograde trophic influences. The nature of these influences may be either chemical or electrical, or both (15, 16).

HVC lesions in a 20-day-old male, however, have much different effects on RA’s development from MAN lesions performed at the same age. In comparison with previous work charting the normal development of the RA in males (11), it is clear that early HVC lesions did not simply reduce the volume and cell size of the ipsilateral RA but rather prevented the surge in growth that would normally occur.

**Fig. 5.** Bar graphs quantifying the effects of ibotenic acid lesions on RA soma size and neuronal density. (A) HVC lesions on 20-day-old males. (B) MAN lesions on 20-day-old males. (C) HVC and MAN lesions on 20-day-old males. (D) Lesions of both HVC and MAN done on adult males. All young-lesioned birds were examined as adults (>90 days), and the adult-lesioned birds were examined after a 2-week survival period. Data shown are means ± SEM.

RA volume following ipsilateral HVC and MAN lesions. In conjunction with this severe atrophy of the nucleus, RA cell size also shrank considerably (Figs. 3C and 5C). On the lesioned side, the mean RA cell size was 22.5 ± 17.7 μm², while the unlesioned side of the RA mean cell size was 181.8 ± 30.4 μm². Lesioning both inputs early in the RA’s development had a severe effect on reducing the size of the ipsilateral RA neurons such that they wound up being only 12% of the size of the RA neurons on the unlesioned side. Another dramatic effect of such early dual lesions was on the neuronal density within the highly atrophied RA (Figs. 3C and 5C). The ipsilateral mean RA cell density was 20.5 ± 2.05 × 10⁴ cells per mm³, or more than four times denser when compared to the unlesioned side mean RA cell density of 5.24 ± 0.633 × 10⁴ cells per mm³. Cell number in RA also dropped by >73% following the early lesions (Fig. 4C). On the ipsilateral side, cell number in RA went down to 3039 ± 979, while the unlesioned side had a mean RA cell number of 11,433 ± 1227.

It is clear from the data that lesions of both HVC and MAN in a 20-day-old male bird drastically reduce the ipsilateral RA volume, cell size, cell number, and interneuronal spacing.
later in development (at 35 days posthatch). The onset of this rapid growth of RA volume and soma size, as well as the reduction of cell density, is sharply timed to coincide with the sudden ingrowth of HVc axons into the RA (11, 19). When HVc axons are prevented from innervating the RA, as in the early HVc lesion experiments, the RA remains in its juvenile, undifferentiated state permanently into adulthood—that is, when comparing RA’s volume, cell size, and density of a normal 20-day-old male with the adult ipsilateral RA of an early HVc-lesioned male, all corresponding measurements are very similar (11). The smaller values of the ipsilateral RA, therefore, reflect the inability of that RA to further develop in the absence of HVc afferent influence.

Ablating both afferent inputs to the RA had different effects on the RA depending on whether the lesions were done on young birds or adults. Dual lesions in 20-day-old males induced massive cell death in the RA and the nucleus greatly atrophied to the point where it became similar in appearance to the highly atrophied RA of an adult female. This would explain the earlier findings that a knife cut placed between the HVc and RA in a young male also had a drastic effect on the integrity of the RA (18). Apparently, axons from both HVc and MAN were severed by these cuts, and the disruption of both afferent influences by this procedure, as in the chemical lesion of both input nuclei, resulted in the collapse of the ipsilateral RA. But the same dual lesions of the HVc and MAN in adult males decreased the ipsilateral RA volume by only 38% in comparison to the 93% drop in volume seen after the lesions were done in young birds. Neuronal cell size in the ipsilateral RA was smaller by only 6% in the adult-lesioned birds but was reduced by 88% if the lesions were done in young birds. RA cell number differed by <1% between the ipsilateral and contralateral RA of adult-lesioned males but resulted in a >73% reduction in the ipsilateral RA if the lesions were again done on 20-day-old males. Clearly, there is an age-dependent sensitive period when unilateral lesions of both HVc and MAN have a much more drastic effect on the development of the RA. Once HVc axons synapse on RA neurons, however, a permanent change is imparted to those cells such that their size and number remain stable, even after complete deafferentation.

The normal development of the RA in males is apparently more complicated than we once thought and seems to consist of three distinct phases. In the first phase, before 30 days posthatch, the MAN is seen to be critically important as the sole afferent support of RA neurons, which are still somewhat small in size as is the nuclear volume. The second phase occurs from 35 to 45 days and is distinguished by the sudden ingrowth of HVc terminals into the RA and the coincident sharp growth of RA volume, cell size, and interneuronal spacing. The third phase is characterized by a drop in RA volume and cell size and occurs from 45 days to around 60 days, after which time the cytoarchitecture of the RA remains fairly stable (11).

Early lesions of L-MAN or area X cause abnormal song development, because they may disrupt the process of matching vocal output with the template of a tutor song (4, 6). It is, however, necessary to consider that ablations of these nuclei during the final phase of RA development may interfere with the formation of normal synaptic connections between the HVc and RA, because significant rearrangement of synaptic inputs from the HVc and L-MAN takes place during this period (19). Thus, the defective song may be due to abnormalities in the motor pathway instead of failure in template matching. It is important to discriminate between these possibilities.

Finally, we point out the general significance of the findings reported here. Neurons may die because either their targets or their afferent inputs are missing under normal, pathological, or experimental conditions (20). Dual afferent inputs are necessary for normal development in several systems, including, for example, ocular dominance columns (21), nucleus laminaris in chicks (22), and lateral motor columns in chicks (23). The essential sources are bilateral sensory inputs in the first and second cases and a combination of peripheral sensory and central descending inputs in the third case. To our knowledge, the need for inputs from two ipsilateral forebrain areas for the normal differentiation of another telencephalic nucleus has not been previously documented. Multiple central afferent innervation may be an important regulatory mechanism in the development of complex brain pathways.

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