Telencephalic Neurons Monosynaptically Link Brainstem and Forebrain Premotor Networks Necessary for Song

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Birdsong, like human speech, is a series of learned vocal gestures resulting from the coordination of vocal and respiratory brainstem networks under the control of the telencephalon. The song motor circuit includes premotor and motor cortical analogs, known as HVC (used as a proper name) and RA (the robust nucleus of the arcopallium), respectively. Previous studies showed that HVC projects to RA and that RA projection neurons (PNs) topographically innervate brainstem vocal-motor and respiratory networks. The idea that singing-related activity flows between HVC and RA in a strictly feedforward manner is a central component of all models of song production. In contrast to this prevailing view of song motor circuit organization, we show that RA sends a reciprocal projection directly to HVC. Lentiviral labeling of RA PN axons and transgene tagging of RA PN synaptic terminals reveal a direct projection from RA to HVC. Retrograde tracing from HVC demonstrates that this projection originates exclusively from neurons in dorsocaudal regions of RA. Using dual retrograde tracer injections, we further show that many of these RA_{HVC} neurons also innervate the brainstem nucleus retroambiguus, which is premotor to expiratory motoneurons, thereby identifying a population of RA PNs positioned to coordinate activity at higher and lower levels of the song motor circuit. In combination, our findings identify a previously unknown pathway that may enable a subset of RA neurons to provide song-related signals to the respiratory brainstem but also transmit a copy of this information to song patterning networks in HVC.

Key words: lentivirus; zebra finch; motor control; anterograde; synaptophysin; vocal learning; songbird; avian

Introduction

The central pathway for motor control of learned birdsong has been the subject of intensive investigation in the three decades since its discovery (Nottebohm et al., 1976). The song motor circuit includes feedforward projections between the telencephalic premotor and motor nuclei, HVC (used as a proper name) and the robust nucleus of the arcopallium (RA), respectively, with RA innervating brainstem vocal-respiratory networks directly (see Fig. 1A) (Vicario, 1991; Wild, 1993b, 1994, 2004; Suthers and Margoliash, 2002). RA projection neurons (PNs) project topographically onto their brainstem targets: neurons in the dorsal third of RA project to expiratory-related [nucleus retroambiguus (RAm)] or inspiratory-related [nucleus parambigualis (PAM)] premotor neurons in the medullary ventral respiratory column, whereas neurons in the ventral two-thirds of RA project directly to the tracheosyringeal portion of the hypoglossal motor nucleus (XIIIts), which innervates the muscles of the syrinx, the bird's vocal organ (Vicario,

1991, 1993; Wild, 1993a,b). Robust connections between RAm, PAM, and XIIIts are thought to mediate the coordination of vocal and respiratory muscle activity during singing, under the control of the telencephalon (Sturdy et al., 2003; Kubke et al., 2005).

The current understanding that all RA PNs send their axons out of the telencephalon serves as an organizing principle of song motor circuit anatomy. Emergent from this anatomy is the view that song patterning mechanisms involve the unidirectional flow of information from HVC to RA. Indeed, a hierarchical model of song, in which HVC activity codes for song syllables and RA activity codes for subsyllable notes (Yu and Margoliash, 1996), as well as a feedforward model, in which HVC functions as an autonomous pattern generator, coding for all song components (Hahnloser et al., 2002), presume that HVC functions without motor-related feedback during song production. A more recent recursive model posits that motor-related feedback from the brainstem plays an important role in song production by providing timing signals to HVC (Ashmore et al., 2005). Electrical stimulation of PAM elicits disruption of ongoing song, supporting the idea that an indirect respiratory-related motor feedback from the brainstem can influence HVC activity. Because this recursive pathway relays information from PAM to HVC via a thalamic intermediary (nucleus uvaeformis) (Striedter and Vu, 1998), it may affect HVC activity over relatively long timescales. A direct RA–HVC pathway, however, such as the one suggested by Wild (2004), could provide shorter-latency feedback to HVC that

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might be of special functional importance, given the precise and rapid vocal and respiratory modulations typical of birdsong.

We examined in greater detail axonal projections from RA using a variety of viral, traditional, and physiological tracing methods. After injections of a sensitive viral-mediated tracer into RA, labeled fibers that resemble axon terminals were found in HVC. Injection of a viral-mediated tracer that selectively labels presynaptic terminals showed that a population of RA PNs form synapses in HVC. Consistent with these findings, conventional retrograde tracer injections in HVC confirmed the presence of a subset of neurons in dorsocaudal regions of RA, and some of these neurons were found by dual tracer injections into RAM and HVC also to project to RAM in the lateral medulla. Therefore, a previously unknown pathway may enable a subset of RA neurons to provide song-related signals to the respiratory brainstem but also transmit a copy of this information to song patterning networks in HVC.

Materials and Methods

All experiments were conducted on adult male zebra finches (105–300 d after hatch) in accordance with protocols approved by the Duke University Institutional Animal Care and Use Committee and by the University of Auckland Animal Ethics Committee.

Vector construction

Standard molecular cloning methods were used to optimize promoter-reporter constructs for use in songbirds. As starting material, we used a self-inactivating FUGW lentivirus containing an ubiquitin-enhanced green fluorescent protein (GFP) promoter-reporter construct (Lois et al., 2002). We replaced the ubiquitin promoter with a Rous sarcoma virus (RSV) long terminal repeat (LTR) amplified from the RSV-cat plasmid (Dr. Garcia-Blanco, Duke University, Durham, NC). We developed two constructs under the control of the RSV promoter, one driving the expression of monomeric Cherry fluorescent protein (Shaner et al., 2004) and another driving the expression of synaptophysin-GFP (Grinevich et al., 2005), resulting in the constructs FRChW and FRSynGW.

Lentivirus production

Lentivirus vectors were made by transfecting 6×10^6 293FT cells with 5 μ g of the vesicular stomatitis virus glycoprotein (VSVg) envelope encoding plasmid, 15 μ g of the delta-8.9 packaging plasmid, and 20 μ g of promoter-reporter plasmid using Lipofectamine. After 72 h, supernatant was harvested from three 10 cm culture plates, filtered at 0.45 μ m, and pelleted by ultracentrifugation at 26,000 rpm for 2 h at 4°C. After resuspension, serially diluted lentivirus was used to transduce 293FT cells; 72 h later, labeled 293FT cells were counted to calculate the viral titer. Lentiviruses with titers ranging from 1×10^9 to 1×10^{12} TU/ml were used in this study.

To test whether insertion of the RSV LTR rescued the ability for lentiviral replication, we transduced 293FT cells with FRChW and then transduced these cells with both VSVg and delta-8.9. We then harvested the medium and applied it to virgin 293FT cells to test whether new viral particles, capable of viral infection, had been produced. The lack of fluorescent labeling in virgin 293FT cells indicated that our RSV-containing constructs were replication incompetent (Miyoshi et al., 1998).

Viral and retrograde tracer injections

Injections were made into RA (10 birds), HVC (22 birds), or RAM and HVC (5 birds) in adult (105–300 d after hatch) male zebra finches. Birds were anesthetized with either isoflurane (2% in O₂) or an equal parts mixture of ketamine (50 mg/kg) and xylazine (20 mg/kg). Stereotaxis combined with multiunit electrophysiological recordings of spontaneous activity was used to target song system nuclei. For injections into RA, the bird's head was positioned so that the pipette did not traverse the borders of HVC, which lies anterior and dorsal to RA. Lentivirus injections (32.2 nl/injection for ~30 injections for a total of ~1 μ l) were made using a Nanoject II (Drummond Scientific, Broomall, PA). Injections of herpes simplex virus-1 (HSV-1) amplicon (gift from Dr. Edward Calla-

way, The Salk Institute for Biological Studies, San Diego, CA) were made in a similar manner. Fast Blue [Sigma (St. Louis, MO) or Polysciences (Warrington, PA)], biotinylated dextran amine (BDA) (10,000 molecular weight; Invitrogen, Carlsbad, CA), or Texas Red-conjugated dextran amine (Invitrogen) was injected into HVC using either air pressure (Picospritzer II, Parker; General Valve, Fairfield, NJ) applied to a glass micropipette (inner diameter of ~15 μ m) or a Nanoject II, whereas AlexaFluor 555-conjugated cholera toxin B-chain (Invitrogen) was injected into RAM using the Picospritzer. The glass micropipette was positioned in RAM after electrophysiological recording of expiratory-related multiunit activity in the nucleus, using a tungsten microelectrode (2 M Ω ; FHC, Bowdoinham, ME) and conventional signal filtering and amplification. The signal of anterograde FRChW labeling was enhanced by staining with anti-red fluorescent protein (RFP) antibody (AB3216, 1:500; Millipore Bioscience Research Reagents, Temecula, CA) visualized with goat anti-rabbit AlexaFluor 594 secondary antibody (1:200; Invitrogen).

Neuronal culture

Cortical neuron cultures were prepared from embryonic day 18 rat embryos as described previously (Ehlers, 2000). Cells were transduced with 1 μ l of FRSyGW at 7 d *in vitro*, followed by immunocytochemistry at 17 d *in vitro* using either postsynaptic density 95 (PSD-95) (MAB1596, 1:500; Millipore Bioscience Research Reagents) or vesicular glutamate transporter (VGLUT1) (135 511, 1:1000; Synaptic Systems, Göttingen, Germany). Goat anti-mouse cyanine 3 was used for visualization (AP124C, 1:1000; Millipore Bioscience Research Reagents).

Brain slices and *in vitro* electrophysiology. Sagittal brain slices through HVC and RA of adult male zebra finches were cut at 400 μ m and transferred to a holding chamber (room temperature). Intracellular recordings were made using an interface-type chamber (30°C; Medical Systems, Greenvale, NY). Artificial CSF consisted of the following (in mM): 119 NaCl, 2.5 KCl, 1.3 MgCl₂, 2.5 CaCl₂, 1 NaH₂PO₄, 26.2 NaHCO₃, and 11 glucose (equilibrated with 95% O₂/5% CO₂). Equiosmolar sucrose was substituted for NaCl during slicing.

Sharp electrodes (borosilicate glass, BF100; Sutter Instruments, Novato, CA) were pulled to yield a resistance of 100–200 M Ω when filled with 2 M potassium acetate and 5% Neurobiotin. A motorized microdrive (model 860A; Newport Scientific, Irvine, CA) was used to lower electrodes into RA, which was visible under epi-illumination. Brief (~1 ms) capacitance overcompensation was used to “ring” the electrode to achieve entry into the cell. An AxoClamp 2B intracellular amplifier (Molecular Devices, Sunnyvale, CA) was used in bridge mode to record intracellular membrane potentials, which were low-pass filtered at 3 kHz, digitized at 10 kHz, and stored on a personal computer hard drive using custom software developed by former laboratory members Fred Livingston and Rob Neumann. Only cells with resting potentials negative of –50 mV and overshooting spikes were used for analysis. A concentric bipolar stimulating electrode (FHC) was placed in the fiber tract between HVC and RA, ~0.5–1.5 mm dorsal to RA, which was discernible under epi-illumination as an arc of myelinated axons extending between the two nuclei. Antidromic action potentials were evoked using brief (100 μ s) currents of 50–1000 μ A. To rule out the possibility of confusing EPSPs with antidromically activated action potentials, all recordings were performed in the presence of excitatory synaptic blockers 2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[*f*]quinoxaline (NBQX) (14 μ M) and D-APV (50 μ M). In two cases, once an RA neuron was antidromically identified, the stimulating electrode was moved anterior to the HVC–RA fiber tract to confirm that the evoked antidromic action potential was only evoked by stimulation in the HVC–RA fiber tract and could not be attributed to passive current spread to RA. Cells were filled with Neurobiotin using positive currents (0.5–1 nA, 500 ms at 1 Hz, 15–20 min).

Histology. Birds were deeply anesthetized with Equithesin and transcardially perfused with 0.9% saline for 3 min, followed by 4% paraformaldehyde (PFA) in 25 mM sodium phosphate buffer for 30 min. The brain was removed and postfixed in 4% PFA with 30% sucrose overnight, blocked and sliced into sagittal or transverse sections on a freezing microtome at 40–50 μ m, either processed for immunohistochemistry or directly mounted onto glass slides, and coverslipped. Some sections were

Figure 1. Lentivirus-based pathway tracing in the song motor circuit. **A**, A schematic of the zebra finch brain, cut parasagittally, highlighting relevant song-related brain circuits. The anterior forebrain pathway, which drives motor variability, originates in HVC and terminates in RA (black). The descending song motor pathway, which controls song production, originates in HVC and terminates in syringeal and respiratory motoneurons in the medulla and spinal cord (red). Inset, PN in RA are topographically organized with neurons in dorsal RA innervating expiratory (RAm) and inspiratory (PAm) premotor neurons and neurons in middle and ventral RA innervating the Xlts. The RA PNs are not thought to project to HVC; however, recent preliminary evidence has suggested otherwise (Wild, 2004). **B**, Lentivirus drives strong mCherry expression in song system neurons, illustrated by the pattern of labeling seen in RA neurons 2 weeks after lentiviral injection. **C, D**, Lentivirus labeling of RA neurons allows visualization of their axonal arbors in RAm, which are several millimeters distal to the injection site, demonstrating that this lentivirus can be used as an anterograde pathway tracer in songbirds. AreaX, Used as proper name; DLM, medial nucleus of the dorsolateral thalamus. Scale bars: **B**, 40 μ m; **C, D**, 100 μ m.

immunohistochemically stained with anti-RFP antibody (AB3216, 1:500; Millipore Bioscience Research Reagents) visualized with goat anti-rabbit AlexaFluor 594 secondary antibody (1:200; Invitrogen) and/or anti-parvalbumin monoclonal antibody (1:1000; Swant, Bellinzona, Switzerland) visualized with goat anti-mouse AlexaFluor 488 secondary antibody (1:200; Invitrogen). After staining, the tissue sections were rinsed in PBS, mounted onto glass slides, air dried, and coverslipped using Fluormount-G (Electron Microscopy Sciences, Hatfield, PA).

Data acquisition. Confocal images were acquired with a Carl Zeiss (Thornwood, NY) LSM 510 upright microscope with argon (458, 488, and 514 nm) and helium–neon 1 (543 nm) and Coherent (Santa Clara, CA) Enterprise UV (351 and 364 nm) lasers (see Figs. 1B, C, 2–5, 6C, D, 7) or using Carl Zeiss Axioskope II with GFP, rhodamine, and UV bandpass filters (see Figs. 1D, 6D, 8). Isoform reconstructions were made using ImageSurfer (version 1.16). Neuronal density measurements were made from four hemispheres from one male and one female using the optical dissector method (Sterio, 1984; Coggeshall and Lekan, 1996), sampling throughout the rostrocaudal extent of the nucleus.

Results

Targeted lentiviral labeling of RA neurons for anterograde tracing

To more fully characterize the axonal projections from RA, we constructed a lentivirus with the avian-directed promoter RSV,

driving expression of the mCherry fluorescent protein (Shaner et al., 2004). RA neurons were infected with lentivirus by first mapping the borders of RA using multiunit recordings of spontaneous activity; injections (~ 900 nl) were then localized to the ventral portion of RA, which resulted in infected neurons throughout RA. Cases in which injections missed the ventral core of RA typically resulted in extensive labeling within the myelinated outer shell of RA, with only scattered labeling within the nucleus. These cases were not considered for additional analysis.

Analysis of brain sections 2–3 weeks after lentivirus injections revealed strong mCherry protein expression in RA neurons (Fig. 1B, C) ($n = 6$). Intracellular diffusion of fluorescent protein allowed axons from RA to be readily tracked for 10–15 mm into the brainstem, in which they terminated in previously identified targets of RA, including RAm (Fig. 1D), PAm, and Xlts. Neuronal somata labeling was absent in sections through HVC, or lateral magnocellular nucleus of the anterior nidopallium (IMAN), which are known sources of RA afferents (data not shown). This lack of retrograde labeling after injections with VSVg pseudotyped lentivirus parallels previous reports in other systems (Blomer et al., 1997; Grinevich et al., 2005), supporting the conclusion that this lentivirus can be used as an exclusive anterograde pathway tracer. Together, these data indicate that the RSV promoter affords stable fluorescent protein expression, providing exceptional morphological detail of infected neurons. Furthermore, viral pseudotyping with VSVg avoids retrograde labeling, making lentivirus based labeling highly useful for detailed analysis of neuronal circuits in songbirds.

RA neurons project dorsally into HVC

Having demonstrated the utility of lentivirus for anterograde labeling of song system neurons, we next examined whether lentivirus injections in RA resulted in anterograde labeling in HVC. We readily traced axons leaving the dorsal border of RA, traversing the fiber tract between HVC and RA, and exhibiting extensive collateralization within the borders of HVC. (Fig. 2A–C) ($n = 6$ animals). We assessed the possibility that labeled axons dorsal to RA and within HVC result from retrograde labeling of HVC neurons and their axonal collaterals. Sections through HVC were carefully inspected and confirmed a lack of lentivirus-labeled neuronal somata in HVC, supporting the conclusion that labeled axons originated from RA neurons and were not collaterals of HVC neurons.

mCherry-labeled RA axons spanned the extent of HVC and exhibited numerous arborizations and varicosities, suggestive of synaptic terminals (Fig. 2). We counterstained sections with a monoclonal anti-parvalbumin antibody (Swant) to label a predominant subclass of HVC interneurons (Wild et al., 2005) and

Figure 2. RA axons ramify within HVC. *A*, Injections of lentivirus expressing mCherry into RA was used to ask whether RA projects to HVC. *B*, A low-power, confocal image showing RA axons in HVC after lentivirus injection into RA. *C*, The RA axons ramify, forming putative terminal fields, within HVC. Scale bar: *B*, 40 μ m; *C*, 20 μ m.

Figure 3. RA axons in HVC display morphological specializations characteristic of presynaptic terminals. *A*, *B*, Anterogradely labeled axons in HVC exhibit prominent terminal boutons and varicosities (red axons). Parvalbumin-positive HVC interneurons are labeled in green. Insets, Isoform reconstruction from regions outlined in white boxes illustrate presynaptic terminal bouton morphology of RA axons in HVC (arrowheads). Scale bars, 20 μ m.

acquired confocal images of these putative presynaptic specializations (Fig. 3). Z-stacks were used to generate isoform reconstructions of axonal segments (Fig. 3, insets). These confocal images and three-dimensional reconstructions revealed axon morphology characteristic of en passant and bouton terminaux presynaptic terminals, providing the first anatomical evidence for a direct projection from RA to HVC. Furthermore, in some of this material, axons appeared to be closely associated with parvalbumin-positive dendrites (Fig. 3*B*, white box).

RA axons elaborate presynaptic terminals in HVC

To determine whether RA axons made synapses in HVC, we constructed a lentivirus driving the expression of the presynaptically targeted fusion protein, synaptophysin–GFP (Grinevich et al., 2005), under the control of the RSV promoter (FRSyGW). Synaptophysin is a synaptic vesicle protein abundantly expressed at synapses and is a commonly used marker of presynaptic terminals (Wiedenmann and Franke, 1985; De Paola et al., 2003). Previous studies show that transgene expression of synaptophysin–GFP results in the active targeting and accumulation of GFP at presynaptic sites (De Paola et al., 2003; Grinevich et al., 2005), thereby distinguishing presynaptic terminals from adjacent axonal regions.

To determine whether our construct was suitable for localizing presynaptic terminals in songbirds, we tested whether expression of synaptophysin–GFP resulted in targeted labeling of presynaptic terminals with GFP in songbirds and whether synaptophysin–GFP-labeled compartments were tightly associated with presynaptic and postsynaptic proteins. First, we examined whether FRSyGW expression resulted in enhanced transport of GFP to axonal varicosities relative to nontargeted mCherry protein by dually infecting songbird HVC_{RA} neurons with FRSyGW and FRChW, followed by visual inspection of their axon terminals in RA (Fig. 4*A*) (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Axons exclusively labeled with synaptophysin–GFP exhibited enhanced labeling in varicosities relative to mCherry-labeled axons, which showed uniform fluorescence. In axons labeled with both GFP and mCherry, GFP labeling was seen exclusively in axonal varicosities, indicating that synaptophysin–GFP is targeted to axonal varicosities in songbird neurons. Second, using cultured mouse cortical neurons, we examined whether synaptophysin–GFP colocalized with the VGlut1, a protein necessary for loading glutamate into synaptic vesicles at excitatory synapses (Bellocchio et al., 2000). Two weeks after infecting these cultures with lentivirus containing the synaptophysin–GFP construct, we found strong overlap of GFP labeling with anti-VGlut staining in cultured cortical neurons (Fig. 4*B*). Third, we asked whether synaptophysin–GFP-labeled puncta formed close appositions with postsynaptic sites (Fig. 4*C*). We labeled the postsynaptic density with an antibody against PSD-95 in cultured cortical neurons that were previously infected with FRSyGW. Visual inspection using confocal microscopy revealed FRSyGW accumulation in clear apposition with regions stained with anti-PSD-95. In summary, these data

Figure 4. Lentivirus expressing synaptophysin–GFP selectively labels presynaptic terminals. **A**, Dual infection of HVC_{RA} neurons with lentiviruses expressing mCherry and synaptophysin–GFP leads to uniform mCherry labeling of axons, whereas GFP labeling is preferentially targeted to axonal varicosities within the axon terminal field in RA (arrowheads). **B**, Synaptophysin–GFP colocalizes with the presynaptic protein VGLut. **C**, Synaptophysin–GFP-labeled presynaptic sites form tight appositions with PSD-95. Scale bars: **A**, **B**, 5 μ m; **C**, 1 μ m.

demonstrate that FRSyGW provides selective targeting of GFP to axonal compartments in which synaptic release is likely to occur.

Having established the suitability of FRSyGW to selectively label presynaptic terminals, we used it to examine whether RA neurons synapse in HVC. Injections of FRSyGW into RA resulted in strong GFP expression in RA (Fig. 5A) ($n = 3$ birds). Labeling at the injection site was predominantly punctate, attributable to labeling of presynaptic terminals (Fig. 5A), although a few weakly labeled neuronal cell bodies were also visible at the injection site. Synaptophysin–GFP lentivirus injections in RA also resulted in puncta-like labeling in HVC (Fig. 5B–E) (supplemental Fig. 2, available at www.jneurosci.org as supplemental material), as well as the brainstem targets of RA (data not shown). Careful examination of HVC did not reveal retrogradely labeled neurons from these RA injections, indicating that the synaptophysin–GFP-labeled presynaptic terminals in HVC originate from RA. These findings show that a subset of RA neurons directly innervate HVC.

Neurons in dorsal RA project to HVC

Projection neurons in different regions of RA target different vocal-respiratory brainstem structures, with dorsal RA neurons targeting respiratory areas and ventral RA neurons innervating vocal motoneurons in XIIIs (Vicario, 1991; Wild, 1993b). To determine whether HVC-projecting RA neurons (RA_{HVC}) localize to one or both of these regions, we made a series of retrograde tracer injections into HVC using either Fast Blue-labeled ($n = 6$) or Texas Red-labeled dextran amine ($n = 4$). These tracer injections consistently resulted in retrograde labeling restricted to a

band of neurons in dorsal and caudal regions of RA (Fig. 6). The discrete location of RA_{HVC} neurons identified through retrograde labeling overlaps with the distribution of HVC neurons that have been shown previously to project onto brainstem respiratory premotor neurons in RA_m and PA_m (Wild, 1993b; Reinke and Wild, 1998).

We next compared the density of this pathway relative with that of the descending HVC_{RA} pathway. We used the optical dissector method to measure the density of retrogradely labeled neurons in dorsal RA after BDA injections into the right and left HVC and replicated this for HVC_{RA} neurons after bilateral BDA injections in RA ($n = 2$ birds, 4 hemispheres). We found that the density of RA_{HVC} neurons within this dorsal region did not differ significantly from the density of HVC_{RA} neurons (RA_{HVC}, $94,117.1 \pm 9920.85$ neurons/mm³, 2 hemispheres; HVC_{RA}, $112,940.6 \pm 10,405.14$ neurons/mm³, 2 hemispheres; $p = 0.2$, Student's t test), suggesting that RA and HVC are reciprocally and robustly interconnected. We estimated that RA_{HVC} neurons occupy ~ 10 – 25% of the volume of RA. The total volume of RA is reported to be ~ 0.21 mm³ (Gil et al., 2006); therefore, there may be as many as 2000–5000 RA_{HVC} neurons/hemisphere: $94,117$ neurons/mm³ $\times$ (0.21 mm³ $\times$ 0.10 – 0.25) = 1976 – 4941 neurons. Previous studies have estimated there are 30,000–50,000 HVC_{RA}

neurons (Wang et al., 2002), and the volume of HVC has been calculated to be 0.32 mm³ (Gil et al., 2006). This volume estimate multiplied by our neuronal density measurements yields a total of 36,140 HVC_{RA} neurons. Combined, these numbers suggest there may be as many as one RA_{HVC} neuron for every eight HVC_{RA} neurons.

RA_{HVC} neurons have descending collaterals

Retrograde labeling with nongenetic techniques often reveals incomplete neuronal morphology. To better characterize the anatomy of RA_{HVC} neurons, we retrogradely labeled these neurons using HSV-1 amplicons expressing GFP (Fig. 7A, 1 bird). The HSV-1 forms latent infections in neurons and can be used to genetically label neurons local to the injection site as well those afferent to it (Kristensson et al., 1971; Berges et al., 2007). HSV-1 injections in HVC resulted in strong GFP labeling in a small number of RA_{HVC} neurons. Dendrites of these HSV-labeled neurons were confined to within few hundred micrometers of the cell body and stayed within the borders of RA (Fig. 7B,C). Axons could be clearly traced leaving these cells, ascending out of RA, and coursing toward HVC within the HVC–RA fiber tract. Interestingly, a second projection could also be traced from individual neurons, which exited the caudoventral border of RA. Once outside of RA, these axons turned rostrally toward the occipitomesencephalic tract, which connects the pallium with the brainstem. Axons of RA_{HVC} neurons collateralize close to the soma, extending one branch upward toward HVC and a second ventrally, indicating that individual RA_{HVC} neurons likely project to a downstream target of RA.

RA_{HVC} neurons project to the expiratory premotor nucleus RA_m

Given the dorsocaudal location of these RA_{HVC} neurons, the topographic organization of RA PNs and the collateral labeling observed in RA_{HVC} neurons after HSV-1 injections in HVC, we wondered whether RA_{HVC} neurons also innervate premotor respiratory nuclei. To test this idea, we made dual retrograde tracer injections into HVC and RA_m. We targeted injections into RA_m by first mapping its location based on expiratory-locked firing patterns seen during multiunit recordings. We then made injections of cholera toxin B-chain–AlexaFluor 555 into this location, followed by Fast Blue injections into HVC (Fig. 7D) ($n = 5$). Birds were killed, and tissue was processed 4–7 d later. Sections through RA yielded retrograde labeling of RA_{HVC} neurons and RA_{RA_m} neurons in dorsal and caudal aspects of RA (Fig. 7E).

The distribution of RA_{HVC} and RA_{RA_m} neurons extensively overlapped in dorsal regions of RA. RA_{HVC} PNs tended to be confined to the most dorsal aspects of RA, perhaps occupying only the 20–25% of the dorsalmost portion of RA, whereas retrogradely labeled RA_{RA_m} neurons were distributed throughout the full dorsal third of the nucleus (Fig. 7E). In regions containing retrogradely labeled RA_{HVC} neurons, many singly labeled RA_{RA_m} neurons were visible. Conversely, the majority of retrogradely labeled RA_{HVC} neurons appeared to be double labeled by the injection into RA_m. This indicates that, in dorsal RA, some PNs project only to RA_m, some project to both HVC and RA_m, and a less abundant group of cells may project to HVC alone. In an exemplar hemisphere, we examined 130 randomly selected RA neurons retrogradely labeled from a Fast Blue injection in HVC (RA_{HVC} neurons) and found that 98 of these neurons were also labeled from an injection in RA_m with cholera toxin B-chain–AlexaFluor 555. This indicates that as many as 75% of the RA neurons that project HVC also project to the ventral respiratory column.

RA_{HVC} neurons provide rapid input to HVC

Expiratory activity is especially important for determining the fine temporal structure of individual syllables, notes, and their durations (Franz and Goller, 2002). Therefore, the present finding that certain RA neurons that target RA_m also directly innervate HVC is potentially significant. Specifically, expiratory-related song motor activity could reach HVC directly from dorsal RA, and RA_{HVC} neurons could rapidly coordinate activity across brainstem and telencephalic levels of the song motor circuit. However, during singing, both respiratory activity and HVC_{RA} neuronal activity change extremely rapidly (~ 10 ms), raising questions as to whether RA_{HVC} neuronal activity can propagate to HVC on a similarly short timescale. To address this issue, we measured the conduction velocity of RA_{HVC} neurons (Fig. 8A). We made brain slices containing HVC, RA, and portions of the

Figure 5. RA axons synapse in HVC. **A**, GFP labeling 10 d after synaptophysin–GFP lentivirus injection in RA. **B–E**, FRSynGW injections in RA result in labeling of RA presynaptic terminals within the borders of HVC, indicating that at least some neurons in RA monosynaptically project to HVC. Scale bars: **A**, 40 μ m; **B–E**, 10 μ m.

intervening fiber tract. Sharp intracellular recordings were made from neurons in dorsocaudal regions in RA, and brief current pulses were applied to the fiber tracts between HVC and RA in an attempt to evoke antidromic action potentials from RA_{HVC} neurons. Recordings were made in the presence of the excitatory synaptic blockers NBQX and D-APV, to prevent orthodromic activation of excitatory HVC synapses in RA. Using this approach, we found that RA_{HVC} neurons could be identified by their short-latency, time-locked antidromic action potentials in response to electrical stimulation of the RA_{HVC} fiber tract (Fig. 8B) ($n = 6$ cells, 3 birds). Intracellular staining with Neurobiotin and *post hoc* visualization of antidromically identified neurons revealed fine-caliber axons leaving RA and coursing toward HVC (Fig. 8C). We divided the measured distance separating the stimulation electrode from identified RA_{HVC} neurons by the latency of elicited antidromic action potentials, providing a conduction velocity of ~ 1 m/s for RA_{HVC} neurons. Given a path length of ~ 3 mm between RA and HVC and a synaptic delay of ~ 1 ms, we estimate that the conduction time from RA to HVC is ~ 4 ms. This conduction time is similar to the ~ 5 ms conduction time estimated from HVC to RA, suggesting a total reciprocal conduction time of ~ 10 ms. This suggests that RA_{HVC} neurons could participate in reciprocal interactions with HVC on a timescale similar to the observed 5–10 ms intervals between individual notes.

Figure 6. Only neurons in dorsal and caudal regions of RA project to HVC. **A**, Injection of retrograde tracers in HVC was used to localize the distribution of RA_{HVC} neurons. **B**, Injection of Fast Blue into HVC retrogradely labeled neurons in dorsal RA (transverse sections). **C**, Injections of Texas Red-conjugated dextran amines in HVC retrogradely labeled neurons in dorsal and caudal regions of RA (sagittal sections). **D**, Bilateral HVC injections with Texas Red-conjugated dextran amines retrogradely labels neurons in dorsal and caudal aspects of RA bilaterally. A, Anterior; D, dorsal; M, medial. Scale bars, 100 μ m.

Discussion

The rapid sequential vocal gestures typical of birdsong require precisely coordinated respiratory and syringeal muscle activity (Goller and Cooper, 2004; Suthers and Zollinger, 2004). Respiratory and syringeal muscle activity is controlled by a hierarchically organized motor circuit broadly similar to brain circuits for speech production in humans (Doupe and Kuhl, 1999). A traditional view maintained that HVC, which is either near or at the apex of the song motor hierarchy, does not receive direct feedback from lower levels of the song motor network, including the song premotor nucleus RA, the direct synaptic target of HVC

(Nottebohm et al., 1976). In contrast to this view, we identified a population of neurons in dorsal RA that innervate brain-stem expiratory song motor circuitry in RAm and provide synaptic input to the song-patterning network in HVC. The anatomical placement of these $RA_{HVC/RAm}$ neurons makes them well suited to roles in both song patterning and song learning.

Possible role for expiratory feedback in song patterning

The acoustic structure of song changes over a wide range of timescales and is tightly correlated with changes in respiratory patterns (Wild et al., 1998; Franz and Goller, 2002, 2003). Expiratory patterns are especially important learned features of song (Franz and Goller, 2002) because air flow through the syrinx is strongly coupled to song acoustic structure (Fee et al., 1998). We found that a subset of RA neurons that target portions of the lateral medulla known to be important to expiratory control (Wild, 1993a,b) also extend axon collaterals that terminate in HVC. Therefore, the $RA_{HVC/RAm}$ neurons described here likely provide HVC with a copy of descending motor signals that modulate expiratory patterning during singing. This direct feedback arrangement from expiratory-related regions of RA to HVC could help to rapidly and precisely coordinate transitions in song premotor activity in HVC, which previous studies have suggested are accomplished entirely by indirect feedback pathways from the brainstem (Ashmore et al., 2005).

Indeed, the placement of $RA_{HVC/RAm}$ neurons in the song motor circuit suggests that they have the potential to influence HVC activity over short timescales. Chronic recordings made in singing birds suggest that the population of coactive $HV-C_{RA}$ neurons changes every ~ 10 ms during an individual motif (Hahnloser et al., 2002; Kozhevnikov and Fee, 2007), possibly serving as a “clock” for song timing (Fee et al., 2004). In RA, the population of coactive PNs also changes every 5–10 ms (Leonardo and Fee, 2005), presumably reflecting the integration of convergent inputs from $HV-C_{RA}$ neurons. The $RA_{HVC/RAm}$ PNs are positioned to provide HVC with rapid monosynaptic feedback, with an estimated total ($HVC \rightarrow RA \rightarrow HVC$) conduction time of ~ 10 ms, commensurate with the clock speed seen in HVC during song production.

One hint that $RA_{HVC/RAm}$ neurons may influence HVC is the finding that small lesions placed in the dorsal part of RA interfere with song initiation (Ashmore et al., 2005), a process traditionally ascribed to “higher” levels of the song motor circuit, including HVC. Young zebra finches that sustain unilateral lesions of HVC can develop normal song using only the intact contralateral song motor circuit. Ashmore et al. (2008) found that such “cyclopean”

Figure 7. A proportion of RA_{HVC} neurons monosynaptically project to the medullary premotor respiratory column. **A**, HSV-1 amplicon was injected into HVC to retrogradely label RA_{HVC} neurons. **B**, **C**, HSV-1 neurons retrogradely labeled from injections in HVC have axonal collaterals projecting dorsally toward HVC and ventrally toward the occipitomesencephalic tract, which links the forebrain and brainstem. **D**, Injections of Fast Blue (FB) in HVC combined with cholera toxin (CTB)–AlexaFluor 555 in nucleus retroambigialis were used to ask whether individual RA_{HVC} neurons also innervate the medullary premotor respiratory column. **E**, Confocal images through dorsal RA show that many retrogradely identified RA_{HVC} neurons (blue) also project to the medullary respiratory column (red), indicating that a population of neurons in dorsal RA monosynaptically link HVC and medullary components of the song motor circuit. Scale bars: **A–C**, 40 μ m; **E**, 100 μ m.

birds showed significant and persistent impairment in song initiation and disruption of song linearity after small lesions placed in dorsal RA; in contrast, similar sized lesions placed in ventral RA did not affect song initiation and only transiently affected song linearity. Together, these findings suggest that PNs in dorsal RA play a role in coordinating motor signals necessary for song initiation and stereotyped execution, perhaps in part by influencing HVC directly.

Neurons in dorsal RA can potentially influence HVC directly via $RA_{HVC/RAm}$ neurons and indirectly via the respiratory brainstem (Vicario, 1991; Wild, 1993a,b; Striedter and Vu, 1998; Ashmore et al., 2005, 2008). These two pathways between RA and HVC are likely to operate over different timescales and convey different types of information. First, brainstem-mediated feedback to HVC relies on a trisynaptic circuit through the medulla and caudal thalamus (Fig. 9). Feedback to HVC via this route requires ~ 20 ms and therefore is unlikely to affect activity in HVC over the duration of a single putative clock cycle (Striedter and Vu, 1998; Ashmore et al., 2005). Second, the brainstem circuit is derived from RA inputs onto the inspiratory premotor neurons in PAm (Striedter and Vu, 1998), whereas RA_{HVC} neurons project onto expiratory premotor neurons in RAm. Although future studies will need to examine whether there is overlap between medullary inputs from $RA_{HVC/RAm}$ PNs and ascending projection from PAm to nucleus uvaeformis, it is possible that these circuits constitute separate channels, supplying HVC with either rapid expiratory-related motor feedback or

slower inspiratory-related motor feedback. Thus, direct feedback signals from RA to HVC may support timing of rapid, sequential changes in expiratory activity seen during syllable production, whereas the indirect feedback from the brainstem to HVC may act over longer timescales to convey information about inspiratory activity that occurs between individual syllables. Regardless of possible functional specializations of these direct and indirect feedback pathways from RA to HVC, the present identification of $RA_{HVC/RAm}$ neurons points to a substrate for coordinating activity across broad regions of the forebrain and brainstem during singing.

RA_{HVC} neurons provide the anterior forebrain pathway indirect access to HVC

$RA_{HVC/RAm}$ neurons also are pivotally placed to influence in song learning because they could serve to indirectly link the anterior forebrain pathway, which is necessary for song learning (Bottjer et al., 1984; Scharff and Nottebohm, 1991), with song-generating circuits in HVC. The output of the anterior forebrain pathway, the telencephalic song nucleus LMAN, is thought to drive exploratory vocal behavior necessary for song learning through a direct projection to RA (Fig. 1) (Scharff and Nottebohm, 1991; Kao et al., 2005; Olveczky et al., 2005). Convergent inputs from LMAN and HVC onto RA neurons (Canady et al., 1988; Mooney, 1992) and the absence of any known direct or indirect projection from LMAN to HVC underlie the traditional view that RA is the major locus of learning-related synaptic plasticity in the song motor

Figure 8. RA_{HVC} neurons provide short-latency input to HVC. **A**, *In vitro* brain slices containing RA and HVC combined with intracellular recording from antidromically identified RA_{HVC} neurons was used to estimate the conduction velocity of the RA to HVC pathway. **B**, Short-latency, time-locked antidromic action potentials in an RA neuron elicited by stimulation of HVC–RA fiber tract 0.7 mm dorsal to RA, yielding a conduction velocity of ~ 1 m/s. Given a path length of ~ 3 mm between RA and HVC and a synaptic delay of ~ 1 ms, an estimate of total conduction time from RA to HVC is ~ 4 ms. **C**, Intracellular staining of an antidromically identified RA neuron recorded in a brain slice preparation reveals an axon leaving RA dorsally (top) and a fine caliber long-range axon collateral coursing toward HVC (inset). A, Anterior; D, dorsal. Scale bar, 40 μ m.

circuit (Doya and Sejnowski, 1998; Fiete et al., 2007). Our identification of RA_{HVC} PNs provides a route via which LMAN could indirectly influence HVC, an arrangement that could enable the anterior forebrain pathway to modulate song premotor activity at multiple levels within the song motor circuit.

An important goal for future research will be to test the functional role of $RA_{HVC/RAm}$ neurons. Because RA_{HVC} neurons also innervate the brainstem, we cannot distinguish the function of this projection to HVC from the influence these neurons have on brainstem premotor circuits using available ablation or stimulation techniques. Instead, experiments using selective silencing or activation of only RA_{HVC} axon terminals (in HVC) during singing will be required to adequately address the function of this feedback circuit. Although such selective manipulations are currently impractical, the identification of a direct projection from RA to HVC suggests that the development and generation of song sequences involves multiple reciprocally interconnected levels of the song motor circuit.

Implications for mammalian cortical circuits and vocalization

Songbirds are one of the very few animal groups, other than humans, that possess a learned vocal communication system (Doupe and Kuhl, 1999), and only songbirds and humans

Figure 9. Schematic of feedback pathways in the song motor circuit. The song motor circuit includes feedforward projections between the telencephalic premotor and motor nuclei, HVC and RA, respectively, with RA topographically innervating brainstem vocal-respiratory networks directly. Feedback circuits impinging on HVC may play an important role in sensorimotor processing necessary to coordinated, sequential motor activity. HVC is known to receive two potential sources of song-related feedback (shown in black), including long-latency auditory feedback via the multisynaptic auditory circuit and song motor feedback through a trisynaptic circuit relaying through the inspiratory premotor medullary nucleus PAm. Here we identify a third direct, monosynaptic source of song motor feedback from neurons in dorsal RA (cell shown in blue, projection shown in red). Given that these neurons are shown to also project onto expiratory premotor medullary neurons in RAm, this feedback pathway likely provides HVC with a copy of descending expiratory-related signals, which may be important for coordinating the precise and rapid vocal and respiratory modulations typical of birdsong. Timing estimates shown reflect the total time required for a signal in RA to propagate back to HVC via three different feedback pathways in the song motor circuit.

possess a telencephalic source of direct projections to vocal motoneurons (Kuypers, 1958; Nottebohm et al., 1976; Jürgens, 2002). A potentially significant difference between songbirds and humans, in the context of respiratory-vocal control, is that the songbird telencephalic nucleus RA innervates both vocal motoneurons and respiratory premotor neurons in the medulla (Wild, 1993b), albeit via different neurons, whereas according to Kuypers, the human motor cortex does not project on respiratory premotor neurons (Holstege and Ehling, 1996, p 164). Nevertheless, recent functional magnetic resonance imaging evidence suggests that the control of phonation and voluntary expiration involved in human speech production is derived from overlapping primary sensorimotor cortical regions (Loucks et al., 2007).

Although it is not known whether these overlapping regions in humans contain neurons that, like dorsal RA neurons in songbirds, have collaterals that both descend to the brainstem and ascend to higher centers, in rats some primary motor

cortex pyramidal neurons in layer V have axons that target the brainstem and collaterals that target the primary somatosensory cortex (Veinante and Deschenes, 2003). This cortical architecture potentially provides the somatosensory cortex with a copy of neural signals being conveyed to the brainstem. More generally in mammals, the primary motor cortex exhibits strong reciprocal connections with other cortical circuits, including premotor and primary sensory cortices (Pandya and Vignolo, 1971; Jones et al., 1978; Strick and Kim, 1978; Vogt and Pandya, 1978). Our findings show that RA, which is analogous to vocal motor cortex in humans, forms reciprocal connections with HVC, which is thought to serve both cortical premotor and sensory roles important to singing. The general architectural parallels between mammals and birds indicate that feedback projections from deep layers of motor cortex or their ventral pallial equivalents in birds may play an important role in sensorimotor processing necessary to coordinated, sequential motor activity.

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