Multiple Cell Types Distinguished by Physiological, Pharmacological, and Anatomic Properties in Nucleus HVc of the Adult Zebra Finch

PATRICK DUTAR, HUAN M. VU, AND DAVID J. PERKEL
Department of Neuroscience, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6074

INTRODUCTION

Nucleus HVc [used here as the full name of the nucleus, following Fortune and Margoliash (1995)] of the songbird is essential for singing and forms a part of the forebrain central pattern generator for song production (Nottebohm et al. 1976; Vu et al. 1994). HVc neurons show premotor firing before singing behavior (McCasland and Konishi 1981; Yu and Margoliash 1996), and disruption of HVc firing during singing can reset the song rhythm (Vu et al. 1994). In addition, HVc neurons have highly selective auditory properties, responding strongly to complex acoustic stimuli, particularly the bird’s own song (Margoliash 1986). HVc is thus a critically important station for sensorimotor integration during song learning and production.

Anatomic studies of HVc in canary (Nixdorf et al. 1989) and in zebra finch (Katz and Gurney 1981; Lewicki and Konishi 1996; Lewicki 1995) indicate that there are several morphologically distinguishable neuron types. Different subpopulations of neurons project to HVc’s two targets, the anterior forebrain auditory region called area X, and the premotor robust nucleus of the archistriatum (RA) (Fortune and Margoliash 1995; Nottebohm et al. 1976). It is thus reasonable to hypothesize that different HVc cell types participate in different functions. Indeed, Katz and Gurney (1981) suggested that HVc neurons that project to area X (X-projecting cells) were more responsive to auditory stimuli than cells projecting to the robust nucleus of the archistriatum (RA-projecting cells), and the same trend was reported by Lewicki (1996). Recent immunohistochemical staining for Fos, the protein product of the immediate early gene c-fos, suggests that the RA-projecting cells are active while birds sing and thus that the two populations of HVc projection neurons have different functional properties (Kimpo and Doupe 1997). However, expression of the immediate early gene zenk is elevated in area X following singing, even in deafened birds, suggesting that a motor or efference copy signal is sent to area X as well (Jarvis and Nottebohm 1997).

Intracellular recordings shed light on the mechanisms of auditory selectivity in HVc neurons (Katz and Gurney 1981; Lewicki 1996; Lewicki and Konishi 1995), and in vitro studies identified slow hyperpolarizing potentials (Kubota and Saito 1991; Schmidt and Perkel 1998). However, the basic cellular properties of the different HVc neuron types remain unclear. We used intracellular recording to examine the intrinsic firing properties, synaptic responses, and pharmacological sensitivity of these cells in brain slices. In addition, we asked whether neurons projecting to different targets have different functional properties. Indeed, the two populations of projection neurons differ strikingly in their characteristics. We report here three cell types distinguished by their soma size, dendritic extent, and axonal projection. Thus HVc neurons projecting to area X have large somata, show little spike-frequency adaptation, a hyperpolarizing response to the metabotropic glutamate receptor (mGluR) agonist (1S,3R)-trans-1-amino-cyclopentane-1,3-dicarboxylic acid (ACP.D), and exhibit a slow inhibitory postsynaptic potential (IPSP) following tetanic stimulation. Those HVc neurons projecting to motor nucleus RA have smaller somata, show strong accommodation, are not consistently hyperpolarized by ACPD, and exhibit no slow IPSP. A third, rarely recorded class of neurons fire in a sustained fashion at very high-frequency and may be interneurons. Thus the neuronal classes within HVc have different functional properties, which may be important for carrying specific information to their postsynaptic targets.
mGlur agonists. These distinct functional properties may be important for the different functions performed by subcircuits within HVc and the rest of the song system.

METHODS

Preparation of slices and electrophysiological recording

Adult male zebra finches (≥120 days old) were obtained from a local supplier or were bred in our colony at the University of Pennsylvania. Animals were housed three or four per cage in a room on a 13:11 h, light:dark cycle. It is likely that they were in breeding condition and singing before slicing, but their behavioral state was not systematically verified by audio recordings. Slices were prepared as described by Schmidt and Perkel (1998), and the procedures were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. Briefly, a bird was anesthetized with halothane and decapitated. The brain was rapidly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) that was prewarmed with 95% O2-5% CO2. The composition of the ACSF was (in mM) 119 NaCl, 2.5 KCl, 1 NaH2PO4, 26.2 NaHCO3, 11 d-glucose, 2.5 CaCl2, and 1.3 MgSO4. Parasagittal slices (300- to 400-µm thick) were prepared with a vibrating microtome and stored submerged in bubbled ACSF. For recording, a slice was transferred to a chamber where it was submerged and superfused at 1–2 ml/min with prewarmed ACSF.

Conventional intracellular recordings were obtained from neurons within a region that could be identified as HVc in the unstained, living slice. The electrodes were filled with 4 M potassium acetate, 20 mM KCl. Membrane potential was amplified with the use of an Axoclamp 2B (Axon Instruments, Foster City, CA). Signals were filtered at 1–5 kHz, digitized at twice the filter cutoff frequency, and stored on a computer hard disk. Acquisition and analysis were carried out with the use of a program written in Labview (M. Farries and D. J. Perkel, personal communication).

The fast afterhyperpolarization (AHP) following each action potential was measured as follows: peak amplitude was the difference between the peak hyperpolarization and the potential immediately before the initiation of the action potential (take-off point), the time-to-peak was measured from the point when the membrane potential hyperpolarized past the take-off point, and the duration was measured at one-half the AHP peak amplitude. Synaptic responses were elicited by electrical stimulation of HVc with stainless-steel bipolar stimulating electrodes (Frederick Haer, Brunswick, ME). High-frequency stimulation trains (50 pulses of 100 µs duration delivered at 100 Hz) were applied to induce the slow hyperpolarization. Results are expressed as means ± SE. The statistical test used was Student’s t-test.

Chemicals used in this study included 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), d(-)-2-amino-5-phosphonovaleric acid (APV), bicusculine methiodide (BMI; all from Research Biochemicals, Natick, MA), carbachol, bafilofen, serotonin (5-HT), picrotoxin (all from Sigma Chemical, St. Louis, MO), (1S,3R)-trans-1-amino-3-cyclopentane-1,3-dicarboxylic acid (ACPD), and (2S,1′,S,2′S)-2-(carboxycyclopentyl)glycine (1-CGG-1) (Tocris Cookson, Ballwin, MO), CGP35348 (gift from CIBA-Geigy, Basel, Germany), and dimethylsulfoxide (DMSO; Fisher Scientific, Pittsburgh, PA). Drugs were added to the superfusion medium by dilution of a stock solution made in water. Stock solutions of CNQX were made in DMSO at a final concentration of DMSO (0.1–0.2%); which did not affect the neuronal responses. Bicusculine was dissolved directly into the ACSF.

Visualization of the neurons

In some experiments, recording electrodes were filled with 2–4% neurobiotin (Vector Laboratories, Burlingame, CA) dissolved in the recording solution. At the end of the recording, neurobiotin was injected into the neuron by passing depolarizing pulses (1 Hz, 600- to 700-ms duration, 0.3- to 1-nA intensity). We then allowed a survival time for the neuron and slice of 30 min to 1 h to permit the diffusion of neurobiotin into the axon and dendrites. The slice was then fixed in paraformaldehyde (4% in 0.1 M phosphate buffer) and kept at 4°C for ≥4 h. It was then placed in a cold sucrose solution (30% in 0.1 M phosphate buffer). After a few hours or days, the slice was resected at 40- to 60-µm thickness with a freezing microtome. Sections were then processed for visualization of injected neurons with the use of avidin and biotinylated horseradish peroxidase complex (ABC Elite Kit, Vector Laboratories) followed by reaction with diaminobenzidine. Drawings were made with a camera lucida. Soma area was calculated by multiplying one-half the major axis by one-half the minor axis by π.

RESULTS

Electrophysiological studies with the use of intracellular recordings from HVc neurons led to the identification of three main neuronal types having different physiological and pharmacological properties. We first determined that the recorded neurons fell into categories based on physiological properties. We tentatively called these type I, type II, and type III HVc neurons. We then demonstrated that these neurons have different pharmacological properties. Finally, we observed evidence for different anatomic properties. These data are based on stable intracellular recordings lasting up to 6 h from 75 neurons from 57 slices from 57 birds.

Physiological properties

The neurons that we recorded in HVc belong to two main types (and occasionally to a third type) having very different physiological properties.

TYPE I NEURONS. The first type of neuron is illustrated in Fig. 1A. This neuronal population had a low spike threshold, a resting membrane potential of −68.7 ± 1.2 (SE) mV (n = 42), and a high input resistance (161.8 ± 8.8 MΩ, n = 44). This cell type was able to fire several action potentials in response to a depolarizing current pulse with little accommodation of the spike discharge (Fig. 1A1, pulse duration 300 ms). The fast AHP following each spike had a large amplitude (15.2 ± 0.6 mV, n = 36), a slow time-to-peak (12.4 ± 1.6 ms, n = 32), and an especially long duration at half-amplitude (66.8 ± 4.7 ms, n = 32; Figs. 1A and 3B). Most of these neurons (28/37) also showed time-dependent inward rectification in response to hyperpolarizing pulses (Fig. 1A2). This is characterized by a sag in the hyperpolarizing response. The mean value of the sag for this neuronal population (measured as the difference between the peak and asymptotic values divided by the peak value) was 13.1 ± 7.8% (n = 28). Spontaneous synaptic events were rarely encountered.

TYPE II NEURONS. A type II neuron is illustrated in Fig. 1B. Compared with the first type, it had a very hyperpolarized resting membrane potential (−80.9 ± 1.5 mV, n = 27) and a low input resistance (87.1 ± 8.2 MΩ, n = 30) and was able to fire only one or two action potentials in response to a depolarizing pulse (Fig. 1B1, pulse duration 300 ms). These values significantly differed from those of type I cells (P < 10−4 and P < 10−4, respectively). These neurons
FIG. 1. Firing properties of the different types of HVc neurons. A: type I neuron. A1: 300-ms depolarizing current pulse (0.2 nA) applied to the neuron induced repetitive firing of action potentials. Each action potential was followed by a large, long-lasting afterhyperpolarization (AHP). These AHPs prevented high-frequency firing. A2: hyperpolarizing current pulse (-0.2 nA) elicited a voltage deflection having a pronounced time-dependent inward rectification characterized by a sag in the voltage response and a rebound overshoot (average of 4 consecutive sweeps). Resting membrane potential: -72 mV.

B: type II neuron. B1: 300-ms depolarizing current pulse (0.2 nA) induced an action potential followed by a short-duration AHP. It was common to elicit a few action potentials per pulse immediately following penetration of the cell, but after a few minutes the neuron reached a very negative resting membrane potential and gave this typical response with usually a single action potential. Increasing the amplitude of the depolarizing pulse did not increase the number of spikes to >2. B2: applying a negative constant current pulse (-0.2 nA) induced a hyperpolarizing response with no apparent membrane rectification (4 averaged sweeps). Resting membrane potential: -82 mV.

C: type III neuron. C1: this type of neuron, more rarely encountered, was characterized by its ability to discharge at very high frequency in response to a 300-ms depolarizing current pulse. In this example, the discharge frequency was 83 Hz. C2: hyperpolarizing pulse induced a voltage deflection exhibiting time-dependent inward rectification. Resting membrane potential: -65 mV.

were not simply unable to fire repetitively because they fired at 10 Hz when given brief (50 ms) depolarizing pulses at that frequency (n = 4/4). Each spike was followed by a fast AHP that had kinetics different from those of the other neuronal types. Although the amplitude of the AHP in type II cells (12.8 ± 1.5 mV, n = 22) was comparable with that in other neuronal types, in contrast, the time-to-peak (2.6 ± 0.3 ms, n = 21) and the duration at half-amplitude (10.4 ± 2.1 ms, n = 21) of the AHP were much shorter in these neurons. The response to hyperpolarizing current pulses exhibited no trace of time-dependent inward rectification or sag (Fig. 1B2). Spontaneous depolarizing synaptic potentials were frequently observed, but they were not examined further. When we plotted one physiological parameter against another (e.g., number of action potentials induced by a 0.4-nA, 300-ms depolarizing current pulse vs. AHP duration, Fig. 2), we observed that the neurons we tentatively called type I and type II form two separate populations (see also Fig. 6, B and C).

TYPE III NEURONS. The third type of neuron was encountered less frequently (n = 7, Fig. 1C). They were able to produce very high-frequency firing (≈150 spikes/s) in response to a depolarizing pulse (see Figs. 1C1 and 3). To test whether they really fell into a different category from the type I cells, we examined their firing in response to depolarizing stimuli. The number of action potentials elicited in response to a depolarizing pulse (0.2 nA, 300 ms) was 16.5 ± 2.9 (n = 4) for type III neurons compared with 3.6 ± 0.5 (n = 21) for type I cells. This was the primary feature that distinguished type III from type I cells. These neurons had a mean resting membrane potential of -67.6 ± 2.0 mV (n = 7), a membrane input resistance of 107.7 ± 13.2 MΩ (n = 7), and a pronounced time-dependent inward rectification (sag 22.3% ± 4.3, Fig. 1C2). The amplitude, time-to-peak, and duration at half-amplitude of AHPs were respectively 13.2 ± 1.4, 5.1 ± 2.0, and 28.4 ± 7.3 ms (n = 7). Of these seven neurons, three showed some phases of bursting activity.

Pharmacological properties

The pharmacological properties of HVc neurons were studied with the use of two approaches. First, we studied the synaptic pharmacology of neurons following electrical stimulation within HVc. Second, we studied the neuronal effects of exogenously applied neurotransmitter agonists.

Synaptic responses

TYPE I NEURONS. In response to high-frequency stimulation of afferents located within HVc (Schmidt and Perkel 1998),
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aminobutyric acid-B (GABA_B) antagonist CGP35348 (500 μM). As reported previously (Schmidt and Perkel 1998), a portion of this hyperpolarization was sensitive to bicuculline and CGP35348 and thus was mediated by activation of GABA_A and GABA_B receptors. A part of the IPSP was resistant to the GABA antagonists (Fig. 4A2) but was blocked by tetrodotoxin (TTX; Fig. 4A3). Its pharmacological nature is still unknown (Schmidt and Perkel 1998).

TYPE II NEURONS. The synaptic response of these neurons was very different from that of the type I neurons in that tetanic stimulation induced a very large EPSP (amplitude 24.6 ± 2.1 mV and duration 7.72 ± 0.77 s, n = 13), which was not followed by an IPSP (Fig. 4B1). The lack of IPSP could not be explained by the small driving force on potassium because, even during depolarization by steady current injection, there was no slow IPSP (average depolarization 16 mV; n = 10/10). The amplitude of the slow EPSP was reduced to 44.1 ± 5% (n = 4) of control in the presence of CNQX (20 μM) and APV (50 μM). A component of the slow EPSP (12.8 ± 2.3 mV; n = 4) was resistant to APV and CNQX, and its nature has not yet been identified (Fig. 4B2).

The difference in the duration of the EPSP between type I and type II neurons was statistically significant (P < 0.0001, t-test) probably because IPSPs in type I neurons curtail the EPSP duration.

TYPE III NEURONS. The number of recorded neurons is small. However, these neurons exhibited an EPSP/IPSP sequence in response to stimulation within HVc. In contrast with type I neurons, the IPSP was totally blocked by application of a cocktail of GABA_A and GABA_B antagonists (not illustrated).

Response to drug application

Our results demonstrate that the different neuronal types differ in their sensitivity to exogenously applied neurotrans-

![Fig. 2](http://jn.physiology.org/)

**Fig. 2.** Types I and II neurons have distinct physiological properties. Scatter plot of the number of action potentials induced by a 300-ms long, 0.4-nA depolarizing current pulse against the duration of the fast AHP, measured at half-amplitude. The neurons of type I (●) and type II (○) fell into two separate populations. Note the different scales for the fast AHP duration for the 2 cell types.

type I neurons exhibited a slow excitatory postsynaptic potential (EPSP; amplitude 19.3 ± 1.3 mV and duration 2.78 ± 0.19 s; n = 19), followed by a slow IPSP (Fig. 4A1). The IPSP following the EPSP had an amplitude of 6.1 ± 0.5 mV (n = 19).

After blockade of the slow EPSP with the ionotropic glutamate receptor antagonists CNQX (20 μM) and APV (50–100 μM), we investigated the slow IPSP with the use of the γ-aminobutyric acid-A (GABA_A) antagonists picrotoxin (50 μM) or bicuculline methiodide (40 μM) and the γ-
mGluR agonists. We applied agonists of GABA<sub>A</sub> receptors and metabotropic glutamate receptors. GABA<sub>A</sub> receptors are known to cause a dramatic hyperpolarization in HVc neurons (Perkel and Schmidt 1998) and in many other vertebrate neurons (Bowery 1993). Metabotropic glutamate receptors are widely distributed throughout the vertebrate nervous system and affect most neuronal types tested (Pin and Duvoisin 1995; Schoepp and Conn 1993).

### TYPE I AND TYPE II NEURONS

Both cell types were reliably hyperpolarized by superfusion of the GABA<sub>A</sub> receptor agonist baclofen (30 µM). The hyperpolarization was associated with a decrease in input resistance. The baclofen-induced hyperpolarization was stronger in type I neurons (−14.0 ± 1.4 mV; n = 14; Fig. 5, top panel) than in type II neurons (−6.6 ± 0.7 mV; n = 17; Fig. 5, middle panel). The difference between type I and type II neurons was statistically significant (P < 0.005). This difference can be explained, at least in part, by the lower resting membrane potential and input resistance of type II neurons.

In contrast, the two neuronal types were very different in their respective sensitivity to mGluR agonists (ACPD and l-CCG-I) and to 5-HT (Figs. 5 and 6A). Type I neurons were strongly hyperpolarized by 5-HT (−10.8 ± 1.6 mV; n = 10), ACPD (−12.7 ± 0.9 mV; n = 24), or l-CCG-I (−14.7 ± 1.3 mV; n = 13). Type II neurons were less sensitive to 5-HT (−2.5 ± 0.7 mV; n = 6; P < 0.02) and gave variable responses to mGluR agonists (Fig. 6A). They were slightly hyperpolarized, insensitive, or even depolarized by l-CCG-I or ACPD (Fig. 5, middle panel). On average, ACPD depolarized type II neurons by 1.9 ± 0.7 mV (n = 17), and l-CCG-I depolarized these cells by 0.14 ± 1.1 mV (n = 14). In addition, both types of neurons were not consistently affected by the muscarinic cholinergic agonist carbachol (type I, n = 5; type II, n = 3), dopamine (n = 2; n = 2), or norepinephrine (n = 5; n = 3).

As a further test of the distinction between types I and II, we plotted the effect of mGluR agonists versus the duration of the fast AHP (Fig. 6B). The clustering of type I cells and type II cells provides additional evidence that these neurons do not fall on a continuum with respect to these parameters. Additionally, we plotted the response to baclofen versus the response to mGluR agonists for the two cell types (Fig. 6C). Type I cells showed a positive correlation between these two responses, with slope 1.01 ± 0.21 (R<sup>2</sup> = 0.69; P < 0.001; n = 17). Type II neurons, on the other hand, were always hyperpolarized by baclofen but were little affected by mGluR agonists. The slope of the regression line was 0.38 ± 0.25 (R<sup>2</sup> = 0.12; P > 0.15; n = 12), suggesting a poor, if any, correlation between the responses to the two agonists.
Because of the small sample, our results must be interpreted cautiously. The type III neurons tested were hyperpolarized by baclofen (−6.4 ± 1.1 mV, n = 6) but were insensitive to mGluR agonists ACPD (0 mV, n = 4) and L-CCG-I (0.6 ± 0.6 mV, n = 5; Fig. 5, bottom panel). In two cases, 5-HT depolarized the neuron and induced bursting activity.

### Anatomy

Most of the neurons were recorded with the use of neurobiotin filled electrodes (see METHODS). This technique allowed us to stain the neurons and to characterize the morphology of the neurons and the direction of their axonal projections.

#### Type I Neurons

Of 30 recorded neurons having the type I physiological properties, we could find 27 cell bodies labeled. Cell somata were mostly round in shape, and their mean surface area was 145.1 ± 7.7 μm² (n = 26; 1 cell was not adequately labeled for size measurement). They were widely distributed throughout nucleus HVC (Fig. 7A). The neurons had a large, extensively arborized dendritic field with no apparent polarization (Fig. 8). The maximal dendritic extension averaged 253.3 ± 6.4 μm (n = 24). In addition, the dendrites of these neurons had a great number of spines. We found the axons of the neurons leaving nucleus HVC in 15 neurons (Fig. 7B). In each case, the axon left in an anteromedial direction and appeared to be headed toward area X. In one case (Fig. 8A–C), after a single recording, we observed two filled somata and two axons. A more typical case is shown in Fig. 8D, in which a single cell was labeled. No axon of a type I neuron was found heading posteroventrally, i.e., toward nucleus RA. This finding indicates that the physiologically defined type I neurons are in fact the HVC neurons projecting to area X.

#### Type II Neurons

We labeled 13 neurons having type II physiology. Their somatic surface area averaged 106.9 ± 7.8 μm². Thus these neurons were smaller than the type I neurons (P < 0.001). They were widely distributed throughout the nucleus (Fig. 7A). The maximal extension of the dendrites averaged 201.0 ± 9.4 μm, less than for type I neurons (P = 0.005). Like those of type I cells, these dendrites had numerous spines.

These neurons had specific anatomic features that allowed us to distinguish them from type I neurons. First, we were able to find the axons of these neurons outside of HVC in eight cases. In each such case, the axon appeared to head caudoventrally, in the direction of nucleus RA (Fig. 7B). In most cases, it was possible to follow the trajectory of the axons over a distance of several hundred microns and often almost into nucleus RA (Fig. 9). The axons did not appear to ramify within the ‘‘shelf’’ region immediately ventral to HVC. This result demonstrates that the physiologically defined type II neurons are the HVC neurons projecting to nucleus RA. In addition, we observed that this projection was organized in the sagittal plane because it was possible to follow a long part of the course of the axon from HVC to RA in a single slice (see Katz and Gurney 1981; Lewicki 1996). Second, in three cases we were able to find two cell bodies labeled following a single somatic injection (Fig. 9), suggesting a high incidence of coupling in these neurons. In two cases of coupled neurons, it was possible to follow two independent axons, both projecting toward RA.

#### Type III Neurons

We filled only two neurons having type III physiology. These neurons resembled those of type I except that the dendrites were apparently devoid of spines. In addition, numerous fine processes were present throughout HVC, possibly representing extensive axonal divergence within HVC. The mean surface area of the soma was
135.5 ± 12.8 μm². In addition, although we found a well-filled axon within HVC, we were unable to see it leaving HVC, suggesting that this neuron could be an interneuron.

DISCUSSION

We have described at least three types of neurons recorded in nucleus HVC. Although the initial classification was made by intrinsic physiological properties, the presence of the slow IPSP, sensitivity to mGluR activation, and location of axonal projection could each serve to distinguish these cell classes. Thus we have shown that neurons with type I physiological properties project to area X, whereas type II neurons project to RA. X-projecting neurons have a resting potential around –68 mV, show sustained firing with a large AHP following each action potential, exhibit a slow IPSP in response to repetitive synaptic stimulation, hyperpolarize in response to ACPD, and in a few cases show dye coupling. RA-projecting neurons have an apparently quite hyperpolarized resting potential (approximately –80 mV), show dramatic spike-frequency accommodation, have no slow IPSP, are not hyperpolarized by mGluR agonists, and can show dye coupling with other HVC neurons. A third cell type was also observed, which could sustain very rapid firing rates, did not respond to mGluR agonists, and whose axon was not observed to leave HVC.

Kubota and Saito (1991) identified in HVC slices neurons that appear to correspond to the type I (X-projecting) cell reported here. They have similar resting potential, input resistance, and time-dependent inward rectification; in addition, both fire multiple action potentials in response to depolarizing current pulses, and both have a prominent slow AHP following prolonged depolarization (Schmidt and Perkel 1998). The large fast AHP following individual action potentials resembles those of auditory neurons illustrated by Lewicki and Konishi (1995) and Lewicki (1996).

The intrinsic properties of the RA-projecting neurons are somewhat unusual. However, similar properties were reported for the goldfish Mauthner cell (Faber et al. 1989) as well as for bushy cells of the mammalian anteroventral cochlear nucleus (Manis and Marx 1991; Oertel 1983; Wu and Oertel 1994) and for their putative avian homologues (Reyes et al. 1994). A rapidly activated potassium conductance may prevent repetitive firing in response to a sustained current pulse (Manis and Marx 1991; Reyes et al. 1994). These neuronal types are specialized for carrying temporal information with great specificity. To explain repetitive firing in response to rapid, brief depolarizing stimuli, such a rapidly activated potassium conductance would also need to be rapidly deactivated upon hyperpolarization. It is possible that the RA-projecting HVC neurons use a similar strategy, perhaps even a similar potassium conductance, to help generate a temporally precise rhythm.

It appears that the RA-projecting HVC cells in these slices possess a tonically active potassium conductance that maintains a hyperpolarized resting potential and low-input resistance. Perhaps these neurons in vitro are missing some tonic activity pattern or level of neurotransmitter or modulatory substance that maintains the closure of such a potassium channel. Although these neurons generate action potentials and respond to activation of neurotransmitter receptors, it remains possible that some aspect of our recording condi-

FIG. 6. Pharmacology of type I and type II neurons. A: average changes in membrane potential of type I (□) and type II (■) neurons in response to drug application (mean ± SE). Note the substantially smaller responses of type II neurons to baclofen and serotonin (5-HT) and their small depolarization in response to ACPD or insensitivity to (2S,1′S,2′S)-2-(carboxycyclopropyl)glycine (l-CCG-I). Numbers indicate the number of neurons tested. B: scatter plot, for each type I (n = 24) and type II neuron (n = 19) tested of the response to metabotropic glutamate receptor (mGluR) agonist application against the AHP duration, which was a key distinguishing intrinsic property. C: scatter plot, for each type I (n = 17) and type II neuron (n = 12) tested of the response to baclofen against the response to mGluR agonist. Type I neurons showed a correlation of the 2 responses, whereas the type II neurons did not (see text).
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FIG. 7. Distribution of labeled cell bodies and axons. A: different neuronal types have no topographic organization. This schematic representation shows the location of the recorded neurons superimposed onto a canonical drawing of HVc. This distribution was drawn after observation of the cell bodies filled with neurobiotin and reconstruction with a camera lucida. Cell bodies of physiologically defined types I (open triangles) and II (filled circles) neurons were found distributed across the whole nucleus. Three pairs of adjacent circles represent locations where 2 cell bodies were found filled with neurobiotin after a single type II recording was obtained. Only 2 type III neurons (filled squares) were filled with neurobiotin. Neuronal types were intermingled and widely distributed throughout the entire HVc nucleus. B: distribution of labeled axons leaving HVc. Camera lucida drawing of the course of the axons of type I and type II neurons at the boundary of nucleus HVc. Axons of type I neurons were all found anterior to the dotted line and coursed in the anterior or anteroventral direction (toward area X). Axons of type II neurons were found posterior to the dotted line and are directed in the ventroposterior direction (toward nucleus RA). Axonal course is illustrated only in the region of the HVc boundary. Ordinarily we could follow the processes for some distance following their exit from the nucleus. Only 13 type I axons are illustrated here because, in 1 case, coronal sections were made, and we could not superimpose the results on this summary graph, but the axon clearly left HVc in the anteroventral direction (toward area X).

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FIG. 8. Partial reconstruction of a type I neuron intracellularly filled with neurobiotin. A: camera lucida drawing of a parasagittal view of the cell body, the dendritic arborization, and the axonal trajectory. This is a 2-D projection of a partial 3-D reconstruction of the neuron generated from examination of multiple sections. B and C: bright field photomicrograph of parts of this neuron showing, at high-magnification, the cell body (B), and a part of the axon outside HVc (C) heading in the direction of area X. In this case, 2 cell bodies within HVc and 2 axons were filled following a single recording. Other parts of the axon used for the reconstruction illustrated in A are on different sections indicating a lateromedial direction for the HVc → X projection. D: camera lucida drawing of a parasagittal view of another type I neuron. Orientation and scaling are the same as in A.

larized by baclofen or 5-HT, indicating that some neurotransmitter-activated potassium channels are present. Thus, a key distinction between the X- and RA-projecting populations is in the effector to which mGluRs are coupled. This distinction might have practical value for song-system research in that it might serve to help identify X- or RA-projecting cells during single-unit recording in vivo. The differential sensitivity to different neurotransmitters suggests there could be selective modulation of auditory and motor function by neuromodulators.

Thus we have identified electrophysiological differences between the two types of HVc projection neuron. Indeed, these cell classes are strongly differentiated with respect to each other in a variety of properties. Katz and Gurney (1981) reported auditory responses in some neurons projecting to area X but not in RA-projecting cells. Lewicki and Konishi (1995) and Lewicki (1996) noted a similar trend. Kimpo and Doupe (1997) found FOS expression in RA-projecting but not X-projecting neurons following singing, the clearest functional difference reported to date. It is tempting to speculate that the auditory neurons in HVc are those that project to area X, whereas the RA-projecting cells do not respond to auditory stimuli. However, Doupe and Konishi (1991) showed that RA receives HVc-dependent auditory responses in the absence of the lateral portion of the magnocellular nucleus of the anterior neostriatum (L-MAN). This raises the possibility that there may be two types of RA-projecting neurons, auditory and nonauditory. Our results do not support the presence of physiologically distinct populations of RA-projecting neurons; we never recorded RA-projecting neurons with type I properties. Thus if auditory HVc neurons project to RA, then either we did not record from them in this study or auditory neurons do not have a distinct profile of physiological characteristics.

The functional differences observed between the two classes of HVc projection neurons are also associated with anatomic differences. In addition to the clear difference in their axonal projection, which allows us to distinguish X-projecting from RA-projecting neurons, these two populations also differ in their morphology. X-projecting neurons...
FIG. 9. Partial reconstruction of a type II neuron intra-cellularly filled with neurobiotin. A: camera lucida drawing of a parasagittal view of the cell body, the dendritic arborization, and the axonal trajectory. Note the presence of 2 labeled cell bodies for a single neurobiotin injection, suggesting the presence of coupling in this neuronal population. Finally, it was possible to follow the axon toward the nucleus RA in a single slice, indicating that the HVC-RA projection is organized in the parasagittal plane. Insets are bright field photomicrographs of parts of this neuron showing the cell bodies (B) and a part of the axon leaving HVC (C). Note also the different directions of axons in type I (Fig. 8) and type II (this figure) neurons.

have larger somata and larger total dendritic extent than RA-projecting neurons, consistent with findings of Nixdorf et al. (1989) and of Fortune and Margoliash (1995). However, both types of cell possess dendritic spines. The X-projecting cells we recorded appear to correspond to the thick dendrite class of HVC neuron described by Nixdorf et al. (1989) and filled by Benton et al. (1998) in the canary. Fortune and Margoliash (1995) reported a similar cell class retrogradely labeled from area X and also observed a class of smaller neurons. It is possible we did not record from this smaller class of X-projecting neurons.

Our RA-projecting cells appear to correspond to the short dendrite class of Nixdorf et al. (1989). However, we did not observe the heterogeneity described by Fortune and Margoliash (1995) in RA-projecting cells, again perhaps reflecting sampling bias caused by intracellular recording. Our type III neurons may represent their aspiny, putative interneuron class.

Gahr and Garcia-Segura (1996) reported electron microscopic evidence for the presence of gap junctions in female canary HVC. Testosterone induced increases in the number of gap junctions in HVC as well as increased singing. Our observation that RA-projecting cells (and less commonly X-projecting cells) are dye coupled to other HVC cells could be explained by gap junctions within HVC of male zebra finches.
This paper represents a first step toward identifying the cell types in HVC and determining their intrinsic properties. We identified at least three neuronal classes with the use of several physiological and pharmacological criteria, although other classes may emerge with more detailed studies. Neurons projecting to different targets and likely participating in different functions exhibit distinct specializations that are perhaps critical to their computational role. It will be important to link the properties of these neurons in vitro with those in vivo; for example, an extremely important question is whether the RA-projecting neurons have auditory responses and, if so, how they differ from those of X-projecting neurons. This information will be useful in determining the timing of sensory feedback activity throughout the song system, which will be crucial for understanding the processes underlying song learning. In addition, information on the degree of interaction of the two projection pathways will provide insight into whether and how the direct and indirect pathways from HVC to RA function in juveniles to accomplish song learning.

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Address for reprint requests: D. J. Perkel, Dept. of Neuroscience, 215 Stemmler Hall, University of Pennsylvania, Philadelphia, PA 19104-6074.

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