Synaptic Interactions Underlying Song-Selectivity in the Avian Nucleus HVC Revealed by Dual Intracellular Recordings

Merri J. Rosen and Richard Mooney
Department of Neurobiology, Duke University Medical Center, Durham, North Carolina

Submitted 28 January 2005; accepted in final form 13 September 2005

Rosen, Merri J. and Richard Mooney. Synthetic interactions underlying song-selectivity in the avian nucleus HVC revealed by dual intracellular recordings. J Neurophysiol 95: 1158 –1175, 2006; doi:10.1152/jn.00100.2005. Stimulus-dependent synaptic interactions underlying selective sensory representations in neural circuits specialized for sensory processing and sensorimotor integration remain poorly understood. The songbird telencephalic nucleus HVC is a sensorimotor area essential to learned vocal control with one projection neuron (PN) type (HVC RA) innervating a song premotor pathway, another PN (HVC X) innervating a basal ganglia pathway essential to vocal plasticity, and interneurons (HVC m). Playback of the bird’s own song (BOS), but not other songs, evokes action potential bursts from both PNs, but HVC RA and HVC X display distinct BOS-evoked subthreshold responses. To characterize synaptic interactions underlying HVC’s BOS-selective responses and assess stimulus-evoked changes in functional interactions between HVC neurons, we made simultaneous in vivo intracellular recordings from various HVC neuron pairs in urethan-anesthetized zebra finches. Spike-triggered averaging revealed that all HVC neuron types receive common excitation and that the onset of this excitation occurs during a narrower time window in projection neurons during BOS playback. To distinguish local from extrinsic contributions to HVC subthreshold response patterns, we inactivated the HVC local circuit with GABA or occluded inhibition in single HVC X cells. After either treatment, BOS-evoked responses in HVC X neurons became purely depolarizing and subthreshold responses of HVC X and HVC RA cells became remarkably similar to one another while retaining BOS selectivity. Therefore both PN types receive a common extrinsic source of BOS-selective excitation, and local inhibition specifically alters processing of auditory information in HVC X cells. In HVC, excitatory and inhibitory synaptic interactions are recruited in a stimulus-dependent fashion, affecting auditory representations of the BOS locally and in other song nuclei important to song learning and production.

INTRODUCTION

Selective neuronal responses to sensory stimuli are thought to serve a wide variety of functions, including object recognition (Perez-Orive et al. 2002; Rolls 2000), sensorimotor integration (du Lac and Knudsen 1990; Heiligenberg et al. 1996; Moss and Sinha 2003), and intraspecific communication (Alder and Rose 1998; Gentner and Margoliash 2003; Klug et al. 2002; Ohlemiller et al. 1996; Schneider 1992; Tian et al. 2001; Wang 2000). Delineating the network interactions that give rise to selective neuronal responses requires a real-time analysis of synaptic activity in the presence and absence of relevant stimuli because functional interactions between component neurons may change in a stimulus-dependent fashion (Ahissar et al. 1992; Lampl et al. 1999; Prut et al. 1998; Vaadia et al. 1995). Furthermore, neurons in these networks receive both local and extrinsic sources of synaptic input, either of which could contribute to synaptic interactions underlying selective sensory responses (Carr and Konishi 1990; Faingold et al. 1991; Ferster and Miller 2000; Hartings and Simons 2000; Rausell et al. 1998; Reid and Alonso 1996; Sillito 1975). Therefore a comprehensive analysis of neuronal selectivity requires distinguishing local and extrinsic contributions to stimulus-evoked synaptic activity.

A specialized network of brain nuclei in oscine songbirds mediates learned vocal control and contains neurons strongly and selectively responsive to playback of the bird’s own song (BOS) (Bottjer et al. 1984; Doupe 1997; Margoliash and Konishi 1988; Nottebohm et al. 1976; Viciano and Yohay 1993), providing an attractive system to investigate stimulus-dependent synaptic interactions relevant to learned vocal communication. Among these nuclei, the telencephalic nucleus HVC is an especially important site for analyzing synaptic mechanisms underlying specific representations of self-generated vocal sounds because BOS-evoked firing patterns in HVC PNs are more sparse than in HVC’s major auditory afferent, the interfacial nucleus of the nidopallium (NIf), and because HVC PNs respond more exclusively to the BOS than do neurons in NIf (Coleman and Mooney 2004). Furthermore, HVC contains two different PN types (HVC RA and HVC X) that innervate functionally specialized pathways for song patterning or audition-dependent vocal plasticity, raising the possibility that auditory information transmitted by these two output cell types may be distinct (Fig. 1A) (Bottjer et al. 1984, 1989; Brenowitz 1991; Gentner et al. 2000; Nottebohm et al. 1976). Indeed, although both HVC PNs discharge sparse action potentials to BOS playback (Mooney 2000; Rosen and Mooney 2003), BOS-evoked subthreshold activity differs in the two HVC PNs: HVC RA exhibit sustained synaptic depolarizations, whereas HVC X undergo sustained hyperpolarizations punctuated by brief depolarizing postsynaptic potentials (PSPs) (Mooney 2000). These contrasting subthreshold response patterns likely reflect different synaptic specializations in HVC’s two PN types that may relate to their distinct functional roles and that could modify auditory information transmitted to RA and area X.

Our knowledge of the underlying synaptic mechanisms driving BOS-evoked sub- and suprathreshold activity in HVC remains incomplete. Prior studies suggested that NIf is the major source of direct or indirect auditory excitatory input to...
all HVC neuron types (Cardin and Schmidt 2004; Coleman and Mooney 2004; Janata and Margoliash 1999) and that HVC interneurons (HVCInt) could account for inhibition onto HVCX cells (Mooney 2000; Mooney and Prather 2005). However, these prior studies did not resolve whether all HVC neuron types receive BOS-selective auditory input from extrinsic sources or instead whether some only receive feedforward auditory drive from other HVC neurons. Furthermore, these earlier studies did not directly test whether removing sources of local inhibition either at the network or single-cell level was

FIG. 1. The song circuit and spike-triggered average analysis. A: auditory input to the song circuit (dark gray) arises via nucleus ovoidalis (Ov), field L, nucleus interfacialis (NIf), and the HVC shelf region. HVC contains separate populations of neurons that project to either the vocal-motor pathway (white) or the song modification pathway (gray), which converges back onto the motor pathway at the robust nucleus of the arcopallium (RA). Dotted lines indicate indirect connections. LMAN, lateral magnocellular nucleus of the anterior nidopallium; X, Area X; DLM, medial nucleus of the dorsolateral thalamus; nAM, nucleus ambiguus; nRAm, nucleus retroambigualis; nXIIts, tracheosyringeal portion of the hypoglossal nucleus; syrinx, song production organ. B: to compute spike-triggered averages (STAs), averages of 200-ms time windows around the time of each spike in cell 2 were computed from the median filtered (to remove contaminating spikes) membrane potential of cell 1 (gray bars), yielding traces such as those depicted in black in C and D. To compute control STAs, artificially generated spike trains at frequencies varying from 0.5 to 15 Hz (set to match the average firing frequency of cell 2) were used to create the time points around which the averages were drawn from the membrane potential in cell 1 (white bars). As these time points were not related to the timing of inputs onto the cells, this provided an estimate of the synaptic noise in cell 1. These control traces are depicted in gray in C and D. C: STA depicting common excitatory inputs, defined as the peak of a depolarizing potential (black circle) exceeding the maximum value of the control trace (top gray line) and following the spike time, while its onset precedes the spike time [onset is defined as the average of the time the membrane potential crosses the maximum value of the control trace (white circle) and the time the slope of the membrane potential (the 1st derivative, middle) exceeds $1.3 SD$ (white circle)]. The model on the right shows cells 1 and 2 receiving common excitatory inputs. D: STA depicting lagging inhibition, defined as both the onset of a hyperpolarizing deflection and its peak negativity (black circle, negative to the minimum value of the control trace, bottom gray line) following the spike time [onset is defined as the average of the time the membrane potential crosses the maximum value of the control trace (white circle) and the time the slope of the membrane potential (the 1st derivative, middle) exceeds $1.3 SD$ (white circle)]. The model on the right shows 2 possible relationships that could yield a lagging hyperpolarization. In the top model, cell 2 directly inhibits cell 1, whereas in the bottom model, cell 1 is indirectly inhibited by an intervening neuron that receives inputs common to cell 2. E: 2 examples of unfiltered raw traces from pairs of simultaneously recorded neurons. Top 2 traces: an HVCX cell pair, bottom 2 traces: an HVCX neuron pair.
sufficient to account for differences in subthreshold response patterns normally observed in the two HVC PN types.

To characterize functional synaptic interactions in HVC, we made dual intracellular recordings from pairs of HVC neurons in vivo and used spike-triggered averaging (STA) methods to examine their spontaneous and BOS-evoked synaptic connections. Consistent with the idea that functional interactions in HVC change in a stimulus-dependent manner, we found that all HVC neurons receive common excitation that becomes more tightly correlated across projection neurons during BOS playback. To determine the extent to which the common excitation seen across all HVC neuron types arises from HVC’s local network or from HVC’s afferents and to establish that BOS-evoked inhibition in HVCX cells derived from local sources, we compared BOS-evoked synaptic activity in HVCX and HVCRA cells while pharmacologically inactivating the entire HVC circuit or while selectively disrupting G-protein-coupled inhibition in HVCX cells. Either treatment abolished song-evoked hyperpolarizations in HVCX cells and increased the similarity of BOS-selective subthreshold response patterns in the two PNs. Therefore the two HVC PNs receive part of their common excitation from a BOS-selective extrinsic source, and BOS playback selectively recruits local inhibition onto HVCX neurons. These results indicate that the functional connectivity within HVC is altered distinctly for each PN type in a stimulus-dependent manner, which may enable HVC to transform information received from a common source (i.e., NIF) into two distinct representations transmitted to premotor and basal ganglia pathways.

METHODS

As most techniques used here have been extensively described previously (Rosen and Mooney 2000, 2003), only a brief description is provided.

Subjects and stimuli

Experiments used 56 adult (>120 posthatch-days) male zebra finches (Taeniopygia guttata) in accordance with a protocol approved by the Duke University Institutional Animal Care and Use Committee. Prior to the experiment, birds were placed in a small recording chamber with a female, and song was recorded and edited to include two exemplary motifs using LabView software (all custom software made for this study was written by M. Rosen and R. Balu). Stimuli were 1.5–3.0 s in duration and included the BOS and reversed BOS (BOS-REV) i.e., song played backward, perturbing local and global temporal order, while maintaining spectral information). Prior studies have shown that HVC neurons are differentially responsive to these two stimuli both at a suprathreshold and subthreshold synaptic level (Margoliash 1983, 1986; Mooney 2000).

Preparatory surgery, in vivo electrophysiology, song presentation, and drug application

On the day of electrophysiological recording, birds were anesthetized with 20% urethan in dH2O (90 μl total), further anesthetized with halothane for 1 min, and placed in a stereotaxic device, and a stainless steel post was mounted to the rostral skull with dental cement and reinforced with cyanoacrylate. Birds were immobilized via the mounted post in a sound-attenuating chamber on an air table and reinforced with cyanoacrylate. Birds were immobilized via the mounted post in a sound-attenuating chamber on an air table and reinforced with cyanoacrylate. The electrode was lowered into the nucleus (~50–600 μm) using a one-dimensional hydraulic manipulator (Soma Scientific). For dual intracellular recordings, two AxoClamp 2B intracellular amplifiers were used in bridge mode to measure intracellular potentials, which were low-pass filtered at 3 kHz, digitized at 10 kHz, and stored on a PC. HVC neuronal types were identified on-line by their characteristic spike shape and firing patterns in response to positive current injection (Mooney 2000). HVC shelf neurons were identified by the location ventral to the nucleus or by their DC-evoked action potential response, which were different from HVC neurons. Cells were tested with 10–30 iterations (at ~70 dB) of the auditory stimuli if their resting potentials were negative of ~55 mV and robust spontaneous synaptic activity was present. As noted in the results for certain cells, tonic negative or positive currents were injected through the recording electrode to shift the resting membrane potential of the cell.

For GABA inactivation experiments, a second micropipette [10–20 μm tip, filled with 250 mM GABA (RBI, Natick, MA) dissolved in 0.9% NaCl] was lowered at a 30° angle through a second craniotomy to a point just ventral to HVC. The GABA was pressure-ejected with a Picospritzer (General Valve) in 10- to 200-ms pulses at 40 psi. GABA was applied during each interstimulus interval while recording, as it washed out very quickly (10–120 s) otherwise. Concentrated GABA application strongly activates chloride and potassium conductances, shunting positive currents, thus greatly reducing excitatory synaptic drive. During GABA treatment, remaining synaptic activity in HVC was greatly reduced but still detectable, and the cells from which we recorded were unable to spike, thus removing their action-potential dependent contributions to local circuit activity. Effectiveness of the GABA inactivation was assessed during and after the application period by monitoring changes in spontaneous PSP amplitude as well as by monitoring the ability of a positive current pulse to evoke action potentials from the impaled cell. To ensure effective inactivation of the entirety of HVC, we used parameters of GABA application that were previously effective in entirely inactivating the song nucleus LMAN, which is of similar size to HVC. For LMAN, we confirmed that the whole nucleus was inactivated by recording from cells in disparate locations across the nucleus during GABA application (Rosen and Mooney 2000). This spatial verification was not possible in HVC as the nucleus is close to the surface and the size of the craniotomy had to be kept to a minimum to maintain stable recordings. However, the positioning of the GABA pipette immediately ventral to HVC where diffusion should encompass all of HVC, along with application of identical concentrations and similar quantities of GABA as those used effectively on LMAN, suggest that HVC was effectively inactivated by this treatment.

Data analysis

SPIKE-TRIGGERED AVERAGES. Briefly, a spike-triggered average (STAs) of one of the two cell’s membrane potentials was calculated by averaging the median-filtered neuronal membrane potential of one cell relative to action potential activity in the other cell that exceeded a user-set trigger threshold (Fig. 1B); STAs were calculated for a time window ±200 ms relative to the trigger event. (Median-filtering the membrane potential removes high-frequency events such as spikes by replacing each point by the median value of the surrounding 50 points,
equivalent to 5 ms at a 10-kHz sampling rate.) These STAs could reveal membrane potential deflections likely to reflect excitatory or inhibitory synaptic events associated with the timing of spikes in the other neuron of the pair (Fig. 1, C and D). To determine significance, spontaneous and stimulus-evoked STAs were compared with control STAs calculated by averaging the cell’s membrane potential with respect to simulated regular spike trains of 1–15 Hz (set to match the average spontaneous or stimulus-evoked firing rate of the spiking neuron). These control STAs indicate the maximum amplitude over which uncorrelated events would appear, effectively capturing the variability inherent in the membrane potential of each cell, which was usually ~1 mV. Deflections in the raw STA that exceeded the maximum or minimum of the control STAs were classified as significant events that could be used to further assess peaks and onsets of the raw STA; these STAs were generally >2 mV, well above the level of the controls (Fig. 1, C and D). The onset times of STA peaks were estimated by calculating the cumulative sum of the mean STA from 20 ms before and 30 ms after spiking in the other cell and designating the peak onset to be 5% of the maximum response (Coleman and Mooney 2004) (Fig. 1, C and D, average of white circles). The onset times of STA troughs, which fell outside this time window, were estimated as the average of the time at which the membrane potential leading up to the trough minimum crossed the mean resting potential of the prespike portion of the STA (V_rest) and the maximum of the cumulative sum of the mean STA between a chosen time point clearly prior to the membrane potential descent and the time point of the trough minimum (these 2 measures often yielded identical onset times). The timing of the maxima or minima of STAs (i.e., the STA peaks or troughs) were measured relative to the trigger spike time. STAs were calculated only for those traces in which both cells remained stable and healthy (spike height was maintained and resting V_m remained negative to ~50 mV). Two examples of typical paired recordings are depicted in Fig. 1E. The prespike portion of the STA depicts the mean resting potential (V_rest) of the cell the membrane potential of which was being averaged. The amplitude of an STA peak or trough is dependent on the difference between V_rest and the reversal potential of the activated currents. Therefore amplitude comparisons were always made between prestimulus and stimulus epochs that were immediately sequential to avoid any effects of any slow fluctuations in V_rest.

To fully characterize the nature of functional interactions observed for various neuron-pair types, STAs were calculated separately during silence (i.e., spontaneous activity), auditory stimulation with a behaviorally-relevant stimulus (BOS-evoked activity), and auditory stimulation with a control stimulus, BOS-REV (BOS-REV-evoked activity) (e.g., Figs. 4, B and E, and 6). All of the action potentials generated by the trigger cell during stimulus presentation were used to calculate each STA, and STAs for all three stimulus conditions were calculated across all cells within each neuron-pair type. To visualize the values representing the shapes of the STAs for all pairs, scatter plots were constructed where the onset times of the STA peaks/troughs were plotted at 0 amplitude, and these onset times were connected by a line to a point marking the time and amplitude of their corresponding peak or trough. Note that in some cases, significance was achieved for only one peak or trough per STA, thus scatter plots did not always contain equivalent numbers of peaks and troughs. These onset-to-peak scatter plots are included to show the variability across all cell pairs that contributed to the average STA (e.g., the variability of the average STA in Fig. 2B is represented by the scatter plot of contributing onsets and peaks in Fig. 2C).

**FIG. 2.** STAs on HVC_Ra-HVC_Ra neuron pairs reveal common excitatory inputs. A: raw intracellular traces from 2 simultaneously recorded HVC_Ra neurons (spikes truncated), recorded during spontaneous activity, showing largely synchronous excitatory postsynaptic potentials (EPSPs). B: averaged STA of all spiking HVC_Ra, HVC_Ra pairs (n = 12) during spontaneous activity shows common excitation onto HVC_Ra neurons, as the peak onset time precedes and the peak time follows spiking in the other HVC_Ra cell. C, mean peak onset time. D: summary data showing the times of onset (○) and peak (●) for all significant STA peaks during spontaneous activity (onset and peaks from individual STAs are connected with lines). The amplitudes are corrected for resting V_m; the peak onset is plotted at 0 mV, and the peak amplitude is measured from the mean of the control STA (see METHODS). All peaks are depolarizing, and all onset and peak times are consistent with common excitation. D: poststimulus time histograms of suprathreshold responses to 10 presentations of BOS are depicted for 2 simultaneously recorded HVC_Ra neurons (top) as are their median-filtered average membrane potentials (middle) and an oscilloscope showing the amplitude envelope of the BOS (bottom). Note that while the subthreshold responses to song are similar across these 2 neurons, their firing patterns are different. E: during BOS playback, common excitation visible in the STA averaged across all spiking pairs has an onset latency significantly closer to spike time than during spontaneous activity (see Table 1). The amplitude of the STA peak is significantly smaller than during silence, which may be due to the BOS-depolarized membrane potential (e.g., in D), ○, mean peak onset time. F: summary data showing the times of onset (○) and peak (●) peaks. All peaks are depolarizing, and most onset and peak times are consistent with common excitation, although a minority may also receive additional lagging excitatory inputs (e.g., positive onset times).
To analyze differences in stimulus-evoked neuronal interactions, comparisons were made between spontaneous and BOS-evoked STAs. Within-cell t-tests were applied to compare the peak amplitudes and latencies of STAs obtained during spontaneous and BOS-evoked activity and were computed on the subset of cells in which significant peaks/troughs occurred in both conditions. This within-cell comparison was necessary to effectively compare stimulus-induced changes in individual peaks or troughs, which were variable in their timing and amplitude across cells.

For a better estimate of changes in stimulus-evoked neuronal interactions, additional comparisons were made on normalized STAs, where the time-varying effect on the membrane potential due to the auditory stimulus was first removed by normalizing each stimulus-evoked STA. Specifically, the median-filtered, mean membrane potential in response to several repetitions of the stimulus (BOS or BOS-REV) was subtracted from each individual trace, and STAs were calculated as described in the preceding text from these normalized traces. This allowed a more accurate estimate of cellular interactions than that obtained by comparing un-normalized STAs, which would be contaminated by activity driven by the auditory stimulus rather than by cell-cell interactions. To compare normalized traces to one another, BOS-evoked STAs were compared with BOS-REV-evoked STAs as a control stimulus rather than with spontaneous STAs, which could not be similarly normalized. Within-cell t-tests were applied to compare the peak amplitudes and latencies of STAs obtained during BOS-evoked and BOS-REV-evoked activity, and were computed on the subset of cells in which significant peaks/troughs occurred in both stimulus-evoked conditions (Fig. 6, Table 2).

SONG-SELECTIVITY QUANTIFICATION. The suprathreshold responsiveness of cells with spiking activity was calculated by subtracting the firing rates during a 2-s baseline period before each stimulus presentation from the firing rates during each stimulus presentation. To assess subthreshold responsiveness in spiking cells, raw traces were first median-filtered to remove high-frequency events such as action potentials. The subthreshold responsiveness of both nonspiking and median-filtered spiking cells was measured by subtracting (baseline from stimulus) the integrals of the deviations in $V_m$ above the mode of the baseline period. A similar approach was used to measure net hyperpolarizations, where the integrals below the mode of the baseline period were calculated. Net stimulus-evoked hyperpolarizations are reported as negative numbers. Note that a lack of subthreshold responsiveness may indicate that a cell receives no stimulus-evoked STA. Specifically, the median-filtered, mean membrane potential in response to several repetitions of the stimulus (BOS or BOS-REV) was subtracted from each individual trace, and STAs were calculated as described in the preceding text from these normalized traces. This allowed a more accurate estimate of cellular interactions than that obtained by comparing un-normalized STAs, which would be contaminated by activity driven by the auditory stimulus rather than by cell-cell interactions. To compare normalized traces to one another, BOS-evoked STAs were compared with BOS-REV-evoked STAs as a control stimulus rather than with spontaneous STAs, which could not be similarly normalized. Within-cell t-tests were applied to compare the peak amplitudes and latencies of STAs obtained during BOS-evoked and BOS-REV-evoked activity, and were computed on the subset of cells in which significant peaks/troughs occurred in both stimulus-evoked conditions (Fig. 6, Table 2).

RESULTS

In the first section of RESULTS, we use simultaneous in vivo intracellular recordings from HVC neuron pairs and spike-triggered averaging methods both during silence and BOS playback to assess any stimulus-evoked changes in functional synaptic interactions. In the second section, we pharmacologically inactivate the HVC local circuit to confirm that all HVC neuron types receive at least some of their BOS-selective excitation from extrinsic sources. In the final section of RESULTS, we block inhibition onto HVCX neurons either at the single-cell level or by inactivating the HVC local circuit to test whether such inhibition is sufficient to account for the differences in BOS-evoked subthreshold activity patterns normally observed between HVCX and HVCRA cells.
Simultaneous dual intracellular in vivo recordings: all HVC cell types receive common excitatory inputs

Simultaneous intracellular recordings were made from 9 HVC\textsubscript{RA}-HVC\textsubscript{RA} pairs, 11 HVC\textsubscript{X}-HVC\textsubscript{X} pairs, 13 HVC\textsubscript{RA}-HVC\textsubscript{X} pairs, and 4 HVC\textsubscript{X}-HVC\textsubscript{Int} pairs. Examples of spontaneous and BOS-evoked activity from each pair type are depicted in Figs. 2–5. All pair types showed clear evidence of common excitatory inputs, i.e., the onset of a significantly depolarizing membrane potential movement in at least one cell of the pair reliably occurred prior to the spike time in the other cell (Fig. 1C; see METHODS). STAs revealed evidence of common excitation in all pairs of HVC\textsubscript{RA} neurons where at least one member of the pair was suprathreshold \((n = 7 \text{ of } 9 \text{ pairs}; \text{Fig. 2, B and E})\), in all 11 HVC\textsubscript{X}-HVC\textsubscript{X} pairs (Fig. 3, B and E), in 11 of 13 HVC\textsubscript{X}-HVC\textsubscript{RA} pairs (Fig. 4, B and E), and in 3 of 4 HVC\textsubscript{X}-HVC\textsubscript{Int} pairs (Fig. 5, B and E). Scatter plots of individual STA onset times and peak times (see METHODS) show that all positive peaks occurred near or past spike time, and that most positive onsets occurred prior to spike time (for onsets that lagged after the spike, a depolarizing STA onset occurred prior to spike time in the other cell of the pair, indicative of common excitatory input; Figs. 2–5, C and F). These data indicate that the majority of HVC cells receive excitatory inputs from a common source, while a minority may also receive additional lagging excitatory inputs. This excitation may arise extrinsically from NIF, intrinsically via connectivity within HVC, or from a combination of the two.

Simultaneous dual intracellular in vivo recordings: HVC\textsubscript{X} neurons receive lagging inhibitory inputs

In addition to common excitation, these dual recordings helped us further elucidate the nature of BOS-evoked hyperpolarizing activity in HVC\textsubscript{X} cells. Indeed, HVC\textsubscript{X} neurons respond to BOS playback with intermittent, phasic excitation occurring on a background of sustained membrane hyperpolarization, clearly distinguishing these cells from HVC\textsubscript{RA} neurons. Sequential recordings from different HVC cell types in individual birds have shown that the timing of HVC\textsubscript{Int} excitation correlates with this hyperpolarization, indicating that HVC\textsubscript{Int} neurons may provide hyperpolarizing inhibition onto HVC\textsubscript{X} cells (Mooney 2000). Inhibition onto HVC\textsubscript{X} cells from these HVC\textsubscript{Int} neurons also may be functionally recruited by one or both HVC PN types, an organizational feature which would result in negative STAs of HVC\textsubscript{X} membrane potential lagging after spikes in any HVC neuron type. To identify potential direct and/or indirect sources of inhibition onto HVC\textsubscript{X} neurons, we made dual recordings in HVC where at least one cell was of the HVC\textsubscript{X} type and exhibited BOS-evoked hyperpolarization.

BOS-evoked hyperpolarizing responses were detected in 13 of 22 HVC\textsubscript{X} neurons from 11 spiking HVC\textsubscript{X}-HVC\textsubscript{X} pairs, in 8 of 10 HVC\textsubscript{X} neurons from 10 spiking HVC\textsubscript{X}-HVC\textsubscript{RA} pairs (3 additional HVC\textsubscript{RA} neurons were subthreshold), and in 3 of 3 HVC\textsubscript{X} neurons from 3 spiking HVC\textsubscript{X}-HVC\textsubscript{Int} pairs (1 addi-
tional HVC_int neuron was subthreshold), yielding a total of 24 cell pairs for the analysis (see Figs. 3D, 4D, and 5D for BOS-evoked hyperpolarization). Of these 24 cell pairs, STAs measured during both spontaneous and BOS-evoked activity revealed a total of 23 HVCX neurons with significant negative peaks (12 of 13 from HVCX-HVCX pairs, 8 of 8 from HVCX-HVC_RA pairs, and 3 of 3 from HVCX-HVC_int pairs; Figs. 3, B and E, 4, B and E, 5, B and E). The mean peak negativity of these STAs lagged after spikes by 20.24 ± 4.16 ms (raw data visible in scatter plots in Figs. 3, C and F, 4, C and F, 5, C and F; see Table 1 for breakdown by cell type). These data show that the timing of action potential activity in HVC PNs of both types as well as interneurons is correlated with a delayed hyperpolarization in HVCX neurons, and thus all three cell types could provide direct or indirect sources of inhibition onto HVCX cells. Similar functional organization has been revealed recently in paired recordings made from different HVC neuron types in brain slices (Mooney and Prather 2005).

Although we were able to record from only three spiking HVCX-HVC_int pairs (of 4 pairs total, as 1 additional interneuron in an HVCX-HVC_int pair exhibited only subthreshold responses), these three cases showed relatively heterogeneous peak behaviors (Fig. 5). There were significant negative peaks in all three HVCX cells lagging behind HVC_int spikes, but two of the three had longer peak latencies and smaller amplitudes compared with the third (onsets: 7.7, 63.4, 4.0 ms; peaks: 61.8, 101.7, 21.3 ms; amplitudes: −0.9, −0.4, −2.7 mV). These longer latency interactions contrasted with that of the mean peak negativity in HVCX cells, which was 20.24 ± 4.16 ms. One possibility is that only some interneurons recorded here are the actual sources of inhibition onto the HVCX cells perhaps because only some classes of interneurons make inhibitory synapses on HVCX cells. Indeed, based on biochemical markers, HVC interneurons comprise several different types, which by analogy to mammalian cortical circuitry, may be functionally specialized in their postsynaptic contacts (Gulyas et al. 1996; Mooney and Prather 2005).

FIG. 4. STAs in HVC_RA-HVCX neuron pairs show common excitation and delayed inhibition. A: intracellular traces from 2 simultaneously recorded neurons, 1 HVCX and 1 HVC_RA (spikes truncated), recorded during a silent period. B: averaged STAs of HVC_RA-HVCX pairs (n = 13) during spontaneous activity, depicted separately for each cell type. B1: STAs of HVC_RA membrane potential around HVCX spikes from 13 spiking HVCX cells; B2: STAs of HVCX membrane potential around HVC_RA spikes from 10 spiking HVC_RA cells. STAs reveal common excitation onto both HVC_RA and HVCX neurons, and delayed inhibition onto HVCX neurons. Shaded bar, mean peak onset times. C: summary data for STAs separated by cell type, showing the times of onset (○) and peak (●) for individual significant positive and negative peaks (onset and peaks from individual STAs are connected with lines). Positive-going peaks, visible in both HVC_RA and HVCX cells, usually show onset and peak times consistent with common excitation. Negative-going peaks, visible only in HVCX cells, show onset times following spike time, indicating a lagging inhibition. Note that not all cells had both significant positive and negative peaks, accounting for the mismatch in the number of peaks vs. troughs. D: poststimulus time histograms of spiking and median-filtered average membrane potentials to 10 presentations of BOS are depicted for simultaneously recorded HVCX and HVC_RA neurons. The BOS evokes depolarization in the HVC_RA neuron (gray) and both depolarization and hyperpolarization in the HVCX neuron (black). E: onset latencies of common excitation in both cell types is significantly closer to spike time during BOS playback. BOS also increases the amplitude of the delayed negative peak in 63% of HVCX neurons (E2, although this effect is not significant across all pairs, see Table 1). Note the visible song-evoked STA baseline depolarization in HVC_RA neurons (compared with values in B at ~50 ms). Shaded bar, mean peak onset times. F: summary data of individual STAs for each cell type showing the times of onset (○) and peak (●) for all significant BOS-driven STA positive and negative peaks.

J Neurophysiol • VOL 95 • FEBRUARY 2006 • www.jn.org
Simultaneous dual intracellular in vivo recordings: Interactions between neurons are significantly altered during BOS playback

In addition to analyzing the sign of interactions between cells in HVC, the dual recording methods allowed us to assess whether these functional connections changed in a stimulus-dependent manner. Prior studies have shown that stimulus-driven changes in synaptic interactions that may reflect or underlie stimulus processing exist in several systems (Ahissar et al. 1992; Lampl et al. 1999; Prut et al. 1998; Vaadia et al. 1995). Therefore we also examined whether the BOS, a particularly salient behavioral stimulus, might dynamically alter synaptic activity within HVC neurons by measuring the timing and amplitude of STA peaks in cell pairs before and during presentation of the BOS and other auditory stimuli. We compared BOS-evoked STA peaks with STA peaks during two control conditions: spontaneous activity and BOS-REV. The latter comparison allowed us to normalize both BOS and BOS-REV traces by removing stimulus-driven membrane potential movement, with the result that the STAs generated from these normalized traces more accurately represent functional connections between cells. Comparisons of unnormalized STAs evoked by BOS versus spontaneous activity are depicted as average STAs and individual peaks and troughs in Figs. 2–5, B and C, versus E and F. Comparisons of normalized STAs evoked by BOS versus BOS-REV are depicted in Fig. 6, which shows an example of BOS and BOS-REV-evoked supra- and subthreshold activity for a HVCX–HVCRA pair and the resulting normalized STAs from that cell pair, as well as average STAs and individual peaks and troughs across all cell-pairs. The unnormalized BOS versus spontaneous comparison and the normalized BOS versus BOS-REV comparison produced similar results.

While common excitation could be detected during spontaneous, BOS-REV, and BOS-evoked activity, BOS playback significantly shortened the onset time of the excitation in HVCRA–HVCX neuron pairs when compared with BOS-REV (Fig. 6C, top), and marginally shortened the onset time compared with spontaneous activity [Fig. 4, B and C, vs. E and F; normalized BOS-REV = −14.5 ± 0.5 ms, normalized BOS = −12.5 ± 1.1 ms, paired t(13) = 2.8, P = 0.008. Unnormalized spontaneous = −14.9 ± 0.4 ms, Unnormalized BOS = −13.7 ± 0.8 ms, paired (15) = 1.6, P = 0.06], This effect was significant across all cell pair types in BOS versus BOS-REV comparisons (normalized BOS-REV = −15.0 ± 0.2 ms, normalized BOS = −14.3 ± 0.4 ms, paired t(44) = 2.5, P = 0.008). In other cell-pair types (HVC RA–HVCRA, HVCX–HVCX, HVCX–HVCInt), the onset times of excitatory peaks in

FIG. 5. STAs in HVCInt–HVCX neuron pairs show common excitation and delayed inhibition. A: HVCX neuron and an HVCInt, recorded simultaneously during a silent period. B: averaged STAs of all spiking HVCX, HVCInt pairs (n = 3), during spontaneous activity and BOS playback, depicted separately for HVCInt membrane potential around HVCX spikes (B1) and HVCX membrane potential around HVCInt spikes (B2). Common excitation is visible in both cell types and delayed inhibition in HVCX cells. Shaded bar, mean peak onset times. C: summary data showing the times of onset (○) and peak (●) for all significant STA positive and negative peaks. Positive-going peaks in both cell types show onset times suggesting common excitation, whereas lagging inhibition is visible only in HVCX cells. D: poststimulus time histograms to 10 presentations of BOS for simultaneously recorded HVCX and HVCInt cells show largely anti-correlated BOS-evoked spiking across these cells. Median-filtered average membrane potentials depict BOS-evoked depolarization in HVCInt cells (gray) and both depolarization and hyperpolarization in HVCX cells (black). E: averaged STAs during BOS playback show that lagging inhibition increased in amplitude in 2 of 3 cells. Shaded bar, mean peak onset times. F: summary data of individual STAs for each cell type showing the times of onset (○) and peak (●) for all significant BOS-driven STA positive and negative peaks.
BOS-evoked STAs did not differ from those in control STAs. Compared with spontaneous activity, BOS playback also significantly reduced the peak amplitude in HVCRA-HVCRA pairs [unnormalized spontaneous = 6.7 ± 1.6 mV, unnormalized BOS = 4.5 ± 1.1 mV, paired t(8) = 3.4, P = 0.005], and across all cell pair types [unnormalized spontaneous = 3.5 ± 0.4 mV, unnormalized BOS = 2.7 ± 0.3 mV, paired t(46) = 2.7, P = 0.004], perhaps due to the previously described sustained positive shift in membrane potential evoked in these cells by BOS playback (Fig. 2, D and E) (Mooney 2000). Consistent with this idea, the STA membrane potential 200 ms prior to the spike time was significantly more positive in the BOS playback than silent condition in HVCRA-HVCRA pairs [visible in Fig. 2, B vs. E; spontaneous = −67.6 ± 4.9 mV, BOS = −64.2 ± 5.3 mV, paired t(11) = 4.09, P < 0.001]. A breakdown of the timing and magnitude of excitatory peaks by pair-type across stimuli is shown in Tables 1 and 2. Therefore our data overall indicate a significant negative peak in the HVCX-trace STA either emerged or increased in amplitude during BOS playback compared with either BOS-REV or spontaneous activity in 70% of HVCX-HVCRA pairs [a significant increase for BOS vs. BOS-REV comparisons: normalized BOS-REV = −1.1 ± 0.3 mV, normalized BOS = −1.4 ± 0.4 mV, paired t(24) = 2.0, P = 0.04], 71% of HVCX-HVCX pairs [significant increases for both comparisons: normalized BOS-REV = −0.7 ± 0.2 mV, normalized BOS = −0.9 ± 0.2 mV, paired t(13) = 2.0, P = 0.037; unnormalized spontaneous = −0.9 ± 0.2 mV, unnormalized BOS = −1.4 ± 0.3 mV, paired t(16) = 2.1, P = 0.028], and 67% of HVCX-HVCX pairs (effective statistical analysis was not possible as only 3 pairs yielded STAs). A breakdown of the timing, magnitude, and significance levels of these inhibitory deflections by pair type across stimuli is shown in Tables 1 and 2. Therefore our data overall indicate a stimulus-driven increase in the amplitude of inhibition onto HVCX neurons, with some evidence for tighter timing in the peaks of hyperpolarizing STAs.

**GABA inactivation reveals selectivity of afferents and differential effects of local circuitry**

Our STA data identify distinctive subthreshold responses exhibited by HVCRA and HVCX neurons, consistent with common excitatory synaptic inputs onto both cell types and an additional lagging inhibitory synaptic input onto HVCX neurons. However, dual recording experiments are not sufficient to deduce the sources of these excitatory and inhibitory inputs on...
FIG. 6. Comparisons of BOS-evoked and BOS-REV-evoked responses. A: example of BOS- and BOS-REV-evoked supra- and subthreshold responses in both cells of a HVCX-HVCRA pair. Top graphs for each neuron depict poststimulus time histograms (10-ms bin width) for 10 presentations of each stimulus. Bottom graphs depict median-filtered averaged membrane potential responses to each stimulus. Song stimuli are shown below as oscillograms. B: example of normalized STAs computed from the cells depicted in A with BOS-evoked (black) and BOS-REV-evoked (gray) normalized STAs overlaid for comparison. Peak maxima and trough minima are shown with circles. C: mean normalized STAs, calculated from cell pairs with significant peaks in both BOS and BOS-REV conditions. Different subsets of cell pairs were used to calculate changes in STA peaks and changes in STA troughs; those subsets were used to generate the mean peak STA and mean trough STA (see METHODS). The HVCRA trace in B contributed to the mean peak STA; the HVCX trace in B contributed to both the mean peak STA and the mean trough STA. D: summary data of individual STAs showing the times of onset and maxima or minima for all significant BOS-driven (○) and BOS-REV-driven (○) positive and negative peaks.
HVC neurons. Previous experiments have shown that abolishing activity in NIf can suppress much or all spontaneous and song-evoked synaptic activity in HVC, and simultaneous recordings in NIf and HVC suggest that NIf supplies either direct or indirect excitatory input onto all three HVC neuron types (Coleman and Mooney 2004). These experiments could not rule out a model where NIf supplies direct excitatory input to only some HVC cell types and excitatory connections local to HVC supply other HVC neuron types with all of their excitation. In addition, these experiments could not exclude the possibility that NIf supplies qualitatively distinct (auditory) input to different HVC cell types. More specifically, the excitation common to both HVC PN types could be due to common input arising from NIf onto both cell types, with the differences between them due to differences in local (inhibitory) activity, or functionally distinct NIf inputs could segregate onto the two cell types.

These alternate models make specific predictions about the effects of inactivating the HVC local circuit. First, if some HVC cell types receive only indirect auditory input from NIf, inactivating the local HVC circuit should abolish their auditory responses altogether. On the other hand, subthreshold responses should persist and become more similar in HVC neurons on local circuit inactivation if NIf provides a relatively homogeneous source of auditory input to all HVC cell types. Finally, if local inactivation of HVC reveals persistent auditory-evoked excitation in both HVC PNs, then blockade of inhibition in HVC neurons at the single-cell level should also cause subthreshold responses in the two PNs to become more similar.

To test these various ideas, we first made intracellular recordings from individual HVC cells and then eliminated the contribution of HVC’s local circuitry to their synaptic responses by extracellular application of GABA to the nucleus. Consistent with prior studies, we found that before GABA application, all cell types showed sub- and suprathreshold selectivity (as measured using $d’$ analysis, see METHODS) for the BOS with hyperpolarizations readily detectable in the HVC membrane potential responses. On application of GABA to HVC, both spontaneous and stimulus-evoked firing was abolished completely in all HVC cell types, disabling local, action potential-dependent contributions to synaptic activity in HVC. Despite such local circuit inactivation, auditory-evoked synaptic activity persisted in all neuronal types (Fig. 7). Moreover, these synaptic responses remained BOS-selective, confirming that all HVC neuron types receive at least some extrinsic sources of BOS-selective input. Furthermore, these extrinsic inputs are likely to be excitatory, because they depolarized the cell and were not occluded by the GABA application.

One potential confound is that GABA treatment strongly hyperpolarized the resting potential of all HVC neurons and thus might be expected to alter driving forces on inhibitory and excitatory currents. Therefore to meaningfully compare subthreshold selectivity before and during GABA application, it was necessary to tonically hyperpolarize the cell prior to GABA application, thus maintaining equivalent resting membrane potentials before and during GABA treatment. Using this approach, we found that the mean selectivity of subthreshold depolarizing responses of HVC$_X$ cells significantly increased on inactivation of the local circuit [Fig. 7C, left; n = 11; HVC$_X$ hyperpolarized pretreatment: 0.51 ± 0.34, HVC$_X$ GABA-treated: 1.45 ± 0.18; paired t(10) = 2.86, P = 0.009]. In contrast, the $d’$ selectivity of depolarizing responses in HVC$_{RA}$ cells did not change [Fig. 7C, middle; n = 9; HVC$_{RA}$ pretreatment: 1.53 ± 0.43, HVC$_{RA}$ GABA-treated: 1.42 ± 0.21; paired t(8) = 0.21, P = 0.58)], whereas those of HVC$_{INT}$ marginally decreased [Fig. 7C, right; n = 6; HVC$_{INT}$ pretreatment: 1.48 ± 0.49, HVC$_{INT}$ GABA-treated: 0.49 ± 0.31; paired t(5) = 1.52, P = 0.09]. These data suggest that extrinsic inputs onto the various HVC neurons are BOS-selective and that HVC’s local circuit alters that selectivity in a cell-type specific manner. Specifically, the increased selectivity in HVC$_X$ neurons’ subthreshold depolarizing responses indicates that the local circuit normally functions to depress BOS-selective depolarizing responses in these cells.

We quantified changes in HVC$_X$ neuron subthreshold selectivity in two ways. First, as previously mentioned, we used $d’$ analysis to show that HVC$_X$ but not HVC$_{RA}$ cells display altered selectivity on inactivation of the local circuit (Fig. 7C, left vs. middle). Second, we noted that HVC$_{RA}$ and HVC$_X$ $d’$ values spanned the same range during local circuit inactivation [Fig. 7C, left vs. middle, comparing GABA selectivity: $t(19) = 1.04$, $P = 0.31$], whereas the $d’$ values for these two cell types were marginally distinct before inactivation [Fig. 7C, left vs.

<table>
<thead>
<tr>
<th>Pair Type (spiking cell in bold)</th>
<th>Normalized BOS-REV</th>
<th>Normalized BOS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Onset time</td>
</tr>
<tr>
<td>Excitatory peaks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All cells</td>
<td>24</td>
<td>15.6 ± 5.9</td>
</tr>
<tr>
<td>HVC$_{RA}$-HVC$_X$</td>
<td>8</td>
<td>7.6 ± 7.4</td>
</tr>
<tr>
<td>HVC$<em>X$-HVC$</em>{RA}$</td>
<td>14</td>
<td>24.7 ± 7.6</td>
</tr>
<tr>
<td>HVC$<em>X$-HVC$</em>{INT}$</td>
<td>2</td>
<td>30.2 ± 32.3</td>
</tr>
</tbody>
</table>

STAs were normalized for both auditory stimuli (see METHODS), and paired t-tests were calculated on the subset of cell-pairs in which both BOS and the control BOS-REV stimulus elicited significant peaks and/or troughs. Values are mean ± SE. Spiking cells are in boldface. *P ≤ 0.05; **P ≤ 0.01.
middle, comparing pre-GABA selectivity: $t(19) = 2.00, P = 0.059]$. These two analyses indicate that the relative bias of subthreshold responses in the two PNs become more similar when local circuit contributions to HVCX neurons are removed.

We also noted that the shape of the subthreshold response pattern changed following local inactivation and did so in a cell-type specific manner. Qualitatively, with the local circuit inactivated, BOS-evoked depolarizations in HVCX neurons became more sustained (Fig. 7A, 3 vs. 5) and more similar in shape to control responses in HVC RA cells recorded in the same bird (Fig. 7A, 3 vs. 2). In contrast, the overall shape of HVC RA BOS-evoked responses did not change markedly on local circuit inactivation (Fig. 7A, I vs. 2). Notably, the change in HVCX responses was not merely an effect of altered driving forces due to GABA-induced hyperpolarization, as the profile of the HVCX BOS-evoked response during DC-induced hyperpolarization in the absence of GABA (effectively removing the BOS-evoked hyperpolarization) did not closely mimic the profile of the subthreshold depolarizations recorded in the HVC RA cell from the same bird (Fig. 7A, I and 2 vs. 7A5).

To more completely quantify changes in subthreshold response patterns in GABA-inactivated HVCX neurons, we calculated normalized cross-correlations at zero time lag of BOS-evoked median-filtered averaged membrane potential records of HVCX neurons in control conditions and GABA-inactivated HVCX neurons within the same bird (see METHODS). These normalized cross-correlations were significantly higher than normalized cross correlations of membrane potential records from the same HVC RA neuron in control conditions and the same HVCX neuron prior to GABA treatment [with the HVCX cell held in a tonically hyperpolarized state; Fig. 9B, black bars; control hyperpolarized HVCX: $0.14 \pm 0.07$, GABA-treated HVCX: $0.35 \pm 0.10$, $n = 9$, paired $t$-test: $t(8) = 2.2, P = 0.03]$. These results suggest that the shapes of the BOS-evoked responses in the two PNs become significantly more similar when local circuit contributions to HVCX neuron

**FIG. 7.** Pharmacological inactivation of HVC by GABA application alters BOS responses in HVC neurons in a cell-type specific manner. A: responses to BOS and reversed BOS (BOS-REV) for 1 HVCX and 1 HVC RA neuron recorded sequentially in the same bird, before and during GABA application, with a spectrogram of song (bottom). Both cell types retain selectivity to BOS vs. BOS-REV (indicated by $d'$ values) on local circuit inactivation. The shape of the response in HVCX neurons during circuit inactivation becomes similar to that in HVC RA neurons. This change in shape is not simply due to a reversal of the inhibition in HVCX neurons on hyperpolarization, as manually hyperpolarizing the HVCX neuron with tonic negative current injection does not similarly change the shape of the HVCX neuron response (bottom black trace). B: HVC Int neurons also retain selectivity on inactivation of HVC. An example from an HVC Int in another bird where the shape of the response was maintained during GABA application. C: $d'$ measure of selectivity indicates that during local circuit inactivation, HVCX neurons significantly increase their bias for BOS over BOS-REV, whereas HVC Int cells marginally significantly decrease their bias; HVC RA bias does not change with local circuit inactivation. Dotted line, the criterion for significant bias for BOS over BOS-REV.
responses are removed. To estimate an upper limit for mem-
brane potential cross-correlation values between different HVC
neurons, we also calculated subthreshold cross-correlations
within homotypic pairs of either untreated HVCX or untreated
HVCRA neurons within the same bird (the white bars in Fig. 9B).
Notably, these homotypic pair cross-correlation values are
quite similar to the across-PN-type cross-correlation values of
GABA-treated HVCX neurons with control HVCRA neurons
[Fig. 9B, compare right-most black bar with white bars; 
GABA-treated HVCX: 0.35 ± 0.10; HVCX vs. HVCX: 0.28 ±
0.05; HVCRA vs. HVCRA: 0.33 ± 0.03; 1-way ANOVA:
F(3) = 0.78, P = 0.51]. Therefore local circuit activity
accounts for much of the differences in subthreshold response
patterns in the two HVC PN types that are detected by
cross-correlation methods.

Although GABA treatment rendered the subthreshold re-
sponses of HVCX and HVCRA neurons more similar, we also
found that some qualitative differences in subthreshold re-
sponse patterns could persist on local circuit inactivation.
In the example cell pair shown in Fig. 8, the BOS-evoked sub-
threshold responses exhibited by the cells of this bird were
particularly phasic, with a slight offset between the two PN
types in the exact timing of their positive-versus-negative-
going potentials (Fig. 8A, left): a cross-correlation of the
BOS-evoked membrane potentials shows a single negative peak at approximately −50 ms, the latency of this offset (Fig.
8A, right). On pharmacological inactivation of HVC, common
excitation was detected in the HVCRA and HVCX cell pair, as
indicated by the emergence of a positive peak at zero lag/lead
time in the cross-correlation. Nonetheless, the timing of the
potentials across cell types maintained a slight offset (Fig. 8B,
left), confirmed by a major positive peak (the HVCX negative-
going potentials reversed polarity) in the correlation at
approximately −50 ms (Fig. 8B, right). Even so, there was
a slight shift in this maintained offset, from −49 to −59 ms,
indicating a role of local circuitry in determining timing
precision. Therefore both local HVC connectivity and fac-
tors besides such local synaptic interactions, such as the
intrinsic properties of the postsynaptic cells or differences in
the temporal patterning of extrinsic synaptic activity, may
determine the exact timing of phasic excitatory components
in the different PN types.

Blocking inhibition in individual HVCX cells reveals
HVCRA-like excitation

Both dual intracellular recordings and GABA inactivation of
HVC suggest that a major distinction between the inputs onto
HVCRA and HVCX neurons is the addition of a locally-
generated, lagging, BOS-enhanced inhibition onto HVCX cells.
If this is indeed the case, then blocking BOS-enhanced inhibi-
tion within single HVCX neurons should mimic the effects of
blocking all local HVC activity with exogenous GABA applica-
tion. Prior studies have shown that a G-protein-coupled
potassium (i.e., GIRK)-mediated inhibition is a major compo-
nent of song-evoked hyperpolarizations in HVCX cells and that
this form of slow inhibition is likely to be driven by HVCint
cells (personal observations; Mooney and Prather 2005; Rosen
and Mooney 2003). To directly test the extent to which GIRK-
mediated inhibition could account for differences in subthresh-
hold responses in the two HVC PN types, we recorded intracel-
larly from single HVCX neurons with GTPyS or GDPβS in the
recording electrode (see METHODS), nonhydrolyzable analogues
of GTP and GDP, which, respectively, occlude or block GIRK-
mediated inhibition in HVCX cells. With GIRK-mediated inhi-
bition effectively disrupted, we then compared BOS-evoked
subthreshold activity in these treated cells with subthreshold
activity of HVCRA neurons recorded with control solutions in
the same birds. Note that although these drugs have been used
to block or occlude GIRK-mediated inhibition in HVCX cells
in a previous report (Rosen and Mooney 2003), the comparison
of subthreshold responses in treated HVCX neurons with sub-
threshold responses in HVCRA neurons in the same bird is
novel.

We observed that disrupting inhibition in HVCX cells un-
masked an underlying excitation to BOS that was similar in
shape to the excitation evoked by BOS in sequentially-re-
corded HVCRA cells of the same bird (*n* = 50 pairs; example
depicted in Fig. 9A). To quantify this effect, we again calcu-
lated cross-correlations of the BOS-evoked median-filtered

![FIG. 8. Fine timing differences between HVCRA and
HVCX neurons remain intact on inactivation of HVC’s
local circuit. Sequential recordings within a single bird
from an HVCRA and an HVCX neuron before (A) and
during (B) pharmacological inactivation of HVC’s local
circuit. BOS playback, which began at 1.5 s, elicited sharp
peaks of synaptic activity in both cells, excitatory in the
HVCRA cell (gray) and inhibitory in the HVCX cell
(black), which were offset from each other by −50 ms, as
indicated by a negative cross-correlation peak computed
on these traces, depicted on the right, with insets showing
a larger time scale. (Dotted lines show the offset in fine
timing across the peaks in the 2 cells.) During circuit
inactivation, an underlying sustained depolarization
emerged in both cells, indicated by an emergent positive
cross-correlation peak at 0 ms, but the −50 ms offset
between the 2 cells remained and became a positive
correlation as endogenous inhibition onto HVCX was oc-
cluded by GABA. The exact timing of this offset shifted
slightly, from −49 to −59 ms, indicating an impact of
HVC circuitry on PSP precision.](http://jn.physiology.org/withappsec/figa.png)
feedforward inhibition from HVCInt cells that receive excitation similar to that in HVCRA cells, as full-wave rectified control HVCX cells (3rd gray bar) are significantly less similar to HVCRA neurons than with inhibition occluded (4th gray bar; intracellular occlusion or local circuit inactivation) makes their responses similar to significantly increases on GABA inactivation of HVC (paired t-test, \( P = 0.0001 \)). Interestingly, HVCRA-HVCX cross-correlation values following disruption of GIRK-mediated inhibition in single HVCX neurons were quite similar to those obtained following GABA inactivation of the entire HVC network [compare right-most black with right-most gray bars, Fig. 9B; GABA-treated HVCX: 0.35 ± 0.10; inhibition-disrupted HVCX: 0.24 ± 0.04; unpaired t-test, \( t(58) = 1.18, P = 0.24 \)]. This similarity suggests that GIRK-mediated inhibition could account for much of the differences in subthreshold response patterns in the two HVC PNs. Indeed, cross-correlations indicate that the subthreshold depolarizing BOS-evoked responses in GTP\(\beta\)S- or GTP\(\beta\)S-dialyzed HVCX cells are as similar to BOS-evoked responses in control HVCRA cells as the responses recorded across homotypic PN pairs in control conditions are to one another [Fig. 9B, compare rightmost black and gray bars with white bars; GABA-treated HVCX: 0.35 ± 0.10; inhibition-occluded HVCX: 0.26 ± 0.03; HVCX vs. HVCX: 0.28 ± 0.05; HVCRA vs. HVCX: 0.33 ± 0.03; 1-way ANOVA: \( F(3) = 0.78, P = 0.51 \)]. As with the GABA inactivation experiments, these comparisons suggest that selectively removing local inhibitory components of the HVCX neuronal response can decrease, if not entirely abolish, the different response patterns in the two classes of HVC PNs.

We also investigated two models that may account for why the subthreshold response patterns of the two PN types become more similar on disruption of GIRK-mediated inhibition in HVCX cells. One possibility is that the phasic depolarizations in HVCX cells seen at early time points are sufficient to account for the positive correlations seen at later time points, but strong hyperpolarizing epochs at early time points in the recording dominate the cross-correlation values. If so, replacing any hyperpolarizations seen in HVCX cells at early time points in drug treatment with the resting \( V_m \) (i.e., half-wave rectification, see METHODS) should result in positive correlations resembling those seen when inhibition was entirely disrupted. However, we observed that half-wave rectification of the HVCX membrane potential was not sufficient to account for the larger, more positive correlations that emerged when GIRK-mediated inhibition was disrupted [Fig. 9B, 2nd gray bar vs. 4th gray bar; half-wave rectified: \(-0.13 ± 0.03\), inhibition occluded: 0.24 ± 0.04, paired t-test: \( t(50) = 8.4, P < 0.0001 \)]. Another idea is that the hyperpolarizing epochs are due to feedforward inhibition from HVCInt cells that receive similar patterns of excitation to those received by HVCRA cells in control conditions. Indeed, this is likely given that HVCRA neurons have been shown to provide convergent input onto HVCInt cells (Mooney and Prather 2005). In this case, inverting hyperpolarizing epochs via full-wave rectification of the HVCX membrane potential record should result in positive correlations resembling those seen when inhibition was occluded. This was indeed the case: the positive cross correlations between membrane potential records of HVCRA cells and either full-wave rectified records of HVCX cells at early time points or nonrectified records of HVCX cells with inhibition disrupted at later time points were not different from one another [Fig. 9B, 3rd gray bar vs. 4th gray bar; full-wave rectified: 0.30 ± 0.03, inhibition disrupted: 0.24 ± 0.04, \( t(50) = 1.5, P = 0.07 \).]
Along with the results obtained with GABA inactivation, these results support the idea that common excitation to all HVC cell types drives a sustained BOS-evoked depolarization, and that feedforward inhibitory interactions, presumably between HVC_{Int} cells and HVC_{X} cells, results in the characteristic differences in subthreshold response patterns previously noted for the different HVC PN cell types.

**D I S C U S S I O N**

The song nucleus HVC is functionally diverse, serving roles in song patterning (Hahnloser et al. 2002; Yu and Margoliash 1996), perception (Brenowitz 1991), and learning (Gentner et al. 2000). HVC also is anatomically complex, containing two or more interneuron types and two different PN types that give rise to two functionally distinct forebrain pathways (Dutar et al. 1998; Kubota and Taniguchi 1998; Mooney 2000; Rauske et al. 2003; Wild et al. 2005). Notably, many HVC neurons display song-related motor activity and also exhibit heightened selectivity for the BOS relative to areas that provide either direct or indirect auditory input to HVC (Coleman and Mooney 2004; Hahnloser et al. 2002; Janata and Margoliash 1999; Lewicki and Arthur 1996; McCasland 1987; Yu and Margoliash 1996). The present observations indicate that the two PN types receive common excitation directly from a BOS-selective extrinsic source and that differences in their subthreshold responses to BOS playback are generated by locally derived inhibitory input onto HVC_{X} neurons. The present results clarify local and extrinsic contributions to BOS-selective responses in HVC and show that the functional synaptic interactions leading to different subthreshold response patterns are driven in a stimulus-dependent manner, most notably by the BOS.

**Differences between PN types: influences of local HVC circuitry versus extrinsic inputs**

Consistent with its remarkable functional complexity, HVC is a site of pronounced anatomical convergence, with numerous afferents, including NIf, the medial magnocellular nucleus of the anterior nidopallium (mMAN), and the thalamic nucleus uvaefornis (Uva) (Kelley and Nottebohm 1979; Nottebohm et al. 1982; Vates et al. 1996, 1997). Although previous inactivation experiments indicated that much of the BOS-selective activity in HVC arises from NIf (Cardin and Schmidt 2004; Coleman and Mooney 2004), they left unanswered several questions that are addressed by our experiments. First, these prior experiments could not distinguish whether all HVC neuronal types receive BOS-selective input from NIf or instead whether certain HVC neurons receive only feedforward BOS activity from other HVC neurons. Our data reveal that all HVC neuron types maintain BOS-selective synaptic responses when the HVC local circuit is silenced pharmacologically, indicating that NIf axons transmit BOS selective information directly to all HVC neuron types.

Furthermore, the NIf inactivation experiments could not determine whether the characteristically distinct subthreshold activity patterns evoked by the BOS in the two PN cell types arise because they receive distinct extrinsic inputs (i.e., from different neuronal subpopulations within NIf) or instead because a relatively homogenous source of extrinsic auditory input is further shaped via local synaptic interactions within HVC. Our data support the latter idea because local circuit inactivation significantly affected the shape of BOS-evoked responses specifically in HVC_{X} cells: cross-correlation analysis confirmed that subthreshold responses in HVC_{X} cells, either during local circuit inactivation or during inhibitory blockade, closely resembled the purely depolarizing responses normally seen in HVC_{RA} neurons. This effect was not mimicked simply by tonic hyperpolarization of the HVC_{X} cell or half-wave rectification of the HVC_{X} cell membrane potential, indicating that local circuit activity, rather than postsynaptic hyperpolarization resulting from GABA application, accounts for the different subthreshold response patterns seen in HVC PN cells. In summary, BOS playback evokes sustained excitation in both HVC_{RA} and HVC_{X} cells, but inhibition derived from the HVC local circuit strongly masks this excitation in HVC_{X} cells. Thus despite HVC being a site of pronounced anatomical convergence, different HVC cell types appear to receive common extrinsic excitation from NIf, and cell-type distinctions in subthreshold response patterns appear to arise from within HVC.

Indeed, the current study extends previous analyses of the origins of BOS selectivity by determining that the HVC local circuit contributes to the BOS-evoked hyperpolarizations characteristic of HVC_{X} cells. This interaction was suggested by sequential in vivo recordings from multiple HVC neurons, which revealed that HVC_{Int} firing correlates closely with hyperpolarization in HVC_{X} cells (Mooney 2000), and by dual intracellular recordings made in brain slices, which showed that HVC interneurons provide direct inhibitory input onto HVC_{X} cells (Mooney and Prather 2005). Two results from the present study demonstrate that the distinctions between subthreshold responses in PN types derive from local circuit contributions and in particular from inhibitory interactions between interneurons and HVC_{X} cells. First, STA analysis showed that BOS-evoked hyperpolarizations followed firing in most HVC_{Int} neurons and in HVC_{RA} neurons, consistent with either cell type providing direct or indirect inhibition onto HVC_{X}. Second, local circuit inactivation affected the shape and selectivity of BOS-evoked responses in HVC_{X} cells but not HVC_{RA} cells, and these effects were mimicked by disrupting GIRK-mediated inhibition in single HVC_{X} cells. This type of inhibition, which can be elicited in unidentified HVC neurons by tetanic stimulation in brain slices and in HVC_{X} neurons by BOS playback in the intact bird, likely involves GABA-relasing interneurons present in HVC (Mooney 2000; Mooney and Prather 2005; Rosen and Mooney 2003; Schmidt and Perkel 1998; Wild et al. 2005). Indeed, dysnaptic GIRK-mediated inhibitory postsynaptic potentials (IPSPs) as well as faster GABA_{A}-mediated IPSPs can be elicited in HVC_{X} cells by antidromic activation of HVC_{RA} axon collaterals that are local to HVC (personal observations; Mooney and Prather 2005; Vu et al. 1994). The observations made here that the negative STA peaks in HVC_{X} cells followed firing in most HVC_{Int} and HVC_{RA} cells is consistent with the idea that HVC_{RA} cells make excitatory contacts onto certain HVC_{Int} cells, which in turn inhibit HVC_{X} cells, as has been shown in brain slices (Mooney and Prather 2005). Another possibility is that common drive from NIf excites all three cell types, triggering a delayed HVC_{Int}-mediated feedforward inhibition, an arrangement supported by timing data from the present study and from simultaneous NIf-HVC recordings (Coleman and Mooney 2004). It is plausible that both mechanisms...
operate within HVC, perhaps relying on distinct subpopulations of HVC interneurons (Wild et al. 2005).

In addition to providing inhibition, the local HVC circuit also appears to be a source of excitation, given that the selectivity of HVC_{int} cells marginally decreased on local circuit inactivation and that some STA excitatory peak onsets followed spike time in the other cell of the pair. This latter feature is consistent with feedforward excitation (Jack et al. 1971; Matsumura et al. 1996), which has been detected between HVC neurons (Mooney and Prather 2005). One function of such local excitation from PNs could be to drive inhibitory HVC_{int} cells that in turn operate to diminish BOS-selective excitation in HVC_{X} neurons (Rosen and Mooney 2003). Such local feedforward excitation onto interneurons also could explain why the BOS selectivity of subthreshold responses decreases in HVC_{int} cells on local circuit inactivation, whereas depolarizing subthreshold selectivity increases in HVC_{X} cells either when the local circuit is entirely inactivated (these data) or when the inhibition onto individual HVC_{X} cells is disrupted (Rosen and Mooney 2003). Furthermore, the pattern of inhibition in HVC_{X} cells appears to closely mimic excitation in HVC_{RA} cells, as full-wave rectification of HVC_{X} cell membrane potential traces increases their similarity to subthreshold responses in HVC_{RA} cells recorded from the same bird. One implication of the full-wave rectification results is that the extrinsic BOS-selective excitation revealed in HVC_{int} cells most likely arises from the same excitatory NIf inputs received by PNs (Coleman and Mooney 2004). Taken together, these results suggest that both inhibitory and excitatory feedforward synaptic interactions in HVC transform BOS-evoked auditory activity transmitted to downstream pathways important to audition-dependent vocal plasticity.

While our data describe common extrinsic excitation and local inhibition as factors that distinguish cell-type specific BOS-evoked patterns of activity, timing differences in BOS-evoked subthreshold responses in the two PN types could persist on local circuit inactivation (Fig. 8). Indeed, although the STA analysis performed here reveals that much spontaneous and BOS-evoked excitation in all HVC neurons derives from a common source, this source could be extrinsic input from NIf, local HVC excitation, or a combination of both. Beyond the scope of the current study, dual recordings coupled with local circuit inactivation could derive the relative contributions of extrinsic and intrinsic sources to BOS-evoked activity in different HVC neurons, which could be one factor distinguishing sub- and suprathreshold response differences in the various HVC cell types. Cell-type and individual differences could also arise via differential terminal organization of intrinsic and extrinsic synaptic inputs or via common inputs subjected to differential postsynaptic filtering imposed by intrinsic cellular properties. We also cannot rule out other potential sources of common, BOS-selective excitatory input to HVC, including those that arise from mMAN and are thought to operate in a feedback fashion.

**BOS activation tightens synaptic timing and increases inhibitory drive**

One idea is that synaptic interactions between neurons change dynamically to facilitate stimulus processing (Ahissar et al. 1992). Consistent with such dynamic modification occurring in HVC, BOS playback shortened excitatory postsynaptic potential (EPSP) onsets in HVC_{X}-HVC_{RA} pairs and selectively recruited inhibition in HVC_{X} cells, relative to BOS-REV playback or the silent (i.e., spontaneous) state. In other systems, tighter timing of excitatory synaptic activity increases the strength of postsynaptic potentials and spike probability (Deschenes et al. 2003; Kara and Reid 2003; Usrey et al. 1998; Zucker 1989). In HVC, BOS-evoked “narrowing” of common excitation via a feedforward inhibitory mechanism could elicit more temporally precise spiking, contributing to the synchronous firing of HVC neurons within individual birds (personal observations; Hahnloser et al. 2002) and the highly phasic firing of HVC projection neurons (Lewicki 1996; Margoliash 1983; Mooney 2000). Indeed, blocking GIRK-mediated inhibition in HVC_{X} cells degrades the temporal precision of their BOS-evoked spikes (Rosen and Mooney 2003).

Given the importance of inhibition to the temporal precision of action potential activity in single HVC_{X} cells (Rosen and Mooney 2003) and the widespread connections that interneurons make with multiple PNs (Mooney and Prather 2005), a likely role of synaptic processing in HVC is to coordinate the firing of ensembles of HVC PNs. Indeed, neurons in HVC and LMAN (the output of the AFP and the indirect target of HVC_{X} cells) exhibit highly correlated firing in response to song playback (Kimpo et al. 2003) and extensive coordination of HVC_{X} output by local inhibition could be an important part of this process, perhaps because synchronized firing in HVC PNs more effectively drives activity in downstream areas. More generally, context-dependent synchronization of action potentials across neurons can underlie specific motor and sensory events (Lampl et al. 1999; Riehle et al. 1997; Vaadia et al. 1995), and synchronized activity within and across brain regions may recruit functional assemblies of cells serving motor or sensory processing (Engel et al. 2001; Softky and Koch 1993), raising the possibility that such synchronization in the song system is important to singing and song recognition.

Another apparent consequence of local synaptic processing in HVC is that spiking activity in HVC_{RA} and HVC_{X} cells is temporally offset (Mooney 2000) despite the fact that both cell types receive extrinsic excitatory input from a common source (Coleman and Mooney 2004). The importance of this temporal offset is unknown, but in computational models of the song system, the modification and/or maintenance of synaptic connections between HVC_{RA} and RA neurons depends on the precise timing of signals propagating to RA from HVC and LMAN (Abarbanel et al. 2004a,b). Therefore the precise regulation of spike timing differences in HVC_{RA} and HVC_{X} neuronal populations may have important consequences for synaptic modification in the song premotor nucleus RA with potentially important consequences for song learning and maintenance.

**Acknowledgments**

We thank Drs. T. Tucker and M. Coleman for providing thoughtful comments on earlier versions of this manuscript.

**Grants**

This research was supported by National Institute on Deafness and Other Communications Disorders Grant R01 DC-02524 to R. Mooney.
REFERENCES


Gulyas AI, Hajo N, and Freund TF. Interneurons containing calretinin are specialized to control other interneurons in the rat hippocampus. *J Neurosci* 16: 3397–3411, 1996.


