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Slow Synaptic Inhibition in Nucleus HVc of the Adult Zebra Finch

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Nervous systems process information over a broad range of time scales and thus need corresponding cellular mechanisms spanning that range. In the avian song system, long integration times are likely necessary to process auditory feedback of the bird's own vocalizations. For example, in nucleus HVc, a center that contains both auditory and premotor neurons and that is thought to act as a gateway for auditory information into the song system, slow inhibitory mechanisms appear to play an important role in the processing of auditory information. These long-lasting processes include inhibitory potentials thought to shape auditory selectivity and a vocalization-induced inhibition of auditory responses lasting several seconds. To investigate the possible cellular mechanisms of these long-lasting inhibitory processes, we have made intracellular recordings from HVc neurons in slices of adult zebra finch brains and have stimulated extracellularly within HVc. A brief, high-frequency train of stimuli (50 pulses at 100 Hz) could elicit a hyperpolarizing response that lasted 2–20 sec. The slow hyperpolarization (SH) could still be elicited in the presence of glutamate receptor blockers, suggesting that it does not require polysynaptic excitation. Three major components contribute to this activity-induced SH: a long-lasting $GABA_B$ receptor-mediated IPSP, a slow afterhyperpolarization requiring action potentials but not Ca^{2+} influx, and a long-lasting IPSP, the neurotransmitter and receptor of which remain unidentified. These three slow hyperpolarizing events are well placed to contribute to the observed inhibition of HVc neurons after singing and could shape auditory feedback during song learning.

Key words: birdsong; avian; IPSP; $GABA_B$; auditory; motor

In oscine songbirds, nucleus HVc (see Fig. 1) is a crucial forebrain center for organizing singing behavior (Nottebohm et al., 1976; McCasland, 1987; Yu and Margoliash, 1996), and it contributes to generating the motor patterns for song (Vu et al., 1994). HVc also receives auditory input (Fortune and Margoliash, 1995; Vates et al., 1996) and responds to playback of complex auditory stimuli (Katz and Gurney, 1981; Margoliash, 1983; Margoliash and Konishi, 1985; Lewicki, 1996). In many cases, these highly selective auditory responses are thought to occur as a consequence of complex interactions between long-lasting IPSPs and intrinsic bursting properties (Lewicki and Konishi, 1995; Lewicki, 1996). An intriguing link between auditory and motor functions within HVc of adult zebra finches is the observation that auditory responses become inhibited during and after singing (McCasland and Konishi, 1981). Because auditory inputs into other areas of the song system originate from HVc (Williams, 1989; Doupe and Konishi, 1991; Doupe, 1993; Vicario and Yohay, 1993), inhibition of auditory inputs in this nucleus during singing may serve an important role in the temporal gating of auditory feedback into the song system. The slow IPSPs observed during auditory responses as well as the inhibition of auditory responses after singing could have profound effects on the processing of auditory information in the adult as well as during song learning.

Long-lasting inhibition could be caused by the maintained release of a short-lasting inhibitory neurotransmitter (e.g., GABA) under the control of a long-lasting network response involving prolonged firing of action potentials (Wagner et al., 1994), or alternatively by a single long-lasting inhibitory synaptic event. Slow hyperpolarization mediated by $GABA_B$ receptor activation has been observed in many different areas of the mammalian CNS (Newberry and Nicoll, 1984; Connors et al., 1988; Dutar and Nicoll, 1988; Allerton et al., 1989). These IPSPs, however, generally last <1 sec (Alger, 1984; Newberry and Nicoll, 1984). Longer-lasting IPSPs that continue for many seconds have been described in only a few cases and may be mediated by 5-HT, norepinephrine, or acetylcholine (Dodd and Horn, 1983; Egan et al., 1983; Pan et al., 1989). Neuropeptides, which also can cause hyperpolarization (Pepper and Henderson, 1980; Pittman and Siggins, 1981; Williams et al., 1982), have been suggested as potential candidate neurotransmitters for IPSPs that last many seconds; a striking example of a slow peptide-mediated synaptic event has been described by Jan and Jan (1982).

We have recorded intracellularly from adult male zebra finch HVc neurons in brain slices to investigate whether the physiological properties within HVc could account for the long-lasting inhibition observed in HVc *in vivo* (McCasland and Konishi, 1981). High-frequency stimulation, approximating levels of activity observed during singing (McCasland, 1987; Yu and Margoliash, 1996), caused a slow IPSP lasting 2–20 sec. Blockade of glutamatergic excitatory transmission reduced the amplitude of the hyperpolarization but did not alter its slow time course, suggesting an intrinsically slow mechanism that did not require a

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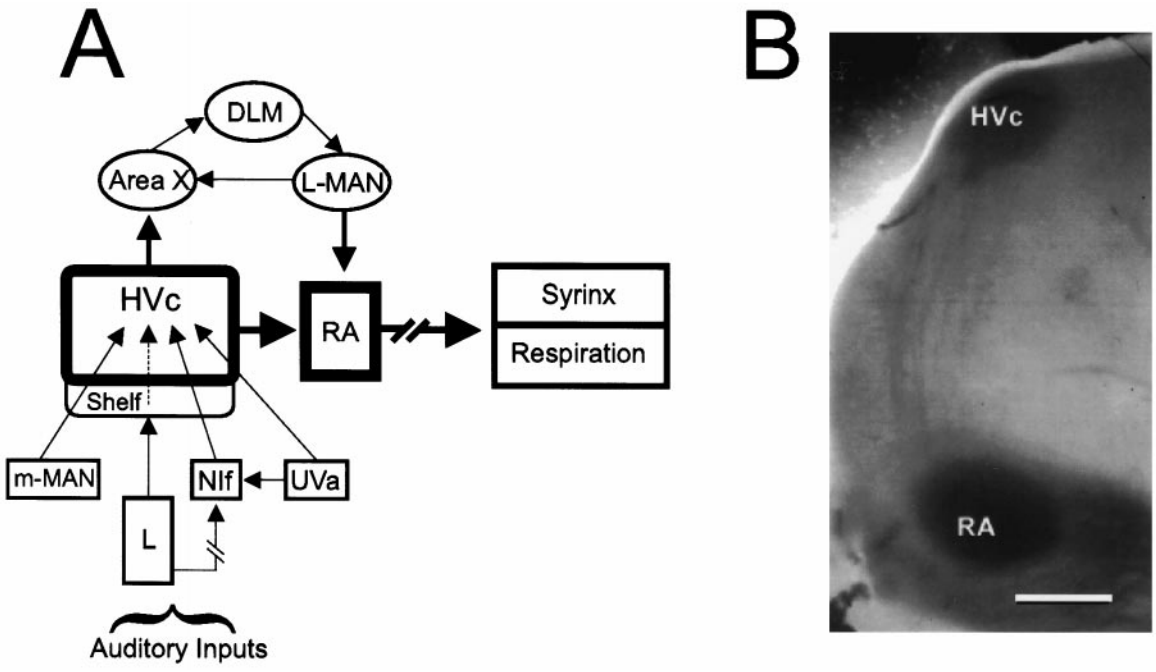

Figure 1. *A*, Schematic diagram of the song system showing the various connections of nucleus HVc. Nucleus *HVc* is part of the motor pathway for song production (*thick outline*), sending a projection to nucleus *RA*. RA projects to brainstem motor and premotor neurons that control the *Syrinx* and muscles of *Respiration*. HVc also projects to *Area X*, which forms part of the anterior forebrain loop, a circuit essential for song learning. Auditory inputs to HVc originate from *NIf* as well as from field *L*, although field L inputs may reach HVc indirectly via the shelf surrounding the nucleus. The *broken line* between field L and NIf indicates that this connection involves several synapses within the auditory forebrain. HVc also receives major inputs from the forebrain nucleus *m-MAN* and the thalamic nucleus *UVa*. *HVc*, used here as the proper name of the nucleus (Brenowitz et al., 1997), also referred to as the high vocal center (HVC); *RA*, nucleus robustus archistriatalis; *UVa*, nucleus uvaeformis; *L*, field L; *NIf*, nucleus interfacialis; *m-MAN*, medial portion of the magnocellular nucleus of the anterior neostriatum; *L-MAN*, lateral portion of the magnocellular nucleus of the anterior neostriatum; *DLM*, medial nucleus of the dorsolateral thalamus. *B*, Photomicrograph of the experimental preparation used in this study. Parasagittal brain slices were cut through the zebra finch brain. Stimulating electrodes were placed near the posterior or anterior pole of the nucleus. Intracellular recordings were obtained from neurons in nucleus HVc, the borders of which were clearly defined even in the unstained tissue. Dorsal is up; anterior is to the right. Scale bar, 500 μm.

polysynaptic network. Pharmacological dissection of this hyperpolarization revealed at least three components of the inhibition.

MATERIALS AND METHODS

Preparation of slices and electrophysiological recording. Adult zebra finches (*Taeniopygia guttata*) were obtained from a breeding colony at Caltech as well as from local suppliers near Los Angeles and Philadelphia. Slices were prepared as described previously (Mooney, 1992; Perkel and Nicoll, 1993). Briefly, birds were anesthetized with halothane or isoflurane and decapitated. The brain was removed rapidly and placed in ice-cold artificial cerebrospinal fluid (ACSF) that had been bubbled with 95% O_2/5% CO_2. Composition of the ACSF was (in mM): NaCl 134, KCl 2.5, NaH_2PO4 1, $NaHCO_3$ 26, D-glucose 12, $CaCl_2$ 2.5, $MgSO_4$ 1.5. The brain was cut midsagittally with a razor blade and parasagittal slices (400 μm thick) were prepared using a vibrating microtome. Slices were stored at room temperature on porous membrane (Nucleopore, Cambridge, MA) at the interface of ACSF and humidified 95% O_2/5% CO_2 or submerged in bubbled ACSF. For recording, a slice was transferred to a submersion chamber where it was superfused with pregassed ACSF.

Intracellular electrodes were filled with 4 M potassium acetate and had DC resistances of 70–140 MΩ. Stable intracellular recordings were routinely obtained from neurons in nucleus HVc, the borders of which were clearly defined even in the unstained tissue. Resting potential and input resistance were closely monitored and remained stable for the duration of the recording, commonly 2–5 hr. One or more stainless steel bipolar stimulating electrodes (impedance 2–5 MΩ) were placed within HVc, usually near the posterior or anterior pole (Fig. 1*B*). We stimulated somata and fibers within HVc (100 μsec duration; constant voltage, 5–60 V) because input pathways to HVc are not segregated well enough to stimulate one specifically. Moreover, stimulation outside of HVc would likely cause antidromic activation of HVc outputs, which exit across a broad region (Nottebohm et al., 1982).

Signals were amplified using an Axoclamp 2A (Axon Instruments, Foster City, CA), low-pass filtered, and digitized at twice the filter cut-off frequency. Intracellular records are presented here as the average of two to three consecutive traces. Analysis included fitting a single exponential function to the decay phase of a synaptic potential using a least-squares algorithm. Average values of response amplitudes or best-fit time constants are presented as the mean ± SD. The Student's *t* test, paired or unpaired, was used as indicated.

Materials. Chemicals were obtained from Sigma (St. Louis, MO), except as noted. DMSO was purchased from Fisher Scientific (Pittsburgh, PA). The AMPA glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), the NMDA glutamate receptor antagonist DL-2-amino-5-phosphonovaleric acid (APV), and the opiate antagonist naloxone were obtained from Research Biochemicals (Natick, MA). The $GABA_B$ receptor antagonist CGP 35348 was a gift from CIBA-Geigy (Basel, Switzerland). Except as noted, all drugs were added to the superfusion medium by dilution of a stock solution made in water. Stock solutions of CNQX were made in DMSO, resulting in a final concentration of DMSO of 0.1–0.2%, which when added alone did not affect any of the responses observed. Bicuculline methiodide (BMI) was dissolved directly into the ACSF.

RESULTS

High-frequency stimuli in HVc elicit a slow depolarization/hyperpolarization sequence lasting many seconds

We recorded intracellularly from HVc neurons and activated synaptic inputs via a stimulating electrode placed within nucleus HVc (Fig. 1*B*). In selecting a stimulation pattern, we were guided by reported high firing rates observed during

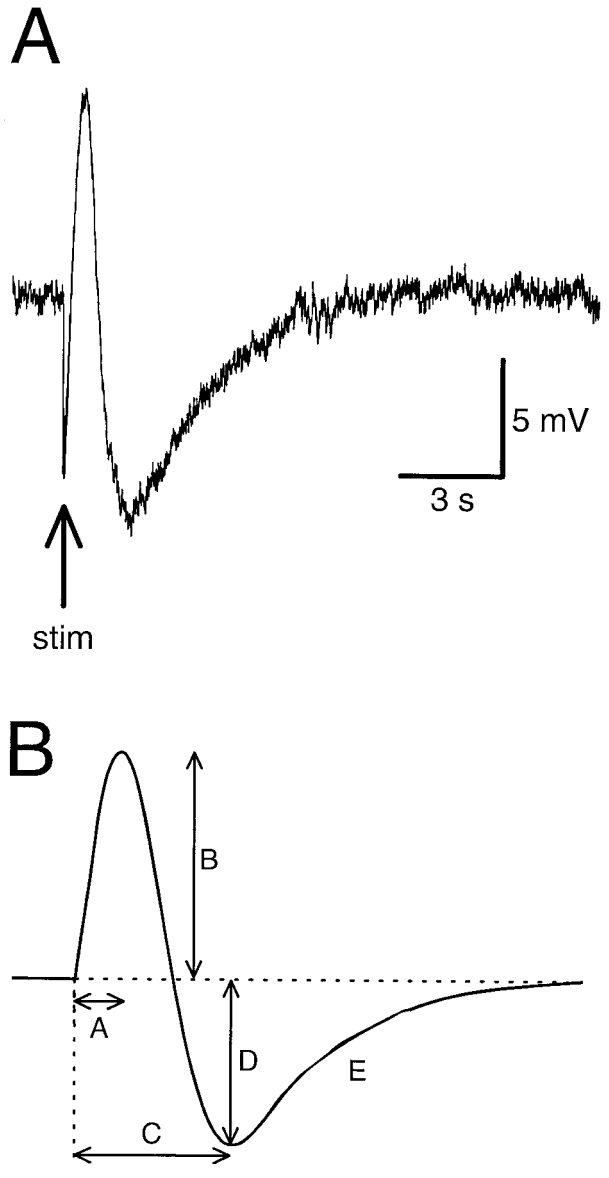

Figure 2. Slow depolarizing/hyperpolarizing sequence after repetitive synaptic stimulation of an HVc neuron. *A*, This sample *trace* illustrates the potential change caused by stimulating within nucleus HVc at 100 Hz for 0.5 sec. Such stimulation caused this HVc neuron to exhibit a slow depolarization followed by a long-lasting hyperpolarization. In this case the decay time constant of the hyperpolarization was 2.6 sec, and the resting membrane potential was −65 mV. *Arrow* indicates onset of stimulation. *B*, This schematic representation of the slow depolarizing/hyperpolarizing sequence illustrates how waveform parameters were measured. *A*, Time-to-peak for the depolarization *B*. Amplitude of peak depolarization. *C*, Time-to-peak hyperpolarization. *D*, Amplitude of peak hyperpolarization. *E*, Decay time constant of the hyperpolarization. See Table 1 for recorded values.

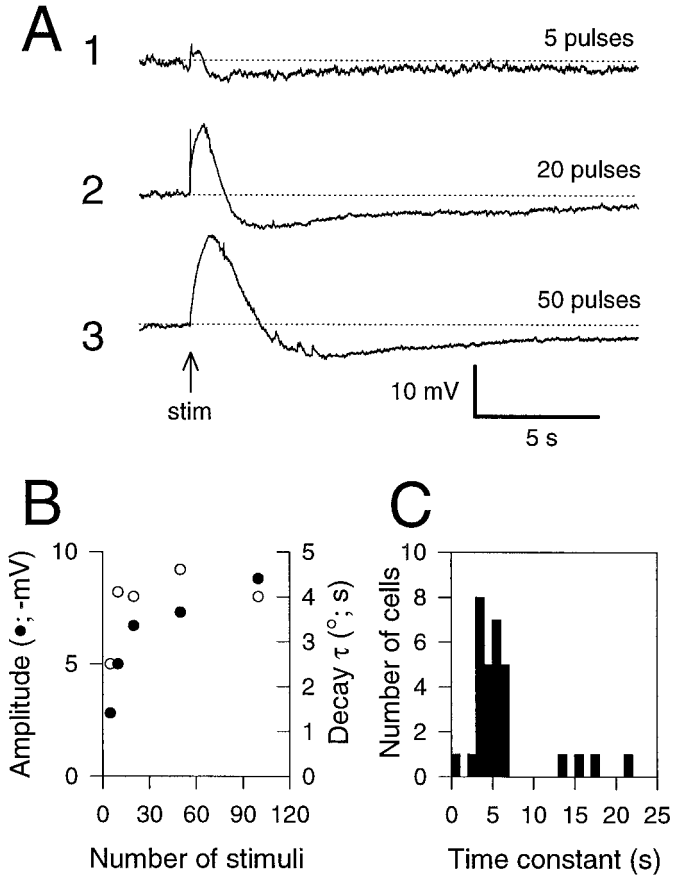

Figure 3. Effect of stimulation pattern on amplitude and time course of the SH. *A*, HVc neurons were subjected to different numbers of stimuli (*A1*, 5 pulses; *A2*, 20 pulses; *A3*, 50 pulses) delivered at 100 Hz. The amplitude and the time course of both the slow depolarization and the hyperpolarization were directly dependent on the number of pulses used in the stimulus. *Dotted line* indicates the resting potential for the cell illustrated (−58 mV). *B*, Peak amplitude and decay time constant of the SH component varied with the number of stimuli. Peak amplitude (*filled circles*) of the SH increased rapidly with the number of stimuli reaching 80% of maximum with a 20-pulse stimulus. Decay time constant (*open circles*) increased from 5 to 10 pulses and then leveled out around 4 sec. *C*, Histogram for all cells recorded showing the range of decay time constants (τ) for the SH obtained using the standard 50-pulse stimulation. Time constants tended to cluster around 5 sec.

singing (McCasland, 1987; Yu and Margoliash, 1996). Single high-frequency trains of synaptic stimuli (50 pulses; 100 Hz) caused a slow depolarization followed by an unusually slow hyperpolarization (SH) lasting up to 20 sec (Figs. 2*A*, 3*C*). The hyperpolarization was often punctuated by small depolarizing deflections (Fig. 2*A*). The overall amplitude of the slow depolarization/hyperpolarization (SDH) appeared to depend on the number of pulses in the stimulus train (Fig. 3*A*). Although we have not performed a systematic analysis of the stimulus parameters, in four of four neurons, the SH amplitude was near maximal with a stimulus train of 20–50 pulses (Fig. 3*B*). Its slow time course was apparent after only five stimuli (Fig. 3*B*). We were able to elicit an SDH in 66% of the cells tested (57/86 neurons). This percentage should be viewed as a rough approximation because it is subject to positioning of the stimulating electrode as well as sampling bias. In cells in which a single train gave rise to an SDH, a similar SDH was also elicited by a more complex stimulus consisting of five pulses at 100 Hz repeated five times at 5 Hz.

We measured the components of the SDH illustrated in Figure 2*B*, and the values are summarized in Table 1. In most of the cells recorded (26/31) (Fig. 3*C*), the decay time constant fell within the range of 2.6–6.9 sec, with a mean of 4.8 ± 1.3 sec for those 26 neurons. One outlier had a decay time constant of 639 msec, whereas four others fell in the range of 13–21 sec.

Table 1. Waveform parameters for the slow IPSP elicited in response to tetanic stimulation

Condition	(A) Time to peak depolarization (msec)	(B) Peak depolarization (mV)	(C) Time to peak hyperpolarization (msec)	(D) Peak hyperpolarization (mV)	(E) Decay time constant (sec)
Control	300 ± 229	9.8 ± 7.8	2600 ± 1200	8.3 ± 3.2	6.3 ± 4.6
CNQX/APV	284 ± 237	7.7 ± 3.5*	801 ± 1000** 2600 ± 1100	3.3 ± 1.7*	4.4 ± 2.3
+BMI	None	None	1200 ± 600*	7.1 ± 3.9***	5.1 ± 4.9

*Significant difference from value measured in control conditions ($p < 0.05$).

**First value for all cells to which CNQX and APV were applied ($n = 20$). Second value for those cells held for BMI application ($n = 8$).

***Significant difference from value measured in the presence of CNQX and APV ($p < 0.02$).

The slow depolarization/hyperpolarization can occur with blocked network excitation

To test whether the SDH response resulted from persistent polysynaptic network activity, we blocked excitatory neurotransmission pharmacologically. Vu and Lewicki (1994) have reported blockade of EPSCs in HVc by ionotropic glutamate receptor antagonists. We used 20 μM CNQX to block AMPA receptors (Honoré et al., 1988) and 100 μM APV to block NMDA receptors (Davies et al., 1981). This combination of antagonists blocked monosynaptic EPSPs evoked by single-shock stimulation (Fig. 4*A*) and almost always (19/20) reduced the amplitude of the SDH response (Fig. 4*B*). The overall effect of blocking glutamatergic transmission on the SDH response was quite variable. The depolarization was often eliminated (9/14), whereas in the remaining five cells it was reduced to 43 ± 13% of its control value (Table 1). The time-to-peak depolarization was unchanged with ionotropic glutamatergic transmission blocked ($p > 0.89$). The effect of CNQX and APV on the hyperpolarization amplitude was particularly variable. The peak amplitude in the presence of the blockers averaged 60 ± 53% of control ($n = 20$; range, 2–140%) (Fig. 4*C*). The time-to-peak hyperpolarization was shortened. Despite the changes in the amplitude values, the addition of CNQX and APV did not significantly change the decay time constant of the SH ($p > 0.45$; paired t test). Because blockade of excitatory amino acid receptors should eliminate polysynaptic responses, these results suggest that glutamatergic excitatory connections remaining within the slice contribute significantly to both the slow depolarization and hyperpolarization responses. However, because a substantial portion of the SH remained after blockade of ionotropic glutamate receptors, these data also suggest that the hyperpolarizing component of the SDH may result from direct activation of inhibitory neurons. Moreover, the time course of the SH appears not to depend on the activation of an extensive network of excitatory glutamatergic connections.

The slow depolarizing response is mediated by activation of $GABA_A$ receptors

To characterize the slow hyperpolarizing portion of the SDH, we attempted to block the initial slow depolarizing phase of the response. Previous studies have shown that $GABA_A$ receptor activation, which is normally hyperpolarizing, can become depolarizing when neurons are stimulated at high frequency (Andersen et al., 1980; Alger and Nicoll, 1982; Staley et al., 1995). The addition of the $GABA_A$ receptor antagonist BMI (40 μM) (Curtis et al., 1970; Newberry and Nicoll, 1984) to neurons exhibiting a substantial SDH in the presence of CNQX and APV completely blocked the slow depolarizing response in all cases tested (Fig. 5) ($n = 8$). Blockade by BMI left a hyperpolarization that sometimes, but not always, appeared to have two decay phases. The slow phase measured in the presence of CNQX, APV, and BMI had a decay time constant unchanged from that measured before the addition of BMI ($n = 8$; $p > 0.3$; paired t test). Although the decay time course of the SH remained unchanged, the peak amplitude during $GABA_A$ receptor blockade was larger ($n = 8$; $p < 0.05$; paired t test) and occurred sooner after stimulation ($n = 8$; $p < 0.05$; paired t test) than in the presence of just CNQX and APV (see Table 1).

When the membrane potential was shifted by injecting DC current, the extrapolated reversal potential was approximately −95 mV (data not shown), suggesting the SH is mediated by an increase in potassium conductance. In two cases, the apparent reversal potential was shifted to a depolarized level when the external potassium concentration was raised (data not shown). A more detailed biophysical analysis of the SH will require voltage-clamp techniques with better control of the postsynaptic membrane potential.

Contribution of a long-lasting AHP to the slow hyperpolarization

The SH was in some cases likely to be purely synaptic in nature because it could be elicited in the absence of stimulus-induced antidromic action potentials ($n = 6$) (Fig. 2*A*). In many cases, however, despite the presence of glutamate receptor blockade, stimulation within HVc produced action potentials and created the possibility that a portion of the hyperpolarization was caused by an action potential-induced slow afterhyperpolarization (sAHP). These action potentials were not unexpected, given the high degree of axonal arborization within HVc (Katz and Gurney, 1981; Nottebohm et al., 1982; Nixdorf et al., 1989) and were likely antidromic in nature because they (1) had rapid onset times, (2) persisted in the presence of glutamatergic receptor antagonists, (3) persisted in the presence of hyperpolarizing current, and (4) were not blocked by the calcium channel blocker $CdCl_2$ (100 μM), which eliminated all synaptic transmission.

Slow AHPs in nucleus HVc could be elicited by direct current injection (Kubota and Saito, 1991) (Fig. 6) and were characterized by an initial, rapidly decaying phase followed by a very slow component, the time course of which resembled the decay time constant of the SH observed after extracellular stimulation within HVc (compare Fig. 5 and Fig. 6). The decay time constant of the two hyperpolarizations was directly compared in 15 cells. The value was 5.4 ± 3.9 sec for the SH and 4.3 ± 2.4 sec ($n = 15$; $p > 0.07$; t test) for the sAHP. Thus, on the basis of the time course, in cases in which extracellular stimulation caused antidromic action potentials the sAHP could have contributed to the SH.

In an attempt to discriminate between the synaptic component of the SH and the sAHP, we sought a pharmacological means of

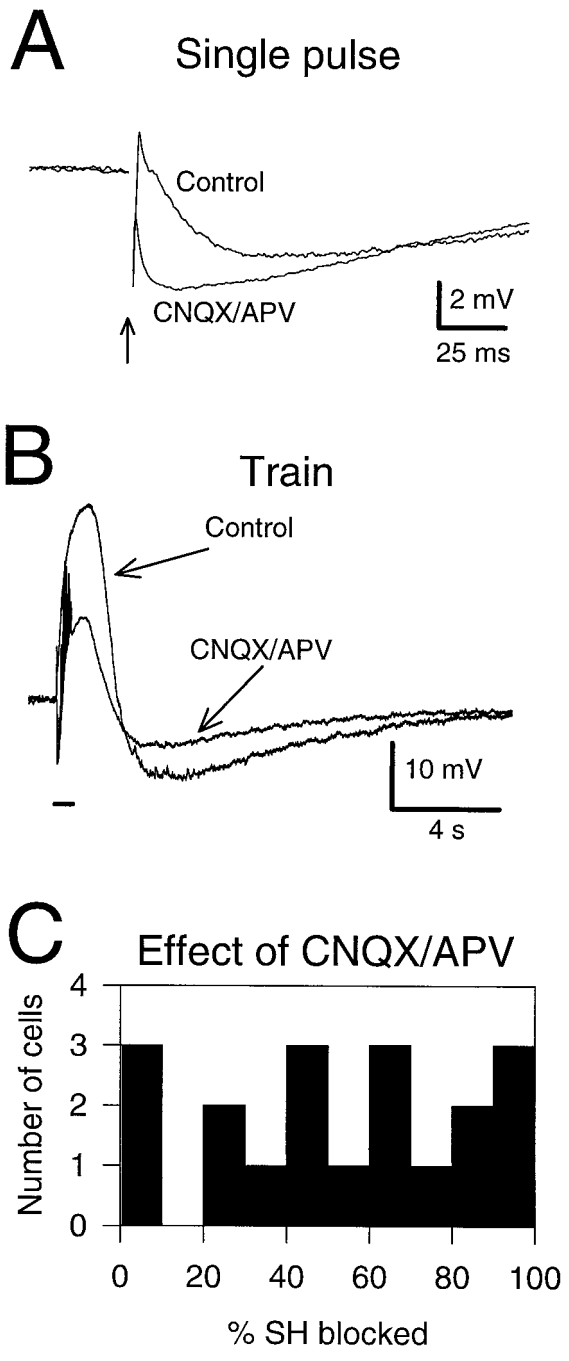

Figure 4. Effect of ionotropic glutamate receptor antagonists on synaptically evoked potentials in nucleus HVc. *A*, A single-shock stimulus typically elicited a rapid onset EPSP followed by an IPSP lasting several hundred milliseconds. Stronger stimulation could elicit an action potential. Bath application of the glutamate antagonists CNQX (20 μM) and APV (100 μM) blocked the EPSP and preserved the slow IPSP. *B*, Bath application of CNQX/APV generally had a wide range of effects on the time course and amplitude of the SH elicited by a fast train of stimuli (50 pulses at 100 Hz). These effects ranged from partial decrease in amplitude of the hyperpolarization to nearly complete blockade. This sample trace illustrates a typical example (different cell from *A*), in which the amplitude of the SH is decreased by ~40% after blockade of excitatory transmission. *C*, This histogram illustrates the range of effects observed after the addition of CNQX/APV. Although addition of glutamate receptor antagonists always reduced the amplitude of the SH, the overall level of reduction was quite variable. Percent reduction of the SH (measured at peak amplitude) ranged from 0 to 98%, with a median reduction near 50%. Two cells in which the SH grew after application of CNQX and APV are represented as 0% blockade here.

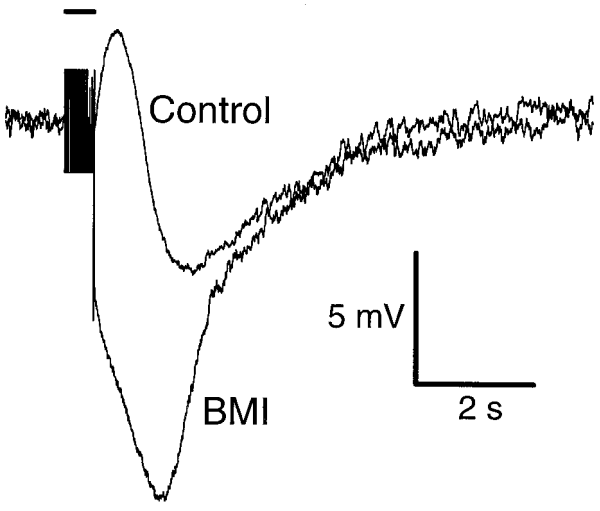

Figure 5. Effect of the $GABA_A$ receptor antagonist bicuculline methiodide (*BMI*) on the depolarizing phase of the synaptically evoked slow depolarization/hyperpolarization sequence. High-frequency stimulation (50 pulses at 100 Hz) was used to obtain an SDH sequence (*Control*) in the presence of CNQX (20 μM) and APV (100 μM). Addition of BMI (40 μM) in the continued presence of CNQX and APV completely blocked the depolarizing phase of the synaptic response. This blockade of a depolarizing response by BMI was not unexpected, given previous reports showing that stimulation at high frequency can cause $GABA_A$ receptor-mediated events to become depolarizing (see Results). The peak amplitude of the hyperpolarization significantly increased after addition of BMI, suggesting that the reversed $GABA_A$ depolarizing phase was masking part of the SH. On average, the slow decay time constant remained unchanged in the presence of BMI. In this case the decay time constants were 1.8 sec (*Control*) and 2.2 sec (*BMI*). The rise to peak hyperpolarization in the presence of BMI often had a characteristic linear shape.

selectively blocking the sAHP. In agreement with Kubota and Saito (1991), we found that in nucleus HVc, the slow phase of the sAHP is insensitive to the broad-spectrum calcium channel blocker cadmium (Fig. 7*A*), suggesting that the conductance is different from that of calcium-dependent sAHPs observed in other systems (for review, see Sah, 1996). This sAHP was also insensitive (Fig. 7*B*) to either norepinephrine (NE) (10 μM; n = 5), or the cholinergic agonist carbachol (10 μM; n = 3), both known modulators of calcium-dependent sAHPs (Nicoll, 1988). We were able to block this sAHP, however, by adding the sodium channel blocker tetrodotoxin (1 μM; n = 7) (Fig. 7*C*) (Kubota and Saito, 1991). This result is consistent with the hypothesis that the sAHP present in HVc neurons is generated by sodium-dependent activation of a potassium conductance (Kubota and Saito, 1991), although other possibilities cannot be excluded. Unfortunately, because TTX would block both sAHP and synaptic-mediated events, this pharmacological agent could not be used to block selectively the sAHP contribution to the SH. To circumvent this problem, we attempted to block sodium channels with the intracellular sodium channel blocker QX-314. This drug did block the action potential-mediated sAHP. Unfortunately, QX-314 also blocked $GABA_B$ receptor-mediated inhibition (Andrade, 1991) (n = 3, data not shown) and could not be used here to remove the sAHP selectively.

These results indicate that HVc neurons contain a slow, TTX-sensitive sAHP with a time course similar to the SH observed after synaptic stimulation. Although we were unable to block the sAHP selectively, we exploited its lack of dependence on calcium.

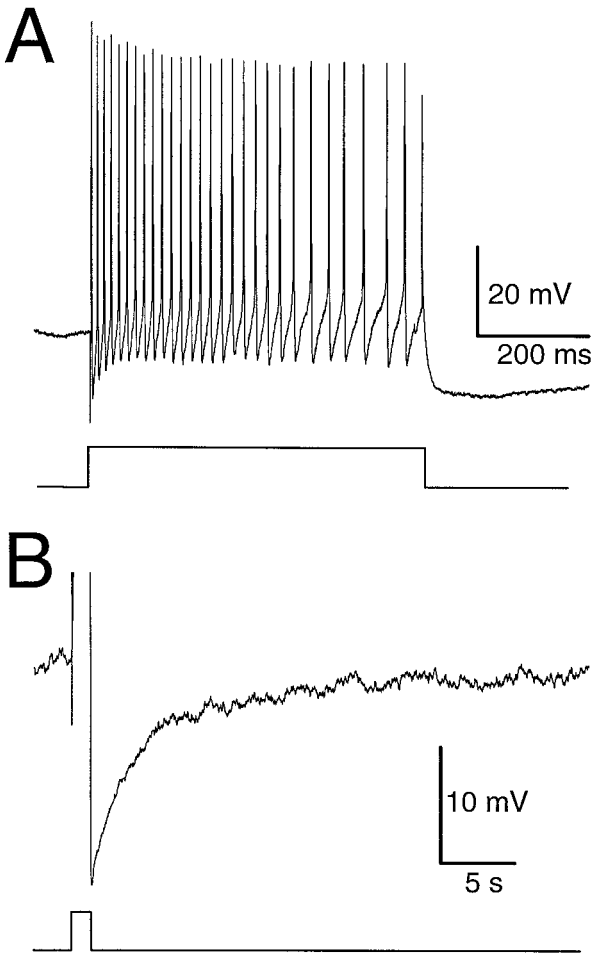

Figure 6. Depolarizing current pulses cause a slow afterhyperpolarization (sAHP). *A*, An example of rapid action potentials elicited by direct current injection into an HVc neuron (0.8 nA, 1 sec). *B*, Such current injection caused an afterhyperpolarization with a relatively short time to peak but slow decay time constant (4.1 sec). Resting membrane potential = −63 mV.

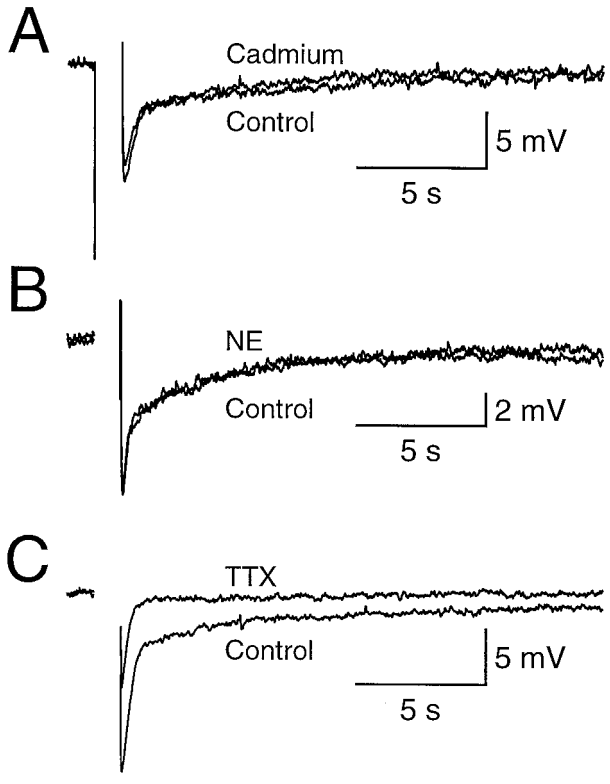

Figure 7. Properties of the sAHP in HVc neurons. *A*, A direct depolarizing current pulse (0.5–2.0 nA; 1 sec) gave rise to a very slowly decaying sAHP. Such depolarization typically elicited 30–40 action potentials during the pulse (not shown). Application of the Ca^{2+} channel blocker $CdCl_2$ (100 μM), known to block sAHPs in other systems, had no effect on either the duration or amplitude of the sAHP. *B*, Norepinephrine (10 μM), which blocks the sAHP in other cell types (Madison and Nicoll, 1982), did not affect the sAHP in HVc. *C*, Bath application of the sodium channel blocker tetrodotoxin (*TTX*) (1 μM) completely blocked the slow phase of the sAHP.

We thus further characterized the SH by separating intrinsic from synaptic components using sensitivity of synaptic transmitter release to cadmium (see Results).

Contributions of $GABA_B$ receptor activation to the slow hyperpolarization

Most neurons contain a multiplicity of potassium channels, the activation of which can lead to hyperpolarization (Nicoll, 1988). In many cases, activation of $GABA_B$ receptors by exogenous application of baclofen causes a hyperpolarization via increased potassium conductance. In hippocampal pyramidal cells, synaptically released GABA acts on $GABA_B$ receptors to cause slow IPSPs that last hundreds of milliseconds (Dutar and Nicoll, 1988). To assess whether HVc neurons are capable of responding to $GABA_B$ receptor activation, we first perfused slices with the $GABA_B$ receptor agonist baclofen (30 μM). Bath application of this agonist caused a marked hyperpolarization in every cell examined (Fig. 8*A*) (11.0 ± 3.8 mV; $n = 14$). This hyperpolarization was associated with a decrease in input resistance, consistent with the increase in potassium conductance observed in other cell types (Fig. 8*B*) (Newberry and Nicoll, 1984; Gahwiler and Brown, 1985; Nicoll, 1988). When the membrane potential was returned to control levels by injection of steady depolarizing current, input resistance was still reduced (Fig. $8B_1,B_3$), indicating that the conductance change was not merely secondary to the change in potential.

In the presence of the $GABA_B$ receptor antagonist CGP 35348 (500 μM) (Olpe et al., 1990), baclofen did not significantly hyperpolarize HVc neurons (−0.6 ± 1.9 mV; $n = 7$; $p > 0.35$). In six cells, responses to baclofen were measured in the presence and absence of CGP 35348. The antagonist reduced the baclofen response by 91 ± 20% ($n = 6$). When baclofen was added after washout of CGP 35348, neurons responded with the characteristic hyperpolarization (Fig. 9) ($n = 4/4$). These findings validate baclofen and CGP 35348 as selective $GABA_B$ receptor compounds in this avian system and indicate that HVc neurons express $GABA_B$ receptors coupled to potassium channels.

To investigate directly the role of $GABA_B$ receptors in generating the slow synaptic hyperpolarization, we examined the effect of CGP 35348 on the SH elicited in the presence of CNQX, APV, and BMI. Bath application of CGP 35348 (500 μM) produced quite variable effects (Fig. 10*B*) ranging from no effect to nearly complete blockade of the SH (Fig. 10*A*). The average reduction in peak amplitude of the SH was 44 ± 27% ($n = 19$). These results demonstrate that a substantial component of the SH can be accounted for by activation of $GABA_B$ receptors and suggest that GABA is able to produce IPSPs that last many seconds. The

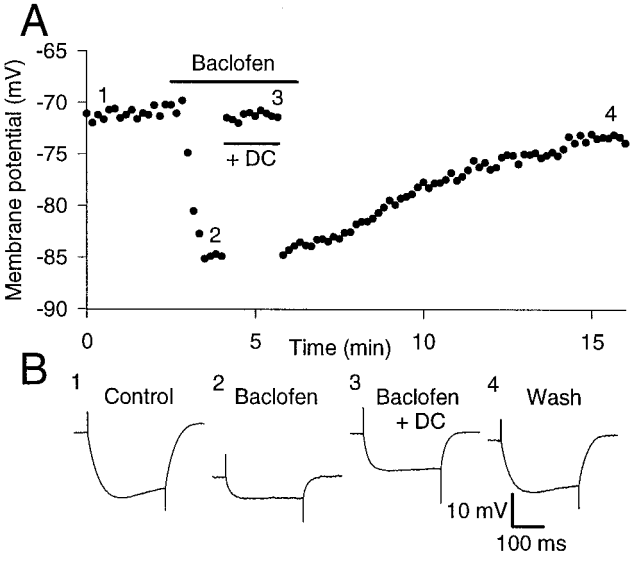

Figure 8. Effect of the $GABA_B$ receptor agonist baclofen on HVc neurons. *A*, Bath application of baclofen (30 μM) caused a reversible hyperpolarization associated with a decrease in input resistance. Input resistance was monitored by passing 300 msec current pulses (−0.1 nA) every 5–20 sec (see *B1–B4*). Input resistance remained decreased even after the membrane potential was shifted back to the original resting potential by DC depolarization (+ *DC*; *B3*). These results suggest that baclofen causes an increase in potassium conductance, a commonly observed effect of activating $GABA_B$ receptors in many other systems.

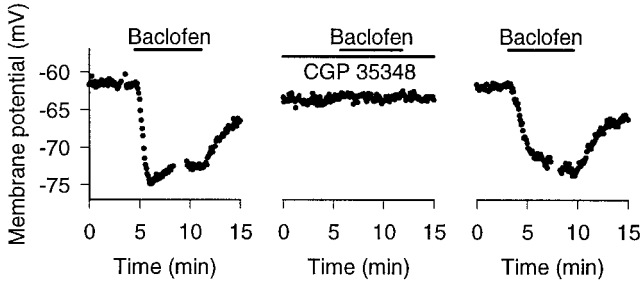

Figure 9. Effect of the $GABA_B$ receptor antagonist CGP 35348 on the hyperpolarizing action of baclofen. To test the effect of CGP 35348 on HVc neurons, neurons were first subjected to bath application of baclofen (30 μM) to ensure that baclofen produced a hyperpolarizing response (*left panel*). After a recovery period of 20 min the membrane potential returned to near the original resting potential. In the presence of CGP 35348 (500 μM), baclofen did not affect the membrane potential (*middle panel*). The small difference in membrane potential between the *left* and *middle panels* was not a common observation. Addition of baclofen after washout of the antagonist produced a normal hyperpolarizing response (*right panel*). *Blank portions* of the traces during baclofen addition indicate time when the membrane potential was returned to the original resting potential (see Fig. 8). These points were omitted for clarity. All traces were obtained from the same neuron over a period of 1 hr.

variable effect of the $GABA_B$ antagonist suggests that $GABA_B$ receptors may contribute a different fraction of the SH in different neurons.

A $GABA_B$ receptor-independent IPSP contributes to the slow hyperpolarization

In many cases, a residual SH resisted blockade by the mixture of CNQX, APV, BMI, and CGP 35348. In an attempt to block the remaining synaptically mediated hyperpolarization, we applied a mixture of receptor antagonists implicated in slow hyperpolarizing responses. The addition of this mixture, consisting of antagonists to opioid receptors (naloxone, 500 μM), muscarinic acetylcholine receptors (atropine, 10 μM), or α_2 noradrenergic receptors (yohimbine, 20 μM) was without effect in reducing either the magnitude or the time course of the hyperpolarization (n = 3; data not shown).

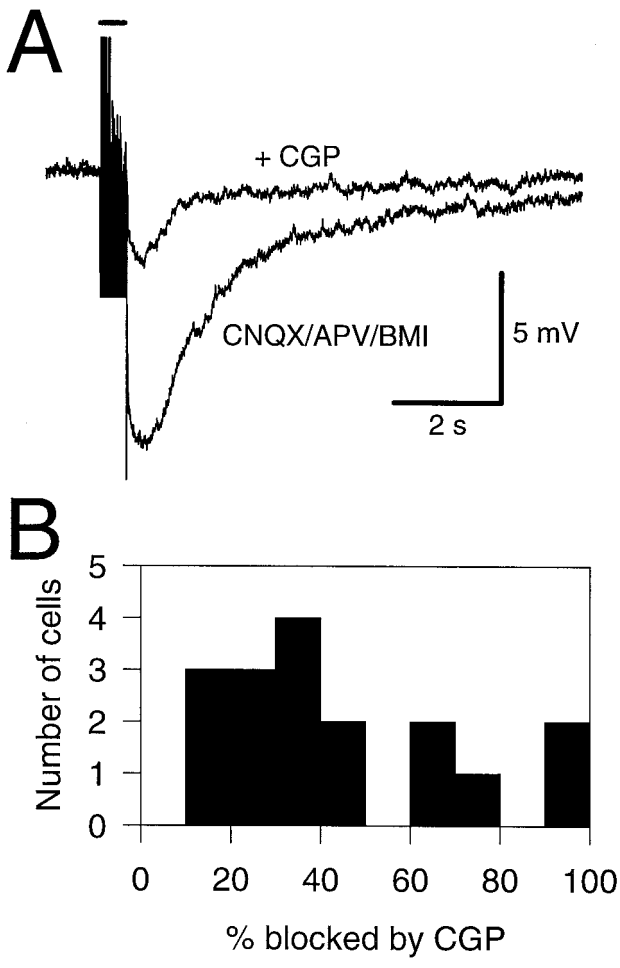

Figure 10. Effect of the $GABA_B$ receptor antagonist CGP 35348 on the stimulus-induced SH. *A*, A brief, high-frequency stimulus train (50 pulses at 100 Hz; indicated by the *horizontal bar above the trace*) was used to elicit a long-lasting SH in the presence of CNQX (20 μM), APV (100 μM), and BMI (40 μM). After several stable stimulation trains (elicited every 2 min), CGP 35348 (500 μM) was added to the mixture of drugs. In this particular example, addition of the $GABA_B$ receptor antagonist blocked a large portion of the SH. The nature of the remaining hyperpolarization could have been caused by a synaptically mediated IPSP or alternatively by an sAHP, because stimulation caused antidromic firing in this neuron. *B*, The effect of CGP 35348 on the SH was quite variable across HVc neurons. This histogram illustrates that nearly all neurons were affected by addition of the $GABA_B$ receptor antagonist. A large proportion of neurons (64%) had their SH reduced by >30% (n = 11/17).

To assess the relative contribution of synaptic transmission to this residual SH, we applied the calcium channel blocker $CdCl_2$, which blocks transmitter release but not the sAHP (Fig. 7*A*). The SH was elicited using our standard stimulation protocol in the presence of antagonists to glutamate and $GABA_A$ receptors (CNQX, APV, and BMI). After several stable baseline traces (Fig. 11*A*), the $GABA_B$ component of the SH was removed by adding CGP 35348 (Fig. 11*B*). After confirming that the antagonistic effect of CGP 35348 had stabilized, $CdCl_2$ (100 μM) was

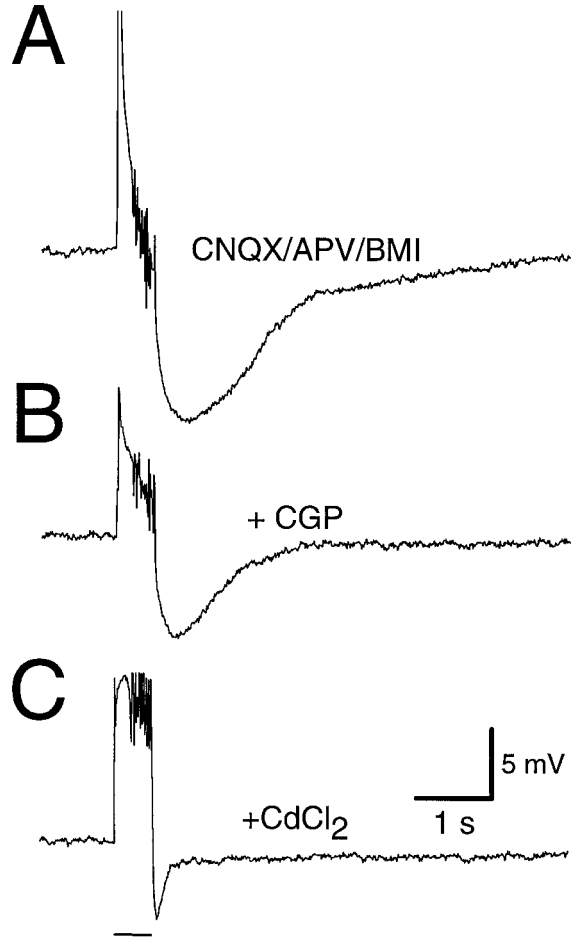

Figure 11. Contribution to the SH of a $GABA_B$ receptor-independent IPSP. *A*, Sample traces showing the presence of an SH in the presence of our standard pharmacological mixture of CNQX (20 μM), APV (100 μM), and BMI (40 μM). Stimulus train consisted of 50 pulses at 100 Hz, at the time indicated by the horizontal bar below the trace in *C*. *B*, Addition of CGP 35348 significantly reduced the magnitude of the SH. *C*, To investigate the nature of the remaining SH, the inorganic calcium channel blocker $CdCl_2$ (100 μM) was added to block all synaptic transmission. Addition of $CdCl_2$ reduced the response to electrical stimulation. The small remaining hyperpolarization was caused by a calcium-insensitive sAHP produced by stimulation-induced antidromic action potentials. In this particular neuron, stimulation caused 38–40 action potentials. All records are from the same cell.

added to the pharmacological mixture. The calcium channel blocker reduced the SH (75 ± 29%; $n = 5$) (Fig. 11*C*), suggesting that a significant portion of the SH is attributable to a $GABA_B$ receptor-independent IPSP. In some cases, a small, brief, residual hyperpolarization remained after the addition of $CdCl_2$ (Fig. 11*C*). This hyperpolarization may have been caused by a calcium-independent sAHP attributable to stimulus-induced antidromic action potentials and could be blocked by subsequent addition of TTX (data not shown).

DISCUSSION

We have described an unusually slow, activity-induced hyperpolarization in neurons of nucleus HVc in the adult zebra finch. At least three components contribute to the SH to varying degrees: (1) an afterhyperpolarization intrinsic to the neuron recorded, (2) a synaptically induced, $GABA_B$ receptor-mediated response, and (3) an unidentified slow IPSP. All three components appear to depend on increased potassium conductance and individually or collectively may play an important role in gating information flow in the song system.

Mechanism of slow hyperpolarization

Intrinsic cell properties

A component of the SH described here is a slow afterhyperpolarization (sAHP) caused by action potentials evoked during stimulation in HVc. These action potentials were probably evoked antidromically by stimulation of the extensive axonal ramifications of HVc neurons within the nucleus (Katz and Gurney, 1981; Nottebohm et al., 1982; Fortune and Margoliash, 1995). Although sAHPs are observed in most neurons studied, the sAHP observed in HVc appears to differ from those observed in most other systems (for review, see Sah, 1996). Kubota and Saito (1991) used multiple trains of action potentials to elicit an sAHP in HVc neurons having a time course similar to or longer than that of sAHPs studied in other systems. The sAHP in HVc is calcium independent and requires Na^+ influx. We have confirmed these findings and shown that the HVc sAHP also differs from other sAHPs, including one other known example of sodium-dependent sAHP (Schwindt et al., 1989), in its apparent insensitivity to modulation by neurotransmitters such as norepinephrine or acetylcholine (Nicoll, 1988; Foehring et al., 1989).

Although the sAHP component of the SH described here may be a consequence of our stimulation paradigm, physiological firing rates (Yu and Margoliash, 1996) could produce a similar sAHP. Slow AHPs in several neuronal types mediate spike-frequency accommodation (Madison and Nicoll, 1984; Sah, 1996) and may influence stimulus-induced spike timing (Hu, 1995). The particularly long time course of the sAHP observed in HVc neurons (typically 1–5 sec for a train of 30–50 action potentials) suggests that it may help modulate neural activity during singing.

Synaptic properties

Although we could not block the sAHP component of the SH selectively, we have shown that one or more slow synaptic events contribute to the SH. In several cases, we were able to elicit a large SH in the complete absence of antidromic action potentials (Fig. 2*A*), indicating that the SH can be caused exclusively by long-lasting synaptic mechanisms. In addition, when antidromic action potentials were present, a substantial proportion of the SH could be blocked by the calcium channel blocker $CdCl_2$. Because cadmium blocks Ca^{2+}-dependent transmitter release and leaves the sAHP intact (Kubota and Saito, 1991; present results), these manipulations indicate that a synaptic component contributes to the SH, even when it is accompanied by antidromic action potentials.

The SH observed here, which lasted up to 20 sec, is unusually long in duration. This slow time course is most likely caused by slow transmitter action, but if it is caused by sustained release of a short-lasting transmitter, intermediary neurons would need to continue firing after stimulation. Such neurons were never recorded in this study, and such postulated excitation would need to persist during blockade of ionotropic glutamate receptors. Alternatively, a circuit consisting of two mutually inhibitory neuronal populations could show prolonged activity (Cropper and Weiss, 1996). Such a scenario might be expected to cause rhythmic bursts of inhibition in the recorded cell. We did not observe oscillatory inhibition, however. Few IPSPs lasting seconds have been described. In most cases these appear to be mediated by serotonin (Pan et al., 1989), norepinephrine (Egan et al., 1983), or acetyl-

choline (Dodd and Horn, 1983). Neuropeptides, although not directly shown to produce IPSPs, have been shown to hyperpolarize neurons in the CNS and may act as transmitters for slow IPSPs (Nicoll et al., 1990).

The CGP 35348-sensitive component of the SH lasts up to 20 sec and appears to require $GABA_B$ receptor activation. Although $GABA_B$ receptors typically cause IPSPs lasting only a few hundred milliseconds (Alger, 1984; Newberry and Nicoll, 1984; Dutar and Nicoll, 1988; Nicoll et al., 1990), they have been shown to mediate a tetanus-induced heterosynaptic presynaptic inhibition in the hippocampus (Isaacson et al., 1993). Because tetanic stimulation can also cause hyperpolarization in hippocampal pyramidal neurons lasting several seconds (Cole and Nicoll, 1984, their Fig. 4), these results suggest that $GABA_B$ receptors may cause slow inhibitory synaptic events lasting many seconds. However, repetitive stimulation can also cause depression of $GABA_B$ receptor-mediated responses. The long duration of inhibition observed here may reflect the stimulation paradigm or an unusually slow action of GABA on HVc neurons, possibly attributable to slow GABA uptake or a long-lasting second messenger system.

In many instances, the addition of the $GABA_B$-receptor antagonist CGP 35348 only partially blocked the SH. By blocking calcium-dependent synaptic transmission with $CdCl_2$, without affecting the sAHP, we were able to show that this residual hyperpolarization consists of a non-$GABA_B$ IPSP. The transmitter and receptor mediating this synaptic component remain elusive, because blockade of muscarinic (atropine), α_2 noradrenergic (yohimbine), or opioid (naloxone) receptors failed to block the IPSP. Further mechanistic analysis of this IPSP is clearly required.

Role of slow hyperpolarization in the song system

Blockade of excitatory transmission by CNQX and APV consistently attenuated or abolished the SH, indicating that a significant portion of the hyperpolarization was generated by neurons intrinsic to HVc that were synaptically excited by our stimulus. However, failure of these drugs to block the SH completely or change its slow time course suggests that monosynaptic inhibitory processes contribute to the observed SH. In this study, we cannot distinguish between monosynaptic IPSPs produced by intrinsic HVc neurons and connections extrinsic to HVc. Such extrinsic inputs to HVc could arise from a number of nuclei, including the thalamic nucleus uvaeformis (Uva) (Nottebohm et al., 1982), the forebrain medial portion of the magnocellular nucleus of the anterior neostriatum (M-MAN) and nucleus interfacialis (NIf) (Nottebohm et al., 1982), the HVc "shelf" (Vates et al., 1996), and possibly the field L complex (Fortune and Margoliash, 1995). Because the physiological nature of these inputs is not known, these connections could provide the excitatory input sufficient to activate inhibitory neurons within HVc, inhibit HVc neurons directly, or provide a mixed excitatory/inhibitory input. The connection from Uva, and possibly NIf (Fig. 1), may be of particular interest in the context of inhibition of auditory responses during singing, because stimulation of Uva has been reported to cause long-lasting inhibition of auditory responses in HVc (Williams, 1989).

The SH often consisted of a combination of sAHP, $GABA_B$-receptor mediated IPSP, and a third unidentified IPSP. The relative contribution of these components to the SH was quite variable. In the present study, we did not attempt to estimate quantitatively the relative contributions to the SH made by the various component mechanisms, because these values likely depend on the details of electrode placement as well as on the class of neuron being recorded and the connections it receives. Identification of the afferent types being stimulated remains challenging because of the confluence from multiple sources. A description of the neuronal properties in HVc may yield interesting insight into the role of this slow inhibition. Nucleus HVc sends out two major projections, to area X and to nucleus robustus archistriatalis (RA) (Nottebohm et al., 1982), originating from separate neuronal populations (Katz and Gurney, 1981; Lewicki, 1996) that may be functionally distinct (Kimpo and Doupe, 1997). The X-projecting neurons have auditory responses (Katz and Gurney, 1981; Lewicki, 1996). We have shown here that only a portion (~60%) of all neurons recorded in HVc exhibit an SH after stimulation within HVc. Recent results (Dutar and Perkel, 1997) suggest that only X-projecting HVc neurons have the SH, providing a clear physiological difference between these neuronal populations.

The SH observed in this study may contribute to generating song selectivity of auditory responses (Margoliash, 1983). Song-selective neurons, which respond most strongly to playback of the bird's own song, were found to become most hyperpolarized during presentation of their preferred stimulus (Lewicki, 1996; Lewicki and Konishi, 1995). This hyperpolarization was interrupted by short, precisely timed bursts of action potentials during specific segments of the song. The hyperpolarization, as well as the bursts, were absent during presentation of less favorable auditory stimuli. Hyperpolarization may permit deinactivation of voltage-dependent conductances, which are thought to cause bursting behavior in certain systems (Crunelli and Leresche, 1991; von Krosigk et al., 1993). These hyperpolarizations, which can be caused by either extrinsic (e.g., $GABA_B$ in the lateral geniculate nucleus) (Crunelli and Leresche, 1991) or intrinsic mechanisms (e.g., sAHP in auditory thalamus) (Hu, 1995), may permit bursts of action potentials and thus provide more precise information than single action potentials (for review, see Lisman, 1996). Long-lasting, stimulus-induced hyperpolarizations may thus play a crucial role in enhancing signal-to-noise ratio by suppressing background noise, and responses to weak inputs, while allowing strong stimuli to produce precisely timed bursts of action potentials (Karlsson et al., 1990; Mainen and Sejnowski, 1995).

The SH could also contribute to the long-lasting inhibition of auditory responses observed during and after singing (McCasland and Konishi, 1981). Because HVc acts as a gateway for auditory information flow into the song system and because song learning requires auditory feedback, inhibition of auditory responses during singing could be regulated during song learning. It will be critical to understand whether and how inhibition in HVc modulates the flow of auditory information into the rest of the song system during different stages of song acquisition. Identification of the cellular basis of this inhibition as well as its development during song learning will illuminate a pivotal aspect of auditory–motor integration.

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