Long-Range Inhibition Within the Zebra Finch Song Nucleus RA Can Coordinate the Firing of Multiple Projection Neurons

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Spiro, John E., Matthew B. Dalva, and Richard Mooney. Long-range inhibition within the zebra finch song nucleus RA can coordinate the firing of multiple projection neurons. J. Neurophysiol. 81: 3007–3020, 1999. The zebra finch forebrain song control nucleus RA (robust nucleus of the archistriatum) generates a phasic and temporally precise neural signal that drives vocal and respiratory motoneurons during singing. RA’s output during singing predicts individual notes, even though afferent drive to RA from the song nucleus HVc is more tonic, and predicts song syllables, independent of the particular notes that comprise the syllable. Therefore RA’s intrinsic circuitry transforms neural activity from HVc into a highly precise premotor output. To understand how RA’s intrinsic circuitry effects this transformation, we characterized RA interneurons and projection neurons using intracellular recordings in brain slices. RA interneurons fired fast action potentials with steep current-frequency relationships and had small somata with thin aspinous processes that extended throughout large portions of the nucleus; the similarity of their fine processes to those labeled with a glutamic acid decarboxylase (GAD) antibody strongly suggests that these interneurons are GABAergic. Electrical stimulation revealed that RA interneurons receive excitatory inputs from RA’s afferents, the lateral magnocellular nucleus of the anterior neostriatum (LMAN) and HVc, and from local axon collaterals of RA projection neurons. To map the functional connections that RA interneurons make onto RA projection neurons, we focally uncaged glutamate, revealing long-range inhibitory connections in RA. Thus these interneurons provide fast feed-forward and feedback inhibition to RA projection neurons and could help contribute the phasic pattern of bursts and pauses that characterizes RA output during singing. Furthermore, selectively activating the inhibitory network phase locks the firing of otherwise unconnected pairs of projection neurons, suggesting that local inhibition could coordinate RA output during singing.

INTRODUCTION

Birdsong, like human speech, requires precise control of vocal and respiratory muscles. The neural circuits for birdsong are localized to a well-defined set of interconnected brain nuclei, affording an opportunity to discover the neural mechanisms enabling this precise vocal control. Here we investigate the intrinsic circuitry of the forebrain nucleus RA (robust nucleus of the archistriatum), the sole output of the telencephalic song circuitry. RA intrinsic circuitry is of general interest because it transforms higher level neural activity from HVc [used here as the full name of the nucleus, following Fortune and Margoliash (1995)] into a highly precise premotor output (Yu and Margoliash 1996).

RA has an obligatory role in the central control of learned song, as has been shown with lesion studies, chronic recordings, and microstimulation experiments (Nottebohm et al. 1976; McCasland 1987; Yu et al. 1994; Yu and Margoliash 1996; see Margoliash 1997 for a review). RA receives synaptic input from the song nucleus HVc and from the lateral magnocellular nucleus of the anterior neostriatum (LMAN), an area essential to normal song development but not to adult song production (Fig. 1A) (Bottjer et al. 1984; Scharff and Nottebohm 1991). The axons of RA projection neurons in ventral RA project topographically to the hypoglossal motoneurons that innervate muscles of the syrinx, the avian song organ, and those in the dorsal RA project to areas in the lateral medulla that control respiration (Fig. 1B) (Vicario 1993, 1994; Wild 1993a,b, 1994).

At rest, RA projection neurons display a tonic, pacemaker-like activity. During singing, this tonic pattern changes to a highly phasic pattern so precise that it predicts note identity (Yu and Margoliash 1996). The input to RA from HVc lacks these qualities; it is instead more tonic and is predictive of syllable identity, independent of the particular notes that make up the syllable (Yu and Margoliash 1996). Because the extrinsic afferents to RA are purely excitatory (Canady et al. 1988; Kubota and Saito 1991; Mooney 1992; Mooney and Konishi 1991), RA inhibitory circuits are likely to transform incoming neural activity to an appropriate output for the brain stem motor circuitry. Indeed, cells staining positive with an antiserum to GAD antibody have been detected in RA (Grisham and Arnold 1994; Sakaguchi 1996), and electrical stimulation of HVc and LMAN axons produces monosynaptic excitatory postsynaptic potentials (EPSPs) and polysynaptic inhibitory postsynaptic potentials (IPSPs) in RA projection neurons; these IPSPs are blocked by the GABA receptor antagonist bicuculline (Mooney 1992). The intrinsic physiology, connectivity, and morphology of RA interneurons, as well as their role in the transformation of afferent activity, however, have remained unknown.

Using high-resistance, dye-filled electrodes, we were able to record directly from interneurons to examine their intrinsic properties and synaptic inputs and to study their morphology. To map the spatial distribution and functional properties of interneuronal input onto RA projection neurons, we used scanning laser photostimulation (Dalva and Katz 1994; Katz and Dalva 1994; Sawatari and Callaway 1996). We show that RA interneurons can provide fast feed-forward and feedback inhibition to RA projection neurons and therefore could help create the pattern of bursts and pauses that characterizes RA output during singing. As activation of the inhibitory network can

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coordinate the firing of multiple projection neurons, and the interneurons extend functional processes across areas of RA that control either breathing or syringeal muscles, inhibition could also serve to coordinate breathing with syringeal control during singing.

Preliminary data have been presented in abstract form (Spiro et al. 1996).

**Methods**

**Tissue Preparation and Solutions**

Photostimulation and intracellular recording and labeling experiments were performed with in vitro brain slices made from male zebra finches (*Taeniopygia guttata*) ranging in age from 26 to 372 posthatch days in accordance with a protocol approved by the Duke University Institutional Animal Care and Use Committee. The details of the slice preparation procedure have been described previously (Mooney 1992; Mooney and Konishi 1991). Briefly, the bird was decapitated following ketamine injection (0.05 ml im) followed by methoxyflurane (Metofane; Mallinckrodt Veterinary, Mundelein, IL) inhalation anesthesia. The brain was removed and chilled in 4°C, equilibrated with 95% O2–5% CO2, then cut in half along the mid sagittal sinus, or blocked transversely for coronal sectioning. A vibratome was then used to cut 200- to 400-μm-thick either sagittal or coronal slices through RA (slice orientation depended on the fiber stimulation protocol used, see Electrophysiological recordings; sharp electrodes), which were immediately transferred to an interface-type holding chamber, where they were maintained at room temperature for 60–90 min.

ACSF consisted of (in mM) 119 NaCl, 2.5 KCl, 1.3 MgCl2, 2.5 CaCl2, 1 NaH2PO4, 26.2 NaHCO3, and 11 glucose. Equimolar sucrose was substituted for NaCl during the tissue preparation stage. All reagents for ACSF were obtained from Mallinkrodt Chemical; α-gluconic acid and picrotoxin were from Sigma, 1,2,3,4-Tetrahydro-2-nitro-3-dioxo-benzo(f)quinoxaline-7-sulfonamide (NBQX), R(−)-2-amino-5-phosphonovaleric acid (D(-)-APV), and lidocaine N-ethyl bromide quaternary salt (QX-314), were from RBI (Natick, MA).

**Electrophysiological recordings; sharp electrodes**

Sharp intracellular recordings of RA interneurons and projection neurons were made in an interface-type chamber (30°C; Medical Systems, Greenvane, NY) using borosilicate glass pipettes (BF-100-50-10, Sutter Instruments, Fountain Valley, CA) pulled to a resistance of 150–200 MΩ and filled with 2 M K-acetate and 5–10% neurobiotin (Vector Laboratories, Burlingame, CA). RA is clearly visible under transillumination, facilitating electrode placement. Interneuron recordings were made in slices from male birds ranging in age from 49 to 168 days post hatch (113 ± 22 days, mean ± SE). Seven interneurons in seven slices from six birds were used for electrophysiological analysis; seven additional interneurons were held too briefly for electrophysiological analysis but were filled adequately with neurobiotin for morphological analysis. Projection neuron recordings were made in slices from birds ranging from 26 to 372 days posthatch age (mean, 158 ± 38 days; not significantly different from the interneuron donor pool, P = 0.4, 2-tailed t-test). Ten cells in nine slices from seven birds were used for electrophysiological analysis. A total of >400 RA neurons were recorded from in the course of this study; projection neurons were encountered at a much higher frequency compared with interneurons (30:1). Intracellular potentials were amplified with an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA) in bridge mode, digitized at 10 kHz (low-pass filtered at 3 kHz) using a National Instruments data acquisition board (AT-MIO-16E2; Austin, TX) controlled by custom LabView software (National Instruments) written by F. Livingston and R. Neumann. In some experiments, concentric bipolar stimulating electrodes [200-μm outer pole (33-gauge stainless steel), 25-μm inner pole (platinum); FHC, Bowdoinham, ME], were placed just dorsal to RA to stimulate the RA-projecting HVC axons orthodromically (100 μs, ∼100 μA, Axon Instruments isolator-10 stimulus isolation unit). Similar bipolar electrodes were also placed in the RA outflow tract to antidromically
stimulate the local axon collaterals of RA projection neurons, or within RA to stimulate interneurons directly. In coronal slices, stimulating electrodes were also used to activate RA-projecting LM AN axons orthodromically (Mooney 1992). Data analyses were performed off-line using LabView and Origin software (Microcal, Northampton, MA). Statistical analyses were performed using JMP IN software (SAS Institute). All values reported are means ± SE.

Input resistance was calculated from the steady-state voltage deflections following the injection of −200-pA current pulses throughout the recording. Because interneurons sometimes did not fire action potentials spontaneously, measurements of spike characteristics for both cell classes were made during ±200-pA current pulses; for each neuron, measurements from at least two action potentials were averaged. Comparisons of spontaneously spiking interneurons (n = 4) and projection neurons (n = 4) revealed similar relationships (data not shown). Action potential width was measured at half maximal amplitude, afterhyperpolarization (AHP) amplitude is relative to action potential threshold voltage, and delay to the peak of AHP is relative to the time of action potential threshold. The AHP recovery reflects the slope of the initial (2 ms) depolarization following the peak of the AHP. The firing frequency versus current relationship was calculated as the slope of a linear fit to a plot of intracellular current injection amplitude versus average instantaneous firing frequency of the first two action potentials.

Cross-correlation analysis

Simultaneous intracellular current-clamp recordings were made from RA projection neurons using the two channels of an Axoclamp 2-B, while a separate site in RA was periodically stimulated with a concentric bipolar stimulating electrode. To isolate monosynaptic IPSPs, excitatory transmission was blocked by adding D-APV (50 μM) and NBQX (5 μM) to the bath. Membrane potential waveforms were passed through a second-order digital Bessel filter, the DC component was subtracted, and then the cross-correlation function was calculated with the use of a 300-ms sliding window between successive windows, using customized software written in Labview by J. Spiro and F. Livingston. The successive correlograms were then used to generate two-dimensional correlograms using Scion Image (Scion Corporation, Frederick, MD) with colors coding for the amplitude of the cross-correlation function.

Scanning laser photostimulation

The techniques used here have been described previously (Katz and Dalva 1994). Briefly, slices from young male zebra finches (30–47 days posthatch; mean, 40 days; 14 cells in 14 slices from 8 birds) were submerged in a recording chamber perfused with ACSF (room temperature) supplemented with 100 μM CNB-caged glutamate [1-glutamyl γ-(α-carboxy-2-nitrobenzyl) ester (Molecular Probes)]. Young tissue was used because of its superior viability in the submersion recording chamber, the increased ease in obtaining whole cell recordings compared with older tissue, and also to avoid light scattering caused by the heavy myelination of adult tissue.

Whole cell recordings from RA projection neurons were made with patch pipettes (4–8 MΩ) filled with an internal solution consisting of (in mM) 100 cesium gluconate, 10 EGTA, 5 MgCl, 40 HEPES, 2 sodium-ATP, 0.3 sodium-GTP, and 1 QX-314, with 0.5% neurobiotin, pH 7.25. Whole cell currents were measured with an Axopatch 1D intracellular amplifier (Axon Instruments), and current traces were digitized at 2–10 kHz after low-pass filtering at half the sampling rate. Data acquisition was performed with a TL-1 interface (Axon Instruments). Custom acquisition and analysis software (M. Dalva) permitted linking of specific electrophysiological responses to specific sites of photostimulation.

The recording chamber was mounted in a fixed position over a Zeiss microscope nose piece resting on a motorized x-y-z translation stage, the position of which was computer controlled. A dissecting microscope was used to guide the placement of patch electrodes within RA. After obtaining a whole cell recording, the laser light was scanned across the tissue systematically (50-μm increments, 2-s delay between flashes), usually over an area that included all of RA, and at least some of the archistriatal tissue immediately surrounding it. Photostimulation (i.e., the release of glutamate from the caged compound) was achieved by illuminating the tissue with a Coherent Argon ion laser (50 mW, continuous wave, model 622) transmitted through a ×40 Nikon fluor objective (1.3 NA) to the specimen. The duration of the illumination was controlled by opening briefly (5–20 ms) a mechanical shutter (Uniblitz) placed in the light path. In most cases, the same region was scanned at least twice, to determine both the reproducibility of the result and also to measure the reversal potential of the evoked postsynaptic currents (PSCs). The numbers of PSCs from repeated scans were averaged to generate both the histograms and the stimulation maps. Fiducial marks were placed in the tissue by injecting fluorescent latex microspheres into several distinct points around RA at the end of the experiment; these spots were then illuminated with laser light, and their positions were recorded along with the photostimulation map. These marks permitted alignment of photostimulation maps with histological sections produced in the neurobiotin processing.

Synaptic events were discriminated and counted using a previously described algorithm (Dalva and Katz 1994). Briefly, the instantaneous derivative was calculated for each point in the first 70 ms after the stimulus. PSCs were defined by two zero crossings and a slope of ≥2 SD larger than the average derivative during the last 150 ms of the trace. If a synaptic event was detected at short latency (50 ms), then all synaptic events were counted that occurred within a 200-ms postflash window.

In some cases, RA was remapped in the presence of picrotoxin (PTX). Blocking GABA A currents with PTX has no effect on the size of photostimulation-evoked glutamatergic currents (M. B. Dalva and L. Katz, unpublished observations). The recirculating perfusion system used for photostimulation experiments prevented wash out of PTX and recovery of photostimulation-evoked inhibitory postsynaptic currents (IPSCs).

Histology of RA neurons and Sholl analysis

Individual RA neurons were filled with neurobiotin using either depolarizing current pulses (~0.5 nA, 500 ms, 50% duty cycle), or simply by diffusion (whole cell recordings). Slices were then fixed in 4% paraformaldehyde in 0.025 M sodium phosphate buffer (PB) for a minimum of 12 h at 4°C. resectioned on a vibratome at 75 μm, and the neurobiotin was then visualized by standard techniques [Vectorstain ABC, dilution of 1:1,000 followed by application of 0.05% diaminobenzidine (DAB), 1.5 × 10−7 H2O2 in PB enhanced with 1% cobalt chloride and 1% nickel ammonium sulfate added to the DAB solution]. A stained cell was categorized as a projection neuron if it had either an axon that left the nucleus or thick, radially symmetrical spinous dendrites, or as an interneuron if it had thin, aspinous neurites simply by diffusion (whole cell recordings). Slices were then illuminated with laser light, and their positions were recorded along with the photostimulation map. These marks permitted alignment of photostimulation maps with histological sections produced in the neurobiotin processing.
collaterals for either cell type because it was difficult to distinguish between these two types of processes for interneurons.

**GAD immunohistochemistry**

Adult zebra finches were anesthetized with a lethal dose (60–80 μl) of Equithesin (0.85 gm of chloral hydrate, 0.21 gm of pentobarbital sodium, 0.42 gm of MgSO₄, 1.8 ml of 100% ethanol, and 8.6 ml of propylene glycol to a total volume of 20 ml with H₂O) and then perfused transcardially with 0.9% saline for 5 min followed by 4% paraformaldehyde in 0.025 M PB for 30 min. The brain was removed from the skull and postfixed in 4% paraformaldehyde and 20% sucrose in 0.025 M PB overnight at 4°C, then blocked sagittally and resectioned at 30 μm on a freezing microtome. Sections were collected into Tris-buffered saline (TBS), blocked in 4% normal rabbit serum (NS) for 30 min at room temperature (RT), rinsed in TBS (3 × 25 min), then incubated in antiserum to GAD (1:2,000 in TBS, 48 h at 4°C) made in sheep against partially purified rat brain GAD. The antiserum was provided from a source (1440 – 4) developed at the National Institutes of Health by Drs. Irwin J. Kopin, Wolfgang Oertel, Donald E. Schmechel, and Marcel Tappaz. Effective use in immunocytochemistry was greatly aided through the laboratory of E. Mugnaini (University of Connecticut, Storrs). The sections were rinsed in TBS (3 × 15 min), then incubated with biotinylated rabbit anti-sheep IgG (Vector) at 1:1,500 in TBS at RT for 60 min, rinsed in TBS (3 × 15 min), and transferred to Vector Elite ABC reagent for 1 h at RT. After a standard DAB reaction (see *Histology of RA neurons and Sholl analysis*), the sections were mounted on subbed slides, dehydrated through alcohols, cleared in xylene, and coverslipped.

**Western blot**

Tissues for Western blot analysis (i.e., cerebellum, forebrain, liver) were removed from an adult male zebra finch previously anesthetized with Metofane and killed by decapitation. The tissues were then homogenized immediately in a 10-fold volume of Ca²⁺ - and Mg²⁺-free PBS (pH 7.4) with 5 mM EGTA, 0.5% sodium dodecyl sulfate (SDS) and DNase (2,000 units). Western blots were performed as detailed in Gutman et al. (1997). Briefly, protein concentration of the homogenates was determined using the DC Protein Assay Kit (Bio-Rad). Samples were incubated in 2 times SDS-Laemmli buffer, and proteins were separated with SDS–polyacrylamide gel electrophoresis (7.5% gel) with 20 μg of protein loaded per lane. They were then transferred to a polyvinylidene fluoride membrane, blocked in Blotto [5% dried milk in TBS, pH 7.6, with 0.05% Tween (Surfact Amps-20, Pierce, Rockford, IL)], and incubated in primary antibody (NIH 1440 anti-GAD, 1:500) for 1 h (dilutions up to 1:7,000 yielded similar results). Membranes were then washed and incubated with secondary antibody for 1 h (HRP-conjugated anti-sheep IgG diluted 1:2,500, Boehringer Mannheim). The secondary antibody was then visualized using a chemiluminescent substrate exposed to Hyperfilm. Similar blots were also made using a commercial anti-GAD antibody (Chemicon AB108, 1:2,000) using an HRP-conjugated anti-rabbit IgG as the secondary (1:5,000).

**RESULTS**

**Interneuron physiology: intrinsic**

We recorded intracellularly from RA interneurons and made direct comparisons of their electrophysiological and morphological properties to those of projection neurons (see Table 1 for all statistical comparisons between interneurons and projection neurons). This new cell type was encountered at very low frequency (~1 of every 30 stable recordings), had intrinsic electrophysiological properties that differed from projection neurons, and was subsequently confirmed morphologically to be a RA interneuron (see *Interneuron morphology*). We recorded from seven of these cells long enough to collect electrophysiological and morphological data; seven other cells were held too briefly to analyze electrophysiologically, but were filled adequately with neurobiotin for morphological analysis.

RA interneurons were characterized by fast action potentials (half-height width 0.40 ± 0.02 ms; Fig. 2, A and B) and by very high-frequency trains of action potentials with varying interspike intervals in response to depolarizing currents (254 Hz/nA; Fig. 2, A and C). RA interneurons fired few or no action potentials at their resting potential (~66 ± 3 mV). In contrast, RA projection neurons fired broader action potentials (0.83 ± 0.11 ms; Fig. 2, A and B), lower frequency (90 Hz/nA), and more regular trains of action potentials in response to depolarizing currents, and displayed spontaneous pacemaker-like action potentials (~15 Hz, Fig. 2, A and C) (Mooney 1992).

Although neither interneuron nor projection neuron action potential trains accommodated significantly with low-amplitude currents, some accommodation occurred with larger currents in both cell types but was not a reliable measure for distinguishing cell type. Although interneurons and projection neurons differed markedly in their responses to similar depolarizing currents, they did not differ significantly in their input impedances (Table 1).

During spike trains, a sigmoidal depolarizing phase preceded each interneuron action potential, whereas a smooth monotonic trajectory preceded each projection neuron action potential (Fig. 2B). Differences were also evident in the slope of the recovery from the peak of the afterhyperpolarization: interneurons recovered with a much steeper slope than did projection neurons (2.4 ± 0.4 vs. 0.4 ± 0.1 mV/ms). These differences in spike shape and the marked difference in firing rates in response to similar depolarizing currents (Fig. 2C).

**TABLE 1. Comparison of intrinsic properties of RA interneurons with RA projection neurons**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Input Resistance, mΩ</th>
<th>V_m, mV</th>
<th>Action Potential Width, ms</th>
<th>Action Potential Height, mV</th>
<th>AHP Amplitude, mV</th>
<th>Delay to Peak of AHP, ms</th>
<th>Slope of AHP Recovery, mV/ms</th>
<th>Firing Frequency/Current, Hz/nA</th>
<th>Soma Area, μm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intereuron</td>
<td>87 ± 12 (7)</td>
<td>−66 ± 3 (7)</td>
<td>0.40 ± 0.02 (7)</td>
<td>47 ± 3 (7)</td>
<td>25 ± 2 (7)</td>
<td>1.7 ± 0.2 (7)</td>
<td>2.4 ± 0.4 (7)</td>
<td>254 (7)</td>
<td>136 ± 12 (14)</td>
</tr>
<tr>
<td>Projection neuron*</td>
<td>121 ± 15 (9)</td>
<td>Tonically active</td>
<td>0.83 ± 0.11 (10)</td>
<td>63 ± 4 (10)</td>
<td>24 ± 1 (10)</td>
<td>4.4 ± 0.4 (10)</td>
<td>0.4 ± 0.1 (10)</td>
<td>90 (10)</td>
<td>204 ± 12 (13)</td>
</tr>
<tr>
<td>P value*</td>
<td>P = 0.1</td>
<td>P &lt; 0.005†</td>
<td>P &lt; 0.007†</td>
<td>P = 0.8</td>
<td>P &lt; 0.0002†</td>
<td>P &lt; 0.0001†</td>
<td>P &lt; 0.0001†</td>
<td>P &lt; 0.0001†</td>
<td>P &lt; 0.0006†</td>
</tr>
</tbody>
</table>

Values are means ± SE with number of neurons in parentheses and ranges in brackets. RA, robust nucleus of the archistriatum; AHP, afterhyperpolarization. * P values determined with a 2-tailed Student’s t-test. † Statistically significant differences. ‡ Statistical significance determined with 2-way ANOVA.

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FIG. 2. RA neurons can be divided into 2 classes, each with distinct intrinsic electrophysiological properties. A: sub- and suprathreshold responses of RA neurons differed in response to intracellular current injection. One class of neurons (with interneuron morphology; see Fig. 3) fired few if any action potentials spontaneously (range = 0–12 spikes over a 1-s interval; mean = 7 Hz for those neurons that were active at the resting potential), but did fire high-frequency trains of action potentials in response to depolarizing currents; the 2nd class (with projection neuron morphology; see Fig. 3) spontaneously fired regularly spaced action potentials (~15 Hz). Below the raw data traces are shown plots of the instantaneous firing frequency in response to different current pulses as a function of time. Note the pronounced variability in interneuron interspike interval relative to that of projection neurons. B: individual projection neuron and interneuron action potentials could be distinguished most readily by their different widths and the contrasting slopes of the rising phase following the peak of the afterhyperpolarization (dotted line indicated by arrow; also see Table 1). Overlaid action potentials in response to +200 pA current were recorded from slices prepared from adult tissue. C: interneurons have a steeper current–action potential firing frequency relationship than do projection neurons. At each current injection amplitude, the instantaneous action potential firing frequency for the 1st 2 spikes was averaged, and the data were pooled across cell types (also see Table 1). The current–action potential firing frequency relationship for each cell type was fit with a line as a means for comparing the 2 cell types (n = 10 projection neurons, r = 0.990; n = 7 interneurons, r = 0.997).
permitted us to readily distinguish between these cell types during recordings.

**Interneuron morphology**

Intracellular staining revealed that these two electrophysiologically distinct cell types also had marked morphological differences. Interneurons had small cell bodies (136 ± 12 μm²) with extensive processes that were thin and aspinous and often appeared beaded (Figs. 3, A and B, and 5A). Despite the great extent of these processes, they did not exit RA. Swellings could be detected in certain processes that resembled presynaptic specializations. In contrast to the interneurons, the projection neurons had a larger average soma size (204 ± 12 μm²), thick spiny dendrites, and an axon that clearly exited along the rostral-ventral border of RA (Fig. 3A, arrow). Projection neuron axons also elaborated a thin axon collateral that branched extensively in a region similar to the parent cell’s dendritic field (Fig. 3A). Sholl analysis confirmed that the extent of an average interneuron’s processes was more than twice that of a typical projection neuron: although in all cases the dendrites and axon collaterals of projection neurons were restricted to within a 200-μm radius of the cell body, interneuronal processes extended in both dorsal-ventral and rostrocaudal directions up to 500 μm away from the cell body (Fig. 4). Thus, in contrast to a projection neuron, a single interneuron extends across large portions of RA. Although subregions of RA project preferentially to syringeal or respiratory motoneurons (Fig. 1), we did not detect any measurable systematic difference in morphology with a neuron’s position in the nucleus.

**GAD immunohistochemistry**

We suspected that the interneurons studied here were GABAergic neurons based on their comparatively smaller soma size relative to projection neurons, consistent with previous studies of GABA-immunoreactive RA neurons (Grisham and Arnold 1994; Sakaguchi 1996). To test this idea, we stained zebra finch tissue for GAD, the synthetic enzyme for GABA, and compared the morphology of immunopositive cells with those interneurons identified physiologically and filled intracellularly with neurobiotin (attempts to label neurobiotin-filled interneurons directly with GAD were unsuccessful).
This antibody labeled cells and processes diffusely throughout the zebra finch brain, including dense labeling of cerebellar Purkinje cells, a well-documented population of GABAergic neurons. This staining produced especially dense labeling of fine processes in RA. These GAD-positive processes, often beaded, strongly resembled the processes that were labeled by neurobiotin during interneuron recordings (Fig. 5), suggesting that the fast-spiking aspinous interneurons that we recorded from here are GABAergic.

Western blots of both homogenized cerebellum and forebrain of an adult male zebra finch were used to provide additional confirmation of the specificity of the antibody in avian tissue (Fig. 5C). As a negative control, liver was subjected to identical analysis. The antibody clearly labeled a protein doublet at 61 and 59 kD in the cerebellum and forebrain, without evidence of labeling in the liver, in agreement with published studies using the same antibody in rat (the species to which it was generated). A doublet of the same molecular weight was also observed using another polyclonal anti-GAD antibody in zebra finch [this study, data not shown, and in a recent report (Luo and Pertel 1999)], and with other anti-GAD antibodies in a variety of other vertebrate species (e.g., Gottlieb et al. 1986).

Interneuron physiology: synaptic

To determine the sources of synaptic input onto RA interneurons directly, we made intracellular recordings from them and stimulated fibers originating from HVc and LMAN, as well as antidromically activating RA projection neuron axon collaterals. Previous studies have shown that electrical stimulation of HVc and LMAN axons can elicit disynaptic IPSPs from RA projection neurons and can trigger the release of ³H-GABA from RA (Mooney 1992; Sakaguchi et al. 1987), suggesting that these extrinsic inputs directly excite GABAergic interneurons in RA. Here, consistent with earlier studies, stimulation of the HVc axons elicited compound PSPs in interneurons (Fig. 6A). Such interneurons could fire an action potential on the shortest latency PSP (mean latency from the stimulus artifact = 2.8 ms, rise time = 7.5 mV/ms; n = 3 cells), which is consistent with the existence of a direct excitatory projection from HVc onto RA interneurons. Similar results were obtained by stimulating LMAN fibers, as well as...
RA projection neuron collaterals (Fig. 6A; mean latencies = 2.1, 1.2 ms; rise times = 7.4, 14.0 mV/ms, respectively). In fact, a single interneuron could receive input from both LMAN and HVc (Fig. 6A).

In addition to excitatory inputs, RA interneurons receive inhibitory inputs, because spontaneous hyperpolarizing synaptic events were commonly observed while the cell was at its resting potential (Fig. 6B). Given the excitatory nature of RA’s afferents (Kubota and Saito 1991; Mooney and Konishi 1991), these inhibitory inputs are likely to arise from other inhibitory neurons within RA. Due to the difficulty in obtaining long, stable recordings from interneurons, and the use of an interface chamber for maximum brain slice viability, however, we were unable to describe the neurotransmitter receptors involved in synaptic transmission onto interneurons in more detail.

In summary, similar to RA projection neurons, RA interneurons receive synaptic inputs from the two sources of afferent drive to RA, HVc, and LMAN, as well as from intrinsic sources, including axon collaterals of projection neurons and other inhibitory interneurons. Thus RA interneurons receive synaptic inputs that could enable them to participate in both feed-forward and feedback processes in RA.

**Photostimulation**

The extensive processes of RA interneurons suggested to us that they could participate in long-range inhibitory processes within RA. To test this idea, photostimulation coupled with whole cell recordings from RA projection neurons was used to assess the spatial extent of RA inhibitory networks. To discriminate evoked inhibitory postsynaptic currents (IPSCs) from excitatory postsynaptic currents (EPSCs), we set the holding potential between −40 and −30 mV, where GABA_A receptor–mediated IPSCs are outward and EPSCs are inward-going.

Photostimulation within RA elicited robust IPSCs from all RA projection neurons tested (14 cells in 14 slices from 8 birds; Fig. 7, A and B). For each cell, we stimulated 585 ± 56 points centered roughly on the recording site and covering an area of 0.54 ± 0.06 mm²; this stimulated area was ~50% greater than the area occupied by RA in these slices (0.35 ± 0.03 mm²). The areas providing synaptic input onto a single projection neuron extended dorsoventrally and rostrocaudally through a major portion of the nucleus. The spatial distribution of locations that evoked IPSCs from all recorded cells is shown in Fig. 8, where the percentages of stimulated sites from which IPSCs could be evoked are plotted as a function of distance from the recording site. Although the density of sites providing input drops off with distance, a given RA neuron can receive synaptic input from a large portion of the nucleus, because >10% of the sites stimulated at 400–500 μm from the recording site still evoked IPSCs. We suspect that spontaneous IPSCs generated false positives at a certain low frequency, as for some sites clearly outside of the borders of RA (Fig. 7). That these are indeed false positives and not indicative of actual...
connections onto RA neurons comes from other lines of evidence (see Fig. 9) (and see Mello et al. 1998), which suggest that sites in the surrounding archistriatum do not make synaptic contacts onto RA projection neurons.

To determine whether the IPSCs elicited by photostimulation were GABA-mediated, we applied the noncompetitive GABA_A receptor antagonist PTX (50 μM) to the slice after first mapping responses in control conditions. Subsequent mapping in the presence of PTX revealed that the evoked IPSCs were blocked completely, confirming that they were mediated by GABA_A receptors (n = 3; Fig. 7C). To measure the effect of 50 μM picrotoxin on the size of evoked events more quantitatively, we made whole cell recordings of identically prepared slices with the same concentration of picrotoxin, but used electrical stimulation of an RA afferent (LMAN) instead of photostimulation to evoke polysynaptic IPSCs. In this configuration (unlike photostimulation), we could measure the amplitude of an evoked IPSC over many trials and compare it with the amplitude evoked after the application of picrotoxin. As with the responses evoked by photostimulation, electrically
evoked IPSCs were essentially completely eliminated 5 min after the introduction of picrotoxin (they were reduced in amplitude by 95 ± 2%; the remaining current was within the noise level of the recordings; n = 3 cells; data not shown). These results indicate that neurons local to RA provide GABAergic inhibitory input onto both near and distant RA projection neurons.

We did not evoke large excitatory responses in the projection neurons with photostimulation, which was somewhat surprising given that local excitatory connections have been demonstrated in RA. For example, putative excitatory synaptic profiles of local origin are seen at the electron microscope (EM) level on RA projection neuron dendrites in canaries (Canady et al. 1988), and antidromic activation of zebra finch RA projection neurons elicits excitatory synaptic currents in other RA projection neurons, presumably through local axon collaterals (Perkel 1995). The lack of evoked excitation can be partially explained by our choice of holding potentials, which increased the driving force for chloride-mediated responses and decreased the excitatory responses. Furthermore, we were able to exclude the possibility that excitatory responses were simply masked by otherwise robust inhibitory inputs, because photostimulation in the presence of GABA_A blockers did not reveal any large excitatory responses.

**Electrical stimulation in RA**

Because the variable latency from the laser flash to the evoked response (see **DISCUSSION**) prevented an unequivocal determination that the long-range responses evoked here by photostimulation were monosynaptic, we also used focal electrical stimulation to map the RA inhibitory circuit. To directly activate monosynaptic IPSPs, and prevent indirect activation of IPSPs by intervening excitatory pathways, slices were bathed in the glutamate receptor antagonists D-APV (50 μM) and NBQX (5 μM). An intracellular recording from a projection neuron was then maintained while a number of sites within RA and in the surrounding archistriatum were stimulated sequentially using a small concentric bipolar electrode (Fig. 9A). Recordings were made from projection neurons near the borders of RA to maximize the potential distance between the stimulating and recording electrodes (n = 3 recordings from 3 slices). IPSPs could be elicited at all sites tested in RA, even those where recording and stimulating electrodes were maximally separated (Fig. 9A, e.g., *position f*). Consistent with a monosynaptic input, these IPSPs were elicited at short latencies and with low stimulus currents. As a further test of their monosynaptic nature, individual sites also were stimulated at a high frequency (50–100 Hz), which would be expected to cause failure in polysynaptic pathways. However, these evoked IPSPs followed the stimulus 1:1, even at high frequency of stimulation (~65 Hz; Fig. 9B; n = 3 cells). At even higher stimulus frequencies the IPSPs did not fail, but rather the deflection of the voltage trace was maintained, and it became difficult to resolve individual PSPs due to capacitive artifacts. To ensure that such electrical stimulation at more distant sites within RA was indeed focal, and not merely activating inhibi-

![Image](http://jn.physiology.org/DownloadedFrom)
itory cells proximal to the recorded cell via current spread, the stimulating electrode was moved to positions closer to the recording electrode, but just outside the borders of RA (i.e., Fig. 9A, positions a and b). At these sites, no PSPs were evoked even with extremely high currents (500–1,000 μA). Taken together with the photostimulation data and the cellular anatomy of the interneurons, these results are consistent with long-range inhibitory circuits in RA.

Local GABAergic inhibition phase locks RA projection neuron firing

The anatomic structure of RA’s inhibitory circuitry could be especially well-suited to coordinate the firing of spatially and functionally distinct sets of RA projection neurons. In other systems, GABAergic inhibitory circuits resembling those described here can function to phase lock the firing of otherwise unconnected neurons (Cobb et al. 1995; Stopfer et al. 1997). To test whether the inhibitory network that we have described here could serve a similar function, we made intracellular recordings from pairs of projection neurons and selectively activated the local inhibitory network with electrical stimulation by including glutamate receptor antagonists in the bath (NBQX, APV; see METHODS). No evidence of synaptic or electrical coupling was observed between these cell pairs (n = 4; for the 3 cell pairs later recovered histologically, the somata were separated by 200–400 μm in the sagittal plane, mean = 283 μm), or between other pairs (n = 20) examined in the absence of the glutamate receptor antagonists. RA projection neurons displayed a spontaneous, pacemaker-like action potential discharge at rest, as previously described in vitro (Mooney 1992; present study) and in vivo (Yu and Margoliash 1996). Here, pairwise recordings revealed that these action potential trains were uncorrelated (n = 4 cell pairs; Fig. 10, 2 bottom traces). In contrast, stimulus trains (~10–20 Hz) generated IPSPs that were strongly phase locked to each stimulus; the effect of the IPSPs was to modulate each projection neuron’s pacemaker-like activity and strongly entrain the subsequent...
action potential. Thus the action potential firing of the two cells became strongly phase locked to each other, resulting in high degrees of cross-correlation ($n = 4$ cell pairs; Fig. 10, 2 top traces). We computed the mean area underneath the rectified cross-correlation function for each dual projection neuron recording for three time points before the stimulus was turned on, and for three time points while the stimulus was on, and compared those numbers as a relative measure of the change in the degree of correlation. For the four cell pairs, the mean increase in area was 63% (range: 5–115%).

**DISCUSSION**

**General conclusions**

Understanding the neural mechanisms for birdsong requires elucidating the cell types and connectivity of vocal premotor areas. The intrinsic circuitry of the song nucleus RA is especially important in this regard because of its role in generating highly precise outputs for the coordinated control of breathing and syringeal muscles during singing (Suthers 1997; Yu and Margoliash 1996). Toward this goal, we have explored RA’s intrinsic circuitry and detected a class of RA interneuron that provides GABAergic inhibition to RA projection neurons. RA interneurons are well suited to transform afferent activity from HVc into an appropriate premotor output because they 1) can provide inhibition that might enable the transition in RA projection neurons from tonic to phasic activity during singing, 2) form long-range connections and thus link disparate parts of RA, and 3) can coordinate the firing of multiple RA projection neurons.

**Evidence for two distinct classes of neurons in RA**

Using sharp intracellular recording in RA, we detected a class of interneuron distinguished electrophysiologically and morphologically from RA projection neurons. Interneurons fired brief action potentials with a steep current-frequency relationship, and fired only sporadically at rest. In contrast, projection neurons fired broader action potentials, had shallower current frequency relationships, and were spontaneously and rhythmically active at rest (see also Mooney 1992). Interneurons had small somata and long aspinous thin processes that projected widely throughout RA without exiting the nucleus. Projection neurons had larger somata, thick spiny dendrites, and an axon that exited the nucleus.

For several reasons we believe that the interneurons we recorded from are GABAergic: consistent with GABA-positive cells in previous studies (Sakaguchi 1996), the average soma area of interneurons recorded here was on average 33% smaller than that of projection neurons. In addition, the interneurons we encountered had thin and beaded processes closely resembling those processes that stain positive for GAD. Finally, the interneurons that we recorded are also remarkably similar in morphological and physiological properties to putative interneurons of another song control nucleus, HVc (Dutar et al. 1998; Kubota and Taniguchi 1998), and to well-described GABAergic interneurons of the mammalian neocortex. Like RA interneurons, neocortical basket cells have dendrites that are aspinous and beaded, fire very short-duration action potentials, and are capable of firing at high frequencies (Azouz et al. 1997; Thomson and Deuchars 1997).

Although the intrinsic properties and morphology of RA neurons supports the classification into at least two cell types, interneuron and projection neuron, this classification is not exhaustive. Based on Golgi material, Gurney (1981) also detected two classes of neuron in the zebra finch RA, spiny and aspinous. In contrast to our observations that the aspinous neurons did not project an axon that exited the nucleus, however, Gurney reported that in several examples, both types of neurons sent an axon outside of the nucleus. Also, in another songbird, the canary, DeVoogd and Nottebohm (1981) distinguished four classes of neurons based on Golgi staining; these included small nonspiny neurons and neurons with thick spiny dendrites, resembling the interneurons and projection neurons described in this report. In addition, however, they reported two other classes of spiny neuron, one like projection neurons that we have filled near the borders of RA, whose dendrites were asymmetric and directed inward toward the center of RA, and another cell class that was moderately spiny with finer dendrites. In short, our classification of RA neurons into projection and interneurons may need to be further expanded. Recent evidence in the mammalian hippocampus suggests that subsets of interneurons defined by morphological, physiological, or pharmacological properties often do not coincide, suggesting that interneurons may not be easily categorized into distinct groups, or the number of groups may be very large (Parra et al. 1998).

**Evidence from photostimulation and electrical stimulation for functional long-range GABAergic inhibition in RA**

The photostimulation data presented here provide direct functional evidence that long-range inhibition acts in RA via GABA$_A$ receptors on projection neurons. Focally uncaging glutamate evoked IPSCs in a projection neuron even when stimulating 400–600 μm from the projection neuron’s cell body. Because inhibitory currents were usually the first evoked responses that we observed, the long-range inhibitory responses elicited here by photostimulation were likely to be monosynaptic. However, as was the case in previous studies in cortex (Dalva and Katz 1994; Sawatari and Callaway 1996), we observed a variable latency from the laser flash to the evoked response, probably due to variability in the time required for uncaged glutamate to diffuse to the presynaptic cell and reach a high enough concentration to cause spiking. This feature, along with low maximal rates of stimulation, makes an unequivocal distinction between mono- versus polysynaptic pathways problematic with this technique. We were also restricted to using juvenile birds for this analysis for technical reasons outlined in Methods. More direct evidence that the long-range inhibition studied here is monosynaptic, and also is present in adult birds, comes from electrical stimulation experiments conducted in the presence of glutamate receptor antagonists, which confirmed that short-latency IPSPs could be evoked from projection neurons even while stimulating at distant sites within RA. These IPSPs could follow at high frequencies of stimulation, characteristic of a monosynaptic connection. When the results of photostimulation and electrical stimulation experiments are taken together with the anatomic structure of RA interneurons, they provide strong evidence for long-range monosynaptic inhibition within RA.

Further studies combining cell fills and EM level analysis
will be needed to develop a more quantitative description of the connections in the nucleus, such as how many projection neurons are contacted by a single interneuron, and the number of interneurons that are contacted by a given projection neuron’s collaterals.

**Roles of inhibitory circuits in RA**

**TRANSITION FROM TONIC TO PHASIC FIRING AND INCREASE IN PRECISION.** Here we show that axons from HVc and LMAN provide direct excitatory input onto RA interneurons, confirming and extending earlier electrophysiological (Mooney 1992) and biochemical (Sakaguchi et al. 1987) evidence for feed-forward GABAergic pathways within RA. These same interneurons also are likely to participate in feedback inhibition, because antidromic activation of RA projection neuron axon collaterals can also elicit short-latency EPSPs. Based on their intrinsic firing properties and synaptic connections, we speculate that RA interneurons transform afferent HVc activity into a more temporally precise pattern of RA projection neuron firing that is then relayed to vocal and respiratory motoneurons. Although RA projection neurons are tonically active when the bird is silent, they display highly phasic bursts of action potentials during singing that alternate with sharp transitions to periods of total silence (Yu and Margoliash 1996). As the pattern of HVc activity during singing lacks this phasic quality (Yu and Margoliash 1996), and HVc terminals in RA are excitatory (this study; Kubota and Saito 1991; Mooney 1992; Mooney and Konishi 1991), local inhibitory interneurons could mediate the tonic to phasic transition in RA. Some longer time scale features of this inhibition could also be achieved by other mechanisms, including slow afterhyperpolarizations of RA projection neurons (Spiro, unpublished observations), and GABA<sub>B</sub> receptor-mediated IPSPs, as detected in HVc (Schmidt and Perkel 1998). Nonetheless, the intrinsic properties of the RA interneurons described here are especially well-suited for the rapid initiation of inhibition. The fast action potentials and steep current-frequency relationships of RA interneurons would allow fast feed-forward inhibition that could sharpen the slower monosynaptic EPSPs evoked by HVc and LMAN axon terminals. This mechanism could reduce jitter by narrowing the time window during which correlated afferent activity drives RA neurons to fire.

**LINKING FUNCTIONALLY SPECIALIZED SUBDOMAINS.** RA interneurons have neurites up to 400 μm long, and projection neurons have dendritic processes that extend 150 μm from their somata (Fig. 4), suggesting that activity in an interneuron could influence a projection neuron up to ~550 μm distant, which is in close agreement with the results achieved with photostimulation and electrical stimulation. These long-range synaptic connections span spatially disparate regions of RA (i.e., dorsal vs. equatorial and ventral RA) that ultimately project to respiratory or syringeal motoneurons. Therefore the interneurons could serve as one neural substrate for coordinating breathing and syringeal muscles observed during singing (see Suthers 1997 for a review). These interneurons might also play a similar role in coordinating different syringeal muscles, because interneurons also link equatorial and ventral regions of RA, which innervate distinct pools of hypoglossal motoneurons that control either ventral or dorsal syringeal muscles (Fig. 1B) (Vicario 1991; Vicario and Nottebohm 1988).

**COORDINATION OF ACTIVITY IN RA.** Beyond a transient suppression of RA projection neuron firing, the present study reveals that RA interneurons can also coordinate the firing of otherwise unconnected and previously unsynchronized output neurons. This role of intrinsic circuitry is directly analogous to that of GABAergic interneurons in other systems (Cobb et al. 1995; Stopfer et al. 1997). For example, using dual recordings of hippocampal pyramidal cells and minimal stimulation in the presence of glutamate receptor blockers to selectively activate inhibitory interneurons, Cobb et al. (1995) demonstrated that interneurons can transiently phase lock previously unsynchronized pyramidal cell firing. In sensory systems, such synchronization has been shown to be important for encoding and discriminating different stimuli (Stopfer et al. 1997). In the vocal premotor system, such synchronized firing could be used to encode and coordinate the integrated movements of the vocal and respiratory systems necessary to birdsong. For example, interneurons receiving excitatory input from HVc during singing could transiently create discrete functional domains of RA projection neurons by phase locking their activity; such an activity pattern could be the neural code for a particular note. A different activity pattern from HVc onto another interneuron might sculpt a new domain of activity for the subsequent note. Such a transient reconfiguration of circuitry is analogous to that which occurs in the stomatogastric ganglion under the influence of various neuromodulators (for review, Harris-Warrick and Marder 1991), albeit at a much slower time scale.

**ROLE IN LEARNING.** Both note duration and structure are learned features of birdsong. Because RA is the first site within the descending vocal motor pathway where temporally precise neural coding for these features emerges, it is strongly implicated as a locus for synaptic modification underlying song learning. Excitatory synapses on the interneurons that we describe here are a potentially important cellular site within RA for these changes, especially if they display use-dependent synaptic plasticity, as described for excitatory inputs onto inhibitory interneurons in other systems, for example in the hippocampus (Maccalferri et al. 1998; McMahon and Kauer 1997). Because single inhibitory interneurons in RA can exert a widespread influence on projection neurons, changes in excitatory drive onto a single neuron could significantly alter the firing pattern of many RA projection neurons, thus strongly influencing song quality. The interaction of LMAN and HVc inputs on RA interneurons during song learning may be an important aspect of such a process.

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