Synaptic interactions between telencephalic neurons innervating descending motor or basal ganglia pathways are essential in the learning, planning, and execution of complex movements. Synaptic interactions within the songbird telencephalic nucleus HVC are implicated in motor and auditory activity associated with learned vocalizations. HVC contains projection neurons (PNs) (HVCRA) that innervate song premotor areas, other PNs (HVCX) that innervate a basal ganglia pathway necessary for vocal plasticity, and interneurons (HVCINT). During singing, HVCRA fire in temporally sparse bursts, possibly because of HVCINT–HVCRA interactions, and a corollary discharge can be detected in the basal ganglia pathway, likely because of synaptic transmission from HVCRA to HVCX cells. During song playback, local interactions, including inhibition onto HVCX cells, shape highly selective responses that distinguish HVC from its auditory afferents. To better understand the synaptic substrate for the motor and auditory properties of HVC, we made intracellular recordings from pairs of HVC neurons in adult male zebra finch brain slices and used spike-triggered averages to assess synaptic connectivity. A major synaptic interaction between the PNs was a disynaptic inhibition from HVCRA to HVCX, which could link song motor signals in the two outputs of HVC neurons in adult male zebra finch brain slices and used spike-triggered averages to assess synaptic connectivity. A major synaptic interaction between the PNs was a disynaptic inhibition from HVCRA to HVCX, which could link song motor signals in the two outputs of HVC neurons in adult male zebra finch brain slices and used spike-triggered averages to assess synaptic connectivity. 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Although the motor and auditory properties of HVC hint at extensive local processing, knowledge of the synaptic interactions between identified HVC neuron types remains incomplete. This gap in understanding exists because axonal and dendritic processes from all three cell types as well as axonal processes from HVC afferents are interwoven with each other, complicating analysis of intrinsic connectivity (Nixdorf, 1989; Fortune and Margoliash, 1995; Foster and Bottjer, 1998; Mooney, 2000). We made intracellular recordings from pairs of identified HVC neurons in brain slices and calculated spike-triggered averages made intracellular recordings from pairs of identified HVC neurons.

<table>
<thead>
<tr>
<th>Pair type</th>
<th>Number of pairs (percentage of total)</th>
<th>Number of connected pairs (percentage of pairs [percentage total pairs])</th>
<th>Type of interaction (number of observations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HVC RA–HVC X</td>
<td>46 (47.9)</td>
<td>6 (13) [6.25]</td>
<td>HVC RA–HVC X IPSP (4)</td>
</tr>
<tr>
<td>HVC RA–HVC C</td>
<td>19 (19.8)</td>
<td>5 (26.3) [5.21]</td>
<td>HVC RA–HVC C dPSP (5)</td>
</tr>
<tr>
<td>HVC C–HVC RA</td>
<td>12 (12.5)</td>
<td>3 (25) [3.12]</td>
<td>HVC C–HVC RA dPSP (1)</td>
</tr>
<tr>
<td>HVC C–HVC X</td>
<td>9 (9.4)</td>
<td>1 (11.1) [1]</td>
<td>Three reciprocally connected pairs</td>
</tr>
<tr>
<td>HVC RA–HVC X</td>
<td>6 (6.2)</td>
<td>2 (33) [2]</td>
<td>All unidirectional IPSPs</td>
</tr>
<tr>
<td>HVC RA–HVC C</td>
<td>4 (4.2)</td>
<td>1 (25) [1]</td>
<td>HVC RA–HVC X IPSP (2)</td>
</tr>
<tr>
<td>Total</td>
<td>96 (100%)</td>
<td>18 (18.7) [NA]</td>
<td>HVC RA–HVC RA dPSP (1)</td>
</tr>
</tbody>
</table>

Acetate and 5% Neurobion (Vector Laboratories, Burlingame, CA). In a few experiments, one of the two recording electrodes contained 5% Lucifer yellow (LY) in a 1 mM lithium chloride solution. Cell penetration was achieved by briefly “ringing” the electrode using capacitance overcompensation, and the cell was then stabilized by passing regular hyperpolarizing current pulses through the recording electrode (~0.5 nA, 500 ms at 1 Hz). Intracellular potentials were amplified with an Axoclamp 2B amplifier (Axon Instruments, Union City, CA) in bridge mode, low-pass filtered at 1–3 kHz, and digitized at 10 kHz. To make paired recordings, we first obtained a stable recording from an HVC neuron and then lowered the second recording electrode to a point typically within ~50 μm of the first electrode and searched for its synaptic partners. This search strategy tended to favor finding pairs in which the first cell obtained was of a type affording a more stable recording, which in our hands tended to be the HVC RA cell type (Table 1) (see Results). Synaptically coupled cells usually could be identified on-line by the appearance of a hyperpolarizing or depolarizing response in one cell locked to the action potential discharge of the other cell, and also by subsequent on-line analysis of STAs (see below). Synaptically coupled cell pairs were most often encountered when the two electrode tips were in close (~50 μm) proximity to one another, and although a detailed count of unconnected pairs was not kept for all experiments, ~10–20% of cell pairs showed evidence of unidirectional or bidirectional synaptic coupling.

Electrophysiological data acquisition and analysis. Data acquisition and analysis for single and paired intracellular recordings were performed using a data acquisition board (AT-MIO-16E2; National Instruments, Austin, TX), controlled by custom Labview software developed by Fred Livingston, Rob Neummann, and Merri Rosen (Duke University, Durham, NC). In paired recordings, one or two action potentials were elicited in turn from each neuron in the pair by passing brief (~10 ms) depolarizing current pulses (+0.5 to 1 nA) through the recording electrode. A software threshold peak detector was used to generate STAs of the membrane potential of the partner cell in the pair. In most cases, current amplitudes and/or the resting membrane potential of the trigger cell were adjusted to elicit only a single action potential per pulse, but in a few cells, two or three spikes were sometimes evoked. In these cases, the STA was calculated off the first spike in the series. STAs were plotted in reference to the time of the trigger spike; note that the zero time for the STA corresponds to the action potential peak and that every cell in the resultant STAs might be slightly leftward-shifted with respect to the actual onset of transmitter release (see Fig. 5B). After collecting 10–40 pulse trials per cell, we then conducted further characterizations of the impaired cells, including their responses to more prolonged depolarizing currents (0.5 s at +0.5 nA). All HVC neurons in this study were identified to type based on their DC-evoked properties, as described previously (Dutar et al., 1998; Mooney, 2000). Briefly, HVC RA neurons fire only to several action potentials to +0.5 nA currents of 0.5 s duration, whereas HVCX neurons fire more regularly with moderate spike-frequency adaptation, and HVC INT fire at high frequency with little or no spike-frequency adaptation (see Fig. 1D). In addition, HVC INT can be distinguished from HVC PN s by their narrower spike widths (~1 vs 2 ms) (Mooney, 2000; Rauske et al., 2003). In many cases, at least one cell in the pair was confirmed to morphological type through intracellular staining and post hoc morphological visualization. Relatively brief recording times (<15 min) prevented thorough filling of both cells in the recorded pair in all but a few cases.

In a subset of paired recordings from synthetically coupled cells, and in all cases in which we recorded from either a single cell or unconnected pairs for pharmacological experiments (see below), we also antidromically activated HVC RA neurons, and thus their axon collaterals within HVC, by passing currents (~25–100 μA for 100 μsec) from an Isolator-10 stimulus isolation unit (Axon Instruments) to the HVC fibers.
that project to the robust nucleus of arcopallium (RA), using a concentric bipolar stimulating electrode (FHC, Brunswick, ME) placed midway between HVC and RA in the region of the caudal telencephalon. HVC axons innervating RA are clearly visible in the brain slice under epi-illumination, appearing as large whorls of whitish fibers (see Fig. 1A). Anatomical studies suggest that these fiber bundles are composed exclusively of HVCRA axons, although a few HVCX axons do travel medial to this area but outside the plane of the slices used here (Mooney, 2000). This mid-point placement was chosen because it is unlikely to activate axons of HVC afferents. Furthermore, antidromic activation of HVCRA neurons was confirmed in some recordings by the appearance of an action potential rising on the shoulder of the stimulus artifact itself. In contrast, as HVCX axons exit rostrally and ventrally from the nucleus, they intermingle with axons arising from several afferents of HVC (i.e., NIF, Uva, and mMAN). This organization renders selective recruitment of HVCX axon collaterals by extracellular stimulation in the brain slice unlikely, and thus we did not attempt to use an antidromic stimulation approach to activate HVCX axon collaterals. Instead, we relied solely on paired recordings to deduce the nature of the synaptic connectivity that HVCX neurons make with the other HVC neuron types.

Several different features of the STA were characterized off-line, including the peak amplitude, the time to peak, and the 25% rise time (i.e., the time to reach 25% of the peak amplitude). In the small minority of cases in which the trigger neuron spiked repetitively and the STA demonstrated a biphasic peak, the first peak was used for these measurements. An ANOVA was used to compare a given STA feature across different cell pair types, followed by Tukey’s post hoc test corrected for multiple comparisons. Values reported are the mean ± SEM, unless noted otherwise.

Synaptic pharmacology. To analyze the types of postsynaptic receptor activations either after antidromic stimulation of HVCRA neurons or in the case of some synthetically coupled pairs, drugs were bath applied to the whole slice after collecting evoked synaptic responses for a 5–10 min baseline period. Picrotoxin (PTX; 50 μM) was used to block inhibitory receptors of the GABA_A subtype, whereas nipecotic acid (NPA-APV; 50 μM) was used to block excitatory transmission mediated by NMDA receptors and 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo(f) quinoxaline-7-sulfonamide disodium (NBQX; 10 μM) was used to block fast excitatory transmission mediated by ionotropic glutamate receptors of the AMPA subtype. The use of an interface chamber, which we prefer for its superior slice viability compared with submersion chambers, especially when recording above room temperature, precluded collecting washouts in most cases (i.e., washout times greatly exceeded 1 h). We used other factors, such as the maintenance of excitatory transmission in the presence of PTX, as well as resting membrane potential and input resistance, to determine the type of synaptic blockade in vivo, but these factors do not reflect a general rundown of the cell or the slice. Drug effects were assessed using paired or unpaired t tests, as appropriate for normally distributed data, and a Mann–Whitney U test for other data sets.

Intracellular staining and imaging. After acquiring electrophysiological data, Neurobiotin was iontophoresed into the cell with positive current pulses (+0.5 to 1 nA, 500 ms per second, for ~15 min). For LY staining, negative current pulses were passed through the electrode (~1 nA, 500 ms at 1 Hz). Slices then were fixed in 4% paraformaldehyde in 25 mm sodium phosphate buffer (PB) overnight at 4°C and then sunk in 30% sucrose in 25 mm PB. Slices then were resectioned at 75–100 μm thickness on a freezing microtome, and Neurobiotin was visualized with a standard avidin–fluorophore reaction using a 1:1000 dilution of avidin–Alexa Fluor 488 or 564 (Molecular Probes, Eugene, OR). Confocal images were generated with a Zeiss (Thornwood, NY) 510 laser scanning microscope, using either a 40X [1.3 numerical aperture (NA)] or 63X (1.4 NA) Zeiss NeoFluar objective and rhodamine/fluorescein filters ([488 and 543 nm excitation wavelengths, emission bandpass filter of 500–540 nm, and emission long-pass filter of 560, sampled in an alternating frame arrangement; optical sections of 1 Airey unit (~1 μm thickness), with Kalman averaging = 2].

Parvalbumin immunoreactivity. In some experiments, we combined intracellular staining with Neurobiotin and immunohistochemical methods to determine which cells in HVC were positive for parvalbumin (PV), a calcium-binding protein previously localized to HVC interneurons (Wild et al., 2005). After cell fills, brain slices were transferred to 4% paraformaldehyde within 30 min, where they remained for a minimum of 24 h before being placed in 30% sucrose for several hours and then sectioned at 50 μm on a freezing microtome. The sections were then incubated in streptavidin–Alexa Fluor 488 in PBS–Triton X-100 overnight, washed in PBS, and incubated overnight again with anti-PV antibody (mouse monoclonal; Swant, Bellinzona, Switzerland) at a dilution of 1:500. All immunohistochemical reactions were performed on free-floating sections at room temperature in PBS containing 0.4% Triton X-100 (PBS-TX). Primary antibody incubations were performed overnight with the inclusion of 2.5% normal horse serum and 0.1% sodium azide. PV was visualized using a biotinylated horse anti-mouse antibody (Vector Laboratories), followed by streptavidin–Alexa Fluor 564 (Molecular Probes). Secondary antibody incubations were 1–2 h in duration in PBS-TX. Between treatments, the sections were washed thoroughly in PBS.

Results

We used blind dual sharp microelectrode recording techniques to record from synthetically coupled pairs of cells in the telen-ephalic song nucleus HVC (Fig. 1A,B). Previous in vitro and in vivo studies have shown that in addition to their morphological differences, HVCRA, HVCX, and HVCINT are readily distin-guished from each other based on their DC-evoked firing prop-erties (Dutart et al., 1998; Mooney, 2000). In the present study, we identified all cells by their DC-evoked action potential responses and/or intracellular staining with Neurobiotin and post hoc visual-ization (Fig. 1C,D) (see Materials and Methods). Synaptic po-tentials were evident as depolarizing or hyperpolarizing mem-brane potential responses in one of the cells immediately after the spontaneous and/or DC-evoked action potentials of the other cell. With respect to both raw and averaged membrane potential records, we refer to spike-evoked responses that are hyperpolariz-ing as IPSPs, depolarizing responses as DPSPs, and depolarizing responses that demonstrably evoked spiking in the postsynaptic cell as EPSPs. In all cases, action potentials were evoked in turn from each of the cells in the pair, allowing us to assess synaptic coupling in both directions. In total, we found 79 neuron pairs that exhibited evidence of either unidirectional or bidirectional synaptic coupling, including 29 HVCRA–HVCX pairs, 20 HVCINT–HVCX pairs, 13 HVCRA–HVCINT pairs, 11 HVCX–HVCRA pairs, 5 HVCRA–HVCX pairs, and 1 HVCINT–HVCRA Pair (n = 53 slices from 30 birds). In this study, the relative abundance of connected neuronal pairs of a given class is dependent on both the true probability of connections within that class and the prob-ability of sampling from that class. Therefore, the major focus of these results is on describing the nature of connections in each class of paired cells rather than the relative prevalence of connect-ions among the different classes.

HVCRA–HVCX pairs

A total of 29 HVCX–HVCRA neuronal pairs exhibited evidence of synaptic coupling. In almost all (26 of 29) pairs of synthetically coupled HVCRA–HVCX neurons, DC-evoked action potentials in the HVCRA neuron triggered a synaptic response in the HVCX neuron. In the vast majority of these cases, action potentials in the HVCRA neuron evoked an IPSP in the HVCX cell (Fig. 2A, Table 2) (25 of 26 cases evoked IPSPs; 1 of 26 cases evoked a DPSP). The amplitudes of these HVCRA–HVCX IPSPs often were sufficiently large (>1 mV) to be visible without averaging. HVCRA STA (see Materials and Methods) of the HVCX neuronal membrane po-tential had an average peak amplitude of ~1.5 ± 0.3 mV, an average time to peak of 15.7 ± 1.0 ms, and a 25% rise time of 4.8 ± 0.4 ms (n = 25) (Fig. 2B, Table 2).
Synaptic coupling from HVCx to HVCRA neurons was detected less frequently. In 7 of 29 pairs of synaptically coupled HVCRA–HVCx neurons, action potentials in the HVCx cell triggered synaptic responses in HVCRA neurons. In four of these cases, action potentials in the HVCx neuron evoked a dEPSP in the HVCRA cell, whereas in the other three cases, IPSPs were elicited (Table 2). Notably, reciprocal connections were detected in 5 of the 29 pairs of synaptically coupled HVCRA–HVCx neurons (data not shown). In all of these cases, action potentials in the HVCRA neuron evoked IPSPs in the HVCx cell; HVCx cell action potentials evoked dEPSPs in three of these pairs and IPSPs in the other two pairs. These results indicate that bidirectional synaptic interactions, including reciprocal inhibitory interactions, can occur between HVCRA and HVCx neurons. Furthermore, these recordings suggest that a dominant pattern of synaptic connectivity between the two HVC PN types involves inhibition from HVCRA to HVCx neurons.

We suspected that the inhibitory interaction between HVCRA and HVCx neurons was mediated by interposed interneurons, given that HVCRA neurons are known to evoke ionotropic glutamate receptor-mediated EPSPs in neurons in the song nucleus RA (Mooney and Konishi, 1991). In this model, the local collaterals of HVCRA neurons excite inhibitory interneurons via ionotropic glutamatergic synapses, which in turn make inhibitory synapses on HVCx neurons. Consistent with this synaptic model, both the mean 25% rise time and the mean time to peak of IPSPs in HVCRA–HVCx cell pairs were significantly longer than those of the IPSPs evoked in HVCx cells by interneurons (see Table 2 for statistical comparisons and the following discussion of synaptic coupling in HVCRA–HVCx cell pairs) (see also Fig. 5B). Furthermore, in some HVCRA–HVCx cell pairs, hyperpolarizing responses in HVCx neurons only were evoked when the HVCRA neuron fired a spike doublet or triplet, possibly reflecting facilitation at an intervening excitatory synapse (Fig. 2C) (*n = 2 cases). These observations provide indirect evidence that HVCRA cells evoke IPSPs in HVCx neurons via a disynaptic mechanism. Another possibility is that the axon collaterals of HVCRA neurons provide monosynaptic inhibition onto HVCx neurons, perhaps via the hyperpolarizing metabotropic glutamate receptors that have been detected within HVC (Schmidt and Perkel, 1998; Dutar et al., 1999, 2000). This monosynaptic model is less likely, given that metabotropic forms of synaptic transmission typically exhibit a much slower onset than observed here for HVCRA–HVCx cell pairs.

These two models can be distinguished by pharmacological methods: HVCRA-evoked IPSPs in HVCx neurons mediated by the disynaptic mechanism will be abolished by ionotropic glutamate receptor blockers, whereas IPSPs mediated by a monosynaptic, metabotropic glutamatergic pathway should not be affected by such treatment. To distinguish between these two outcomes, we recorded intracellularly from HVCx cells and an-
In control conditions, antidromic stimulation of HVCRA bath applied ionotropic glutamate receptor blockers to the slice evoke IPSPs in the HVCX neuron. Cellular recordings show that DC-evoked action potentials in the HVCRA neuron (bottom trace) can robust to enable single HVCRA neurons to

![Figure 2](image)

**Figure 2.** Action potentials in HVCRA neurons evoke IPSPs in HVCX neurons. A. Dual intracellular recordings show that DC-evoked action potentials in the HVCRA neuron (bottom trace) can evoke IPSPs in the HVCX neuron (top trace) for the cell pair shown in A. B. A membrane hyperpolarization followed the HVCRA action potential, indicating that HVCRA neurons directly or indirectly drive IPSPs in the HVCX cell. C. In some HVCRA–HVCX cell pairs, spike doublets in the HVCRA neuron were necessary to drive IPSPs in the HVCX cell (right traces), whereas single spikes failed to evoke any response (left traces), suggestive of disynaptic coupling.

tidromically activated HVCRA axon collaterals en masse and then bath applied ionotropic glutamate receptor blockers to the slice (Fig. 3). In control conditions, antidromic stimulation of HVCRA axons evoked robust IPSPs in all HVCX neurons that we tested (n = 5), and in all cases, these IPSPs were abolished by the bath application of a mixture of ionotropic glutamate receptor blockers, NBQX (10 μM) and d-APV (50 μM) (Fig. 3) (mean ± SD: control, −12.4 ± 4.8 mV; drug, 0.9 ± 1.7 mV; paired t test; n = 5; p = 0.00132). These results support a model in which HVCRA axon collaterals activate excitatory ionotropic glutamate receptors on inhibitory interneurons, ultimately providing a disynaptic inhibitory linkage from HVCRA to HVCX cells. Together with the results of paired recordings, these experiments also suggest that the HVCRA–interneuron excitatory coupling is sufficiently robust to enable single HVCRA neurons to drive disynaptic, feedforward inhibition in HVCX neurons.

**HVCRA–HVCINT pairs**

Paired recordings provided direct evidence of the excitatory nature of the synaptic contacts that HVCRA axon collaterals make onto HVCINT, consistent with the disynaptic model of feedforward inhibition between HVCRA and HVCX cells (Fig. 4A). A total of 13 HVCRA–HVCINT cell pairs revealed evidence of unidirectional or bidirectional synaptic coupling. Seven cases of HVCRA-to-HVCINT coupling were observed, and in six of those cases, DC-evoked action potentials in the HVCRA neuron evoked a fast dPSP in the corre-

### Table 2. Characteristics of synaptic responses in connected HVC neuron pairs

<table>
<thead>
<tr>
<th>Pair type (number of pairs with given PSP)</th>
<th>Peak amplitude (mV)</th>
<th>Time to peak (ms)</th>
<th>25% rise time (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPSPs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HVCRA &gt; HVCX (25 of 29)</td>
<td>−1.5 ± 0.3</td>
<td>15.7 ± 1.0</td>
<td>4.8 ± 0.4</td>
</tr>
<tr>
<td>HVCX &gt; HVCRA (3 of 29)</td>
<td>−1.1 ± 0.1</td>
<td>9.9 ± 1.3</td>
<td>2.6 ± 0.7</td>
</tr>
<tr>
<td>HVCX &gt; HVCRA (11 of 11)</td>
<td>−2.0 ± 0.4</td>
<td>15.4 ± 2.7</td>
<td>5.0 ± 1.7</td>
</tr>
<tr>
<td>HVCXINT &gt; HVCRA (19 of 20)</td>
<td>−1.2 ± 0.2</td>
<td>10.2 ± 0.9⁴</td>
<td>1.4 ± 0.3⁶</td>
</tr>
<tr>
<td>HVCXINT &gt; HVCINT (6 of 13)</td>
<td>−0.9 ± 0.2</td>
<td>10.7 ± 2.5</td>
<td>1.7 ± 1.0⁷</td>
</tr>
<tr>
<td>dIPSPs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HVCRA &gt; HVCX (6 of 13)</td>
<td>2.0 ± 0.4</td>
<td>4.7 ± 0.5</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>HVCX &gt; HVCRA (5 of 20)</td>
<td>1.2 ± 0.3</td>
<td>4.3 ± 0.4⁴</td>
<td>1.0 ± 0.6⁷</td>
</tr>
<tr>
<td>HVCXINT &gt; HVCRA (4 of 4)</td>
<td>2.2 ± 1.1</td>
<td>9.1 ± 2.3</td>
<td>4.0 ± 0.3</td>
</tr>
<tr>
<td>HVCXINT &gt; HVCINT (4 of 29)</td>
<td>0.5 ± 0.1</td>
<td>8.3 ± 0.9</td>
<td>3.2 ± 0.3</td>
</tr>
</tbody>
</table>

⁴ p < 0.05 versus HVCRA > HVCX, by ANOVA.  
⁵ p < 0.05 versus HVCX > HVCXINT, by ANOVA.  
⁶ p > 0.05 versus HVCX > HVCXINT, by ANOVA.  
⁷ p > 0.05 versus HVCRA > HVCXINT, by unpaired t test.
and the existence of reciprocal inhibitory connections from HVC\textsubscript{INT} to HVC\textsubscript{RA} suggests that action potential activity in HVC\textsubscript{RA} neurons could be shaped by inhibitory feedback acting on a spike-by-spike basis.

**HVC\textsubscript{INT}--HVC\textsubscript{X} pairs**

Paired recordings also revealed that interneurons could provide inhibitory input to HVC\textsubscript{X} cells. In almost all synaptically connected HVC\textsubscript{INT}--HVC\textsubscript{X} cell pairs, an action potential in the interneuron evoked an IPSP in the HVC\textsubscript{X} cell (Fig. 5A) (19 of 20 cases; average peak amplitude, $-1.2 \pm 0.2$ mV; average time to peak, $10.2 \pm 0.9$ ms; 25% rise time, $1.4 \pm 0.3$ ms; $n = 19$) (Table 2). In one case, unidirectional coupling from the HVC\textsubscript{X} neuron to the interneuron, in the form of a dPSP, was observed (data not shown). We noted that interneuron-evoked IPSPs in HVC\textsubscript{X} cells had faster rise times and times to peak than did IPSPs recorded in HVC\textsubscript{RA}--HVC\textsubscript{X} cell pairs, although IPSPs of either type otherwise had a similar overall shape and time course (Fig. 5B) (for statistical comparisons, see Table 2). The very short onset latency of

Figure 3. Antidromic stimulation of HVC\textsubscript{RA} axons can be used to characterize the pharmacological nature of the inhibitory interactions between HVC\textsubscript{RA} and HVC\textsubscript{X} cells. A schematic of the slice preparation (top), showing how antidromic stimulation of the HVC\textsubscript{RA} axon fiber bundle (lightning bolt) can be used to activate the HVC microcircuit while recording intracellularly from HVC\textsubscript{X} cells. In this model, local collaterals of the HVC\textsubscript{RA} axon excite HVC\textsubscript{INT}, which ultimately drive IPSPs in the HVC\textsubscript{X} cell. Consistent with this idea, electrical stimulation of the HVC\textsubscript{RA} fibers drives IPSPs in HVC\textsubscript{X} cells (bottom; control), which are blocked by the bath application of non-NMDA glutamate receptor antagonists (NBQX/APV).

**Figure 4.** Dual intracellular recordings provide direct evidence of the excitatory synapses that HVC\textsubscript{RA} neurons make with HVC\textsubscript{INT}. A, Depolarizing current pulses injected into the HVC\textsubscript{RA} neuron (bottom trace) elicit action potentials, which were followed at short latency by subthreshold (left) and suprathreshold (right) EPSPs in the HVC\textsubscript{INT}. B, An STA of HVC\textsubscript{INT} membrane potential triggered off of the HVC\textsubscript{RA} action potential, from the cell pair shown in A. A fast-rising, short-latency-positive STA was detected after the HVC\textsubscript{RA} action potential, consistent with the idea that the HVC\textsubscript{RA} neuron makes an excitatory synapse with the interneuron. C, Longer depolarizing currents could evoke irregular spiking in the HVC\textsubscript{RA} neuron (bottom trace), which were paralleled by dEPSPs in the HVC\textsubscript{INT}. In this case, note that the last six HVC\textsubscript{RA} spikes occurred in doublets and that the second dEPSP was larger than the one immediately preceding it, suggestive of synaptic facilitation.
rons evoke short-latency IPSPs in HVCX cells and are likely to
inhibit interneurons and HVCX cells (Figs. 5 in vivo
responses.

A higher-order excitatory synapse (HVCX cell to HVCX cell) is feasible, but further experiments are needed to clarify these responses. However, the data from these experiments support the view that the fast-spiking interneurons we recorded from here provide monosynaptic inhibitory input to HVCX cells and thus are plausible interneurons in the HVC microcircuit. These experiments also reveal monosynaptic and polysynaptic excitatory pathways from HVCRA to HVCX cells, which are normally suppressed or otherwise masked by GABA-ergic inhibition.

Figure 5. Dual intracellular recordings reveal that HVCINT provide short-latency inhibition onto HVCX cells. A, Raw membrane potential records from a sympathetically coupled interneuron (bottom) and HVCX cell (top). DC-evoked spikes could evoke robust IPSPs in the HVCX cell; note that a spontaneous IPSP, presumably from another interneuron, occurred after the DC-evoked spikes. B, The mean STA from all HVCINT–HVCX cell pairs compared with the mean STA from all HVCRA–HVCX cell pairs, showing the offset in the 25% rise times (horizontal dashed line) (see Table 2). The overall shapes of the STAs in the different cell pairs were very similar, but the HVCRA–HVCX STA was delayed relative to HVCINT–HVCX STA, suggesting that HVCX cells are connected indirectly with HVCX cells. The STA conventions are as in Figures 2 and 4. C, Higher-frequency firing in an HVCINT can drive a sustained hyperpolarization in the HVCX cell. D, HVCX cells in some cases could drive EPSPs in an HVCINT cell. DC-evoked firing in the HVCX cell (bottom) reliably evoked suprathreshold EPSPs in the HVCINT (top trace). The HVCX also evoked IPSPs in the HVCX cell (shown in Fig. 9D), indicating that interneurons and HVCX cells can form reciprocal synaptic connections.

These results also provided evidence of reciprocal connectivity between interneurons and HVCX cells (Figs. 5D) (see Fig. 9D, right) (n = 4 pairs). In all four reciprocally connected pairs, the HVCX neuron action potential evoked a dEPSP in the interneuron; in two pairs, these EPSPs were demonstrably excitatory (Fig. 5D, Table 2) (average peak amplitude, 1.2 ± 0.3 mV; average time to peak, 4.3 ± 0.4 ms; 25% rise time, 1.0 ± 0.6 ms). The rapid onset and time to peak of the dEPSPs were similar to those recorded in HVCRA–HVCINT cell pairs (for statistical comparisons, see Table 2). In all of our reciprocally connected HVCINT–HVCX cell pairs, the interneuron action potential evoked an IPSP in the HVCX neuron. Therefore, HVCX neurons can form strong excitatory synapses onto interneurons that provide them with reciprocal inhibitory input, suggesting that action potential activity in HVCX neurons, as with HVCRA neurons, could be shaped on a spike-by-spike basis by inhibitory feedback.

Pharmacology of HVCINT-mediated inhibition
Previous studies showed that IPSPs in HVC neurons are mediated by several different neurotransmitter receptors, including ionotropic GABA \( \alpha \) receptors and metabotropic GABA and glutamate receptors (Schmidt and Perkel, 1998; Dutar et al., 1999, 2000; Rosen and Mooney, 2003). The relatively fast onset and time to peak of the IPSPs we recorded in HVCINT–HVCX cell pairs suggested that ionotropic GABA \( \alpha \) receptors, and not metabotropic receptors, were involved. We tested this idea in several ways. First, we bath applied PTX (50 \( \mu M \)), a GABA \( \alpha \) receptor blocker, while recording from sympathetically coupled pairs of cells. In two HVCINT–HVCX pairs and one HVCRA–HVCX cell pair, spike-evoked IPSPs in the HVCX cell were abolished by this treatment, indicating that in both types of connections, ionotropic GABA \( \alpha \) receptors mediated the IPSP (Fig. 6A,B) (control, −1.2 ± 0.2 mV; PTX, 0.0 ± 0.1 mV; n = 3; p < 0.03; ANOVA). Second, we evoked fast, short-latency IPSPs in HVCX cells by antidiromically stimulating HVCRA neurons and then bath applied PTX (50 \( \mu M \)). Treatment with PTX consistently and completely abolished the electrically evoked short-latency IPSP and unmasked a short-latency dPSP (Fig. 7A, inset) (n = 12 cases; control, −3.7 ± 0.6 mV; PTX, +4.9 ± 0.3 mV; p = 0.000012). Third, paired recording revealed that interneuron-evoked IPSPs in an HVCX cell were unaffected by ionotropic glutamate receptor blockers (Fig. 6C), although EPSPs in the interneuron and IPSPs in the HVCX cell evoked by antidiromic stimulation of HVCRA neurons were blocked by this treatment (Fig. 6D) (n = 1). These experiments show that GABA \( \alpha \) receptors mediate the fast IPSPs evoked in HVCX cells by both interneurons and HVCRA cells. These experiments also reveal monosynaptic and/or polysynaptic excitatory pathways from HVCRA to HVCX cells, which are normally suppressed or otherwise masked by GABA \( \alpha \) -mediated inhibition.

Prolonged blockade of GABA \( \alpha \) receptors in HVC also unmasked more complex synaptic interactions between HVCRA and HVCX cells. Notably, after 15–20 min of PTX application, antidiromic stimulation of HVCRA neurons evoked larger EPSPs capable of eliciting a high-frequency action potential burst in the HVCX neuron (Fig. 6E, middle). In several cells (n = 3), repetitive bursting followed a single antidiromic stimulus (Fig. 7A). PTX treatment also unmasked evidence for slow inhibitory signaling from HVCRA to HVCX cells. In two cells, a prolonged, multiphasic response was evoked by HVCRA stimulation shortly after applying PTX but before the emergence of the dEPSP (Fig. 7B, left). In addition, prolonged hyperpolarizations sometimes followed the shorter-latency dEPSPs (Fig. 7A,B, right). The shorter-latency EPSPs, as well as associated prolonged hyperpolarizations, were completely abolished by a combination of NBQX and d-APV, indicating they were mediated in part via monosynaptic and/or polysynaptic pathways involving ionotropic glutamate receptors (Figs. 6E, right; 7A) (n = 9 cases; PTX, +8.8 ± 1.7 mV; NBQX/d-APV, −0.1 ± 0.12 mV; p < 0.001; Mann–Whitney U test). In summary, these pharmacological experiments show that fast-spiking interneurons evoke IPSPs in HVCX cells via GABA \( \alpha \) receptors. Furthermore, HVCRA neurons drive excitatory and inhibitory responses in HVCX neurons via synaptic pathways that involve ionotropic glutamate receptors.
Interneurons that evoke IPSPs are PV positive

The interneurons that evoke IPSPs in HVCx cells are fast-spiking cells with varicose dendrites (Fig. 1C,D). Previous immunohistochemical studies indicated that fast-spiking HVC interneurons with varicose dendrites (Fig. 1C,D) are PV positive (PV+/H11001) (Wild et al., 2005) but did not resolve whether PV+/H11001 interneurons are a source of inhibitory input onto HVC PNs. We used anti-PV antibodies and intracellular staining with Neurobiotin to determine whether the interneurons that provided inhibitory input onto HVCX cells were PV+/H11001. In two HVCINT–HVCX pairs, morphologically and physiologically identified fast-spiking interneurons that evoked IPSPs in HVCX cells were PV+/H11001, whereas the corresponding HVCX cells were PV negative (PV+/H11002) (Fig. 8A). We did note, however, that HVCX neurons were sometimes in extremely close apposition to PV+/H11001 cell bodies (Fig. 8B) (n = 2 cases). These results show that PV+/H11001 interneurons provide some of the inhibitory input onto HVC PNs that innervate basal ganglia structures in the songbird brain.

Homotypic synaptic interactions

Paired recordings also revealed synaptic interactions between neurons of the same type. Eleven HVCX pairs showed signs of unidirectional inhibitory synaptic coupling, all in the form of spike-evoked IPSPs (individual data not shown; average peak amplitude, −2.0 ± 0.4 mV; average time to peak, 15.4 ± 2.7 ms; 25% rise time, 5.0 ± 1.7 ms; n = 11) (for mean data, see Table 2). These IPSPs were presumably mediated by intervening interneurons, in part because recordings previously described indicated that HVCX neurons can make excitatory synapses onto HVCINT. Furthermore, the mean 25% rise time of IPSPs evoked in HVCINT–HVCX cell pairs was significantly longer than that of IPSPs evoked with varicose dendrites are PV positive (PV+)(Wild et al., 2005) but did not resolve whether PV+ interneurons are a source of inhibitory input onto HVC PNs. We used anti-PV antibodies and intracellular staining with Neurobiotin to determine whether the interneurons that provided inhibitory input onto HVC PNs were PV+. In two HVCINT–HVCX pairs, morphologically and physiologically identified fast-spiking interneurons that evoked IPSPs in HVCX cells were PV+, whereas the corresponding HVCX cells were PV negative (PV−) (Fig. 8A). We did note, however, that HVCX neurons were sometimes in extremely close apposition to PV+ cell bodies (Fig. 8B) (n = 2 cases). These results show that PV+ interneurons provide some of the inhibitory input onto HVC PNs that innervate basal ganglia structures in the songbird brain.
corded in HVC<sub>INT</sub>–HVC<sub>X</sub>, but not HV-
C<sub>RA</sub>–HVC<sub>X</sub>, cell pairs (for statistical
comparisons, see Table 2). Five HVC<sub>RA</sub>
pairs were also recorded: four exhibited
unidirectional dEPSPs (Table 2), whereas
one showed an IPSP (data not shown). Fin-
ally, one synaptically coupled interneu-
ron pair was detected that exhibited a uni-
directional IPSP (data not shown) (Table
1). Therefore, inhibitory as well as excita-
tory connections serve to synaptically link
homotypic as well as heterotypic pairs of
neurons within HVC.

**Divergent and convergent synaptic
connections in HVC**

The extensive local axonal network of
both PNs and interneurons raises the pos-
sibility of divergent and convergent pat-
terns of synaptic connectivity within
HVC. We were able to detect both diver-
gent and convergent synaptic connections
by recording from one HVC neuron with
a fixed electrode and moving a second
electrode about the slice to sequentially
record from a series of other cells that were either its presynaptic
or postsynaptic partner. In four of these sequential paired record-
nings, the fixed electrode was in an interneuron, whereas the
moveable electrode encountered a series of PNs. Three qualita-
tive observations resulted from these recordings. First, a single
interneuron can evoke IPSPs in both HVC<sub>RA</sub> and HVC<sub>X</sub>
neurons (Fig. 9A). Second, a single interneuron can make divergent inhibi-
tory synapses on several HVC<sub>X</sub> cells (Fig. 9B). Third, a single
interneuron can receive convergent input from two or more HV-
C<sub>RA</sub> Neurons (data not shown). In three other sequential paired
recordings, the fixed electrode was placed in an HVC<sub>X</sub> neuron,
whereas the moveable electrode recorded from HVC<sub>INT</sub> and/or
HVC<sub>RA</sub> neurons. These recordings revealed that both HVC<sub>INT</sub>
and HVC<sub>RA</sub> PNs can provide inhibitory input onto the same
HVC<sub>X</sub> cell (Fig. 9C). Therefore, single interneurons can contact
PNs of both types and can receive convergent excitatory input
from HVC<sub>RA</sub> neurons.

Interneurons also receive convergent excitatory input from
PNs of different types. We examined interactions between the
two cell types by coupling antidromic stimulation of HVC<sub>RA</sub>
neurons while using two electrodes to record from HVC<sub>INT</sub>–
HVC<sub>X</sub> cell pairs. In all six pairs, the HVC<sub>INT</sub> evoked IPSPs in the
HVC<sub>X</sub> cell; in two of these pairs, the HVC<sub>X</sub> cell provided recip-
trocal excitation to the interneuron. In all of these pairs, anti-
dromic activation of the HVC<sub>RA</sub> axon collateral network evoked
an EPSP in the HVC<sub>INT</sub> cell and an IPSP in the HVC<sub>X</sub> cell (Fig.
9D). These results show that the two PN types provide convergent
excitatory input onto single interneurons, which in turn provide
an inhibitory link between the HVC PNs. The various features of
the local synaptic organization of HVC revealed in this study are
summarized in Figure 10.

**Frequency of synaptic connections between different HVC
cell types**

We also performed additional paired recordings to provide an
estimate both of the frequency of pair types that we encountered
using these recording methods and the frequency of synaptic
connections that we detected between neurons of given types. A
total of 96 pairs was obtained, 18 of which displayed either uni-
directional or bidirectional synaptic coupling (~19%; note that
the connected pairs from this sample contributed to the total
pool of connected pairs represented in Table 2 and discussed in
previous sections of Results) (Table 1). Several features of this
sample are notable. First, the vast majority of pairs that we ob-
tained [77 of 96 (~80%)] contained at least one HVC<sub>X</sub> neuron,
likely reflecting the fact that these cells are relatively numerous
and large, and typically afford the most stable recordings with the
sharp electrode methods used in this study. Second, almost two-
thirds [61 of 96 (63%)] of all pairs consisted of HVC<sub>RA</sub> neurons,
which despite their small size are highly abundant (Kirn et al.,
1991; Wild et al., 2005). Perhaps as a result of these various fac-
tors, HVC<sub>RA</sub>–HVC<sub>X</sub> neuron pairs made up almost one-half this
sample [46 of 96 (~48%)]. Finally, in contrast to our overall
larger sample of HVC<sub>RA</sub> and HVC<sub>X</sub> neuron pairs, this smaller
sample exhibited a higher proportion of HVC<sub>X</sub>-to-HVC<sub>RA</sub> syn-
aptic connections [5 of 6 (83%) vs 7 of 29 (24%)] and included three reciprocally connected cell pairs.

**Discussion**

The present study reveals several synaptic features likely to be
important to the song-related motor and auditory functions of
HVC. First, HVC<sub>RA</sub> neurons excite interneurons, which inhibit
HVC<sub>X</sub> neurons, providing a feedforward inhibitory mechanism
linking song premotor and basal ganglia projecting pathways em-
inating from HVC. This feedforward inhibition could help shape
motor-related activity transmitted to the AFP and generate cell
type-specific patterns of auditory activity. Second, interneurons
innervate multiple PNs of both types and thus could coordinate
their activity. Finally, HVC contains reciprocally connected PNs
and interneurons, similar to other pattern-generating networks.

**Feedforward inhibition and excitation from HVC<sub>RA</sub> to
HVC<sub>X</sub> neurons**

These studies show that HVC<sub>X</sub> cells, which innervate basal gan-
glia structures important to vocal plasticity (Nottebohm et al.,
1976, 1982; Bottjer et al., 1984; Scharff and Nottebohm, 1991),
are inhibited directly by interneurons and indirectly by HVC PNs of both types. The IPSPs from HVC_RA to HVC_X cells were most likely mediated via disynaptic, feedforward mechanisms, because: (1) antagonists of ionotropic glutamate receptors blocked all inhibitory synaptic transmission in HVC_X cells evoked by antidromic stimulation of HVC_RA fibers; (2) in paired recordings, GABA_A receptor blockers abolished IPSPs evoked in HVC_X cells by either HVC_INT or HVC_RA; (3) HVC_RA neurons drive fast rise-time EPSPs mediated by ionotropic glutamate receptors on HVC_INT and on their extrinsic targets in the nucleus RA; (4) interneurons drive IPSPs in HVC_X cells with faster rise times than IPSPs driven in HVC_X cells by HVC_RA neurons; and (5) spike doublets in HVC_RA cells could evoke excitatory synaptic facilitation in interneurons and were sometimes required to trigger IPSPs in HVC_X cells. Similar disynaptic mechanisms likely underlie IPSPs detected in pairs of HVC_X cells, because the rise times of these IPSPs were relatively slow, like those in HVC_RA–HVC_X cell pairs, and because HVC_X cells evoke EPSPs in interneurons. Additionally, monosynaptic and polysynaptic excitatory pathways and polysynaptic inhibitory pathways link HVC_RA to HVC_X cells but are normally masked by fast inhibition.

Functional implications of divergent and convergent synaptic connections

Sequential paired recordings revealed that interneurons divergently innervate PNs of both types, an arrangement that could synchronize the firing of multiple HVC cells, as occurs in sleeping birds (Rauske et al., 2003). Although synaptically coupled cell pairs recorded here were typically in close proximity (cf. Feldmeyer et al., 1999), intracellular staining showed that interneuron processes are extensive (Fig. 1C) (Katz and Gurney, 1981; Mooney, 2000; Wild et al., 2005), raising the possibility of a more widespread influence on HVC synchrony. Although none of the paired recordings we obtained displayed evidence of electrotonic coupling, gap junctions have been detected in HVC (Gahr and Garcia-Segura, 1996), affording a potential synchronizing influ-
whether single interneurons synapse on multiple HVCRA neuronal array. An important future goal will be to determine HVCX neurons (dashed lines). These monosynaptic and polysynaptic pathways are dependent on fast excitation mediated by ionotropic glutamate receptors, whereas fast inhibition is mediated by GABAA receptors. Additional polysynaptic and possibly monosynaptic excitatory pathways and polysynaptic inhibitory pathways also provide a synaptic linkage from HVCRA to HVCX neurons (dashed lines). These monosynaptic and polysynaptic pathways are dependent on ionotropic glutamate receptors, presumably involving direct synapses between HVCRA axon collaterals and HVCX neurons and intervening synapses between HVCRA axon collaterals and other HVC interneurons.

Previous in vivo and in vitro studies have shown that HVCX neurons receive remarkably diverse forms of inhibition, including fast IPSPs mediated by GABA_A receptors and slow IPSPs mediated by GABA_B and metabotropic glutamate receptors (Schmidt and Perkel, 1998; Dutar et al., 1999, 2000; Hahnloser et al., 2002; Rosen and Mooney, 2003). This functional diversity may be reflected in part by the diverse calcium-binding protein expression patterns of HVC interneurons, which contain various combinations of PV, calbindin, and calretinin (Wild et al., 2005). The present study shows that at least some fast-spiking interneurons are PV^+ and evoke GABA_A receptor-mediated IPSPs in HVCX cells. This result links previous observations that fast-spiking interneurons are PV^+ (Wild et al., 2005) and that PV^+ cells coexpress the synthetic enzyme for GABA (Zuschratter et al., 1987). Because PV^+ neurons evoke fast, GABA_A-mediated IPSPs in HVCX cells, they functionally resemble PV^+ interneurons in the mammalian cortex, which evoke fast GABA_A-mediated inhibitory synaptic currents in pyramidal neurons (Maccarelli et al., 2000). Interneurons also are the likely source of the slow IPSPs evoked in HVCX cells by antidromically stimulating HVCRA neurons in the presence of PTX. These slow IPSPs could be blocked by antagonists of ionotropic glutamate receptors, suggesting they arise polysynaptically through HVC interneurons, rather than monosynaptically via HVCRA axon collaterals (i.e., via metabotropic glutamate receptors) (Schmidt and Perkel, 1998; Dutar et al., 1999, 2000). An important future goal will be to further characterize the correspondence between morphological, biochemical, and functional properties of different HVC interneuron types.

Relevance to the auditory and motor properties of HVC

The inhibitory and excitatory linkage from HVCRA to HVCX cells could have important consequences for the processing of auditory and song motor activity in HVC. Exquisite auditory selectivity for the BOS is a hallmark of HVC neuronal responses in anesthetized songbirds (Margoliash, 1983; Theunissen and Doupe, 1998), and the HVC local circuit is thought to play a role in shaping this selectivity (Lewicki and Konishi, 1995; Lewicki and Arthur, 1996; Mooney, 2000; Rosen and Mooney, 2003; Coleman and Mooney, 2004). Intracellular recordings from urethane anesthetized zebra finches have shown that BOS playback evokes distinct subthreshold responses in the two HVC PNs, including sustained and mostly subthreshold depolarization in HVCRA neurons and prolonged hyperpolarizing responses punctuated by phasic excitation in HVCX cells (Mooney, 2000). These hyperpolarizing responses help shape the pattern of BOS-evoked firing in HVCX cells and likely arise through local inhibition onto HVCX cells (Rosen and Mooney, 2003). Indeed, inactivating HVC by local application of GABA unmarks prolonged BOS-evoked depolarizations in HVCX cells, pointing to a local source of inhibition (M. Rosen and R. Mooney, unpublished observations). Furthermore, BOS-evoked hyperpolarizations in HVCX cells closely correlate with firing in interneurons (Mooney, 2000), and these interneurons appear to be the same type shown here that drive fast IPSPs in HVCX cells, because both cells are fast spiking, have varicose dendrites, and are PV^+ (Mooney, unpublished observations). However, BOS-evoked hyperpolarizations in HVCX cells involve slow G-protein-mediated potassium currents, with only a cryptic contribution from chloride-mediated currents typical of ionotropic GABA_A receptors (Rosen and Mooney, 2003). Therefore, additional inhibitory pathways normally quiescent in the in vitro preparation must be active during song playback, possibly including the slow inhibitory pathways unmasked by PTX that indirectly link HVCRA to HVCX cells.

Chronic recordings in singing birds reveal that activity in the AFP is closely locked to the acoustical features of the bird’s song.
and persists after deafening, suggesting a motor origin (Hessler and Doupe, 1999; Leonardo, 2002). The inhibitory and excitatory synaptic linkage from HVCRA to HVCX cells seen here suggests mechanisms by which HVC circuitry could shape and convey song motor activity to the AFP. First, monosynaptic excitation and lagging disynaptic inhibition from HVCRA cells could generate tightly correlated phasic excitation in HVCX cells (Pouille and Scanziani, 2001), which may enhance signal propagation in the AFP. Second, inhibition can synchronize neuronal firing (Lyttton and Sejnowski, 1991; Bush and Sejnowski, 1996) and, in HVCX cells, may also trigger burst firing by deinactivation of low-threshold calcium channels (Kubota and Saito, 1991; Rosen and Mooney, 2003), two features that could facilitate transmission of excitatory signals to the AFP. Third, by analogy to mammalian basal ganglia circuitry (Affi, 1994; Wichmann and DeLong, 1996; Reiner, 2002), motor-driven inhibition from HVCRA onto HVCX cells could disinhibit downstream targets in the AFP (Wilson, 1993; Sil’kis, 2002; Nambu, 2004). Indeed, inhibitory synapses in the AFP (i.e., between Area X and the medial nucleus of the dorsolateral thalamus (Luo and Perkel, 2002)) could effect the necessary sign inversion for such disinhibition. Fourth, high-frequency (>100 Hz) firing in the HVCRA neuron sometimes was required to evoke an IPSP in the HVCX cell, apparently because of facilitation at the HVCRA–interneuron synapse. Given the propensity for HVCRA neurons to fire in high-frequency bursts during singing (Hahnloser et al., 2002), such facilitation could be integral to shaping premotor activity in HVCX neurons and thus modulating AFP song motor activity. Finally, we noted that antidromic stimulation of HVCRA neurons evoked an IPSP that was nearly 10-fold greater in amplitude than unitary IPSPs evoked in HVCX neurons by either interneurons or HVCRA cells, suggesting that multiple interneurons converge directly onto single HVCX cells and that multiple HVCRA neurons converge indirectly onto HVCX cells. This pattern of convergence in HVC may enable the activity of a larger ensemble of HVCX neurons to be integrated in single cells projecting to the AFP. Because HVCX neurons fire in a temporally sparse manner during singing and song playback (Mooney, 2000; Hahnloser et al., 2002), such synaptic integration may facilitate larger time scale representations of song in the AFP.

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